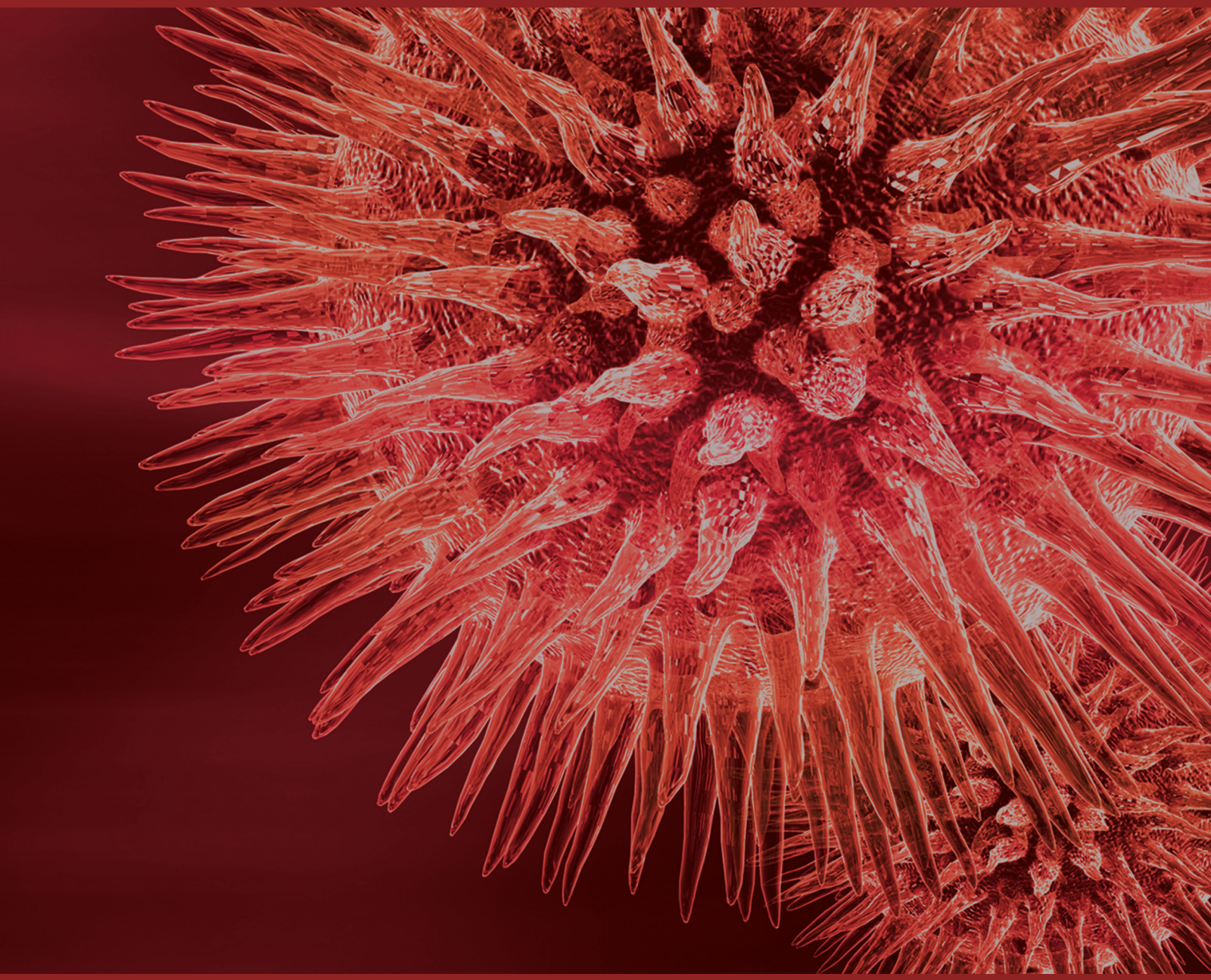
BioMed Research International

Bridging the Gap between Translational and Outcome Research in Cardiovascular Disease

Guest Editors: Giacomo Frati, Umberto Benedetto, Giuseppe Biondi-Zoccai, and Sebastiano Sciarretta



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Editorial

Bridging the Gap between Translational and Outcome Research in Cardiovascular Disease

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The adjective "translational" stems from Ancient Latin "translatio" (transfero, carry over). Translational research is the branch of scientific research aimed at transferring discoveries obtained in basic research investigations to the clinical context. Accordingly, in the field of biomedical sciences the concept of translational research relates to the transfer of scientific knowledge into practical clinical applications in order to improve the management and treatment of human diseases.

Medicine in general is particularly fascinated by the translational concept, probably for its peculiar applied scopes, and cardiology is not an exception. In this regard, the International Society for Cardiovascular Translational Research (http://www.isctr.org/) was founded in 2007 with the objective to coordinate and guide basic and clinical researchers, regulatory authorities, and the medical industry to improve the process of transferring new scientific evidence into clinical applications, promote translational research and divulgate new scientific results to the scientific community, and develop guidelines for conducting translational research studies. In addition, the ISCTR and the American College of Cardiology (ACC) collaborative efforts began in 2010 involving education on translational science pathways.

The reason behind the interest for translation research in the cardiovascular field is obvious. Although huge advances in the treatment of cardiovascular diseases have been made during the last decades, the morbidity and mortality associated with heart diseases are still too high [1]. In particular, the incidence of heart failure continues to increase with a progressively higher overload for national health systems and health care providers involved in the management of this chronic and highly disabling disease. For all these reasons, it is crucial to develop new therapeutic strategies for the prevention and treatment of cardiac diseases, with a particular focus on heart failure. However, the only way to be successful in this difficult task is to implement the efforts devoted to translational research.

One important goal of cardiovascular translational research is the discovery of new biomarkers that could be useful for the diagnosis, prognostic stratification, and therapeutic management of specific cardiac diseases [2]. This can be achieved by testing whether factors, which were previously found to be associated with specific cardiovascular disease models in experimental studies, are indeed useful for the clinical management of patients affected by these illnesses. These biomarkers could be circulating humoral factors or specific cellular subtypes, or preclinical markers of organ damage. For example, it is now well established that a reduction of flow-mediated artery dilation in human subjects is a preclinical sign of endothelial dysfunction and an independent predictor of adverse cardiovascular events [3].

Another important interest of translational research is the elucidation of the genetic basis of cardiovascular diseases. In fact, the clarification of the genetic predisposition to cardiovascular illnesses can help to identify those subjects who need a different clinical management. Genome-wide association studies are continuously providing new insights into the gene variants associated with cardiovascular sicknesses [4]. However, case-cohort studies or longitudinal investigations are also brilliantly identifying new polymorphisms linked to an increased incidence of cardiovascular diseases or to a worse prognosis. For example, recent studies demonstrated that T2238C atrial natriuretic peptide gene variant is independently associated with an increased incidence of adverse cardiovascular events [5].

A fundamental field in cardiovascular research is also represented by the study of cardioprotection [6]. In fact, the discovery of new therapies reducing the amount of myocardial death during an acute myocardial infarction would help to significantly reduce the incidence of subsequent cardiac dysfunction and heart failure. This would be possible only if the mechanisms regulating cardiomyocyte survival and death during myocardial ischemia are elucidated so that appropriate therapeutic targets can be identified. Unfortunately, in the last decades only few of the therapeutic interventions effective in the reduction of myocardial infarction and cardiac remodeling in animal models of ischemia, ischemia/reperfusion, and chronic myocardial infarction resulted to be effective also in patients experiencing a real heart attack. This may be due to the fact that the animal models of myocardial ischemia and infarction currently employed in basic experimental studies do not accurately mimic the pathophysiology of a human myocardial infarction [7]. Future efforts of researchers working on cardioprotection should be devoted to the development of more relevant models of cardiac diseases.

Finally, a fascinating and promising field of translational research is represented by cardiac regeneration. The possibility for physicians to repair a failing heart with stem cells or applied tissue-engineered myocardial patches still represents a new frontier for the treatment of heart failure [8]. Unfortunately, as in the field of cardioprotection, most of the attempts to effectively translate the highly promising experimental results obtained in this field into the clinical setting highlighted mixed results with benefits ranging from absent to transient or, at most, marginal [9, 10]. A part of the delay in the therapeutic advancement in the field of stem cells and cardiac regeneration is caused by the fact that little is still known about the mechanisms to increase stem cell survival after in vivo transplantation and to efficiently induce its transdifferentiation into mature cardiomyocytes. These mechanisms need to be urgently elucidated in future investigations. In addition, the standardization of the procedures for isolation, purification, manipulation, and transplantation of human stem cells used for therapeutic purposes needs to be implemented, despite the fact that the standards of safety and quality for stem cell therapeutic uses are currently defined in the Good Manufacturing Practice (GMP) guidelines (see Eudralex EU guidelines for Good Manufacturing Practice for Medicinal Products for Human and Veterinary Use) [11, 12]. Nonetheless, full compliance with GMP is a mandatory aspect of stem cell-tissue engineering and manufacturing.

The improvement of translational research, however, cannot represent alone the solution for developing new strategies for the cure of cardiovascular diseases. Together with translational research, it would be highly important to implement also clinical and outcome research. These scientific research branches aim to further expand the current knowledge of the prevalence, incidence, impact, and management of cardiovascular abnormal conditions in selected or real-world patients. This would help to identify shortfalls in practice and to develop strategies to improve care. In particular, outcome research is planned to continuously provide new insights into the therapeutic interventions working best for specific types of patients and under specific circumstances.

Given these premises, this special issue aimed at integrating expertise from different disciplines toward the same objective: a deeper understanding of the mechanisms underlying cardiovascular diseases as well as the development of new therapeutic strategies to prevent or treat cardiovascular diseases. In our opinion the result was notable. Among the accepted manuscripts, some studies developed new methods enhancing the cardiovascular transdifferentiation of stem cells or standardized the procedures for the isolation of bone marrow-derived cellular subtypes for the treatment of refractory ischemia. Other manuscripts dealt with the molecular mechanisms underlying ischemia/reperfusion damage or heart failure with preserved ejection fraction. The biomarkers associated with resistant hypertension or aortic aneurism rupture were also studied. In addition, some epidemiologic studies provided new insights into the factors associated with coronary artery disease or with a worse cardiovascular outcome. Finally, the genetic basis of metabolically unhealthy obesity was also investigated.

The editors really hope that the scientific contributions accepted for this special issue may contribute in some extent to the advance of the current knowledge of the pathophysiology, prognosis, and management of cardiovascular diseases.

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Giuseppe Biondi-Zoccai
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Clinical Study

Recent Developments in Minimally Invasive Cardiac Surgery: Evolution or Revolution?

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Intraluminal aortic clamping has been achieved until now by means of a sophisticated device consisting of a three-lumen catheter named Endoclamp, which allows at the same time occlusion of the aorta, antegrade delivering of cardioplegia, and venting through the aortic root. This tool has shown important advantages allowing aortic occlusion and perfusate delivering without a direct contact with ascending aorta reducing meanwhile the risk of traumatic and/or iatrogenic injuries. Recently, a new device (Intraclude catheter) with the same characteristics and properties has been proposed and introduced in clinical practice. The aim of this paper is to investigate the differences between Endoclamp and Intraclude catheters and to analyze the advantages advocated by this new device for intraluminal aortic occlusion since it is noticeable as these new technological tools are gaining more and more attractiveness due to their appraised clinical efficacy.

1. Introduction

Since Bailey reported in 1951 the first surgical treatment of mitral valve with mitral annulus narrowing by external constriction through left thoracotomy [1], several approaches and techniques for mitral valve surgery have been progressively proposed, modified, and refined, especially after the introduction of CPB (cardiopulmonary bypass). LILLEHEI and colleagues reported the first case of mitral valve repair through a right thoracotomy, using femoral artery cannulation for cardiopulmonary bypass (CPB) [2]. With extensive use of CPB in 1960s, median sternotomy became the primary surgical approach to mitral valve considering the undoubted advantages related to its reliability, speed, and worthy exposure of the mitral valve as well as access to the rest of the heart compared to right thoracotomic incision. In the late 90s, the increasing interest for minimally invasive surgery due to patients demand, marketing policy, and new developing technologies stimulated the reconsideration of different left atrial and mitral approaches. At first, parasternal incision and partial sternotomy, following the work of Gillinov and Cosgrove [3], have been the most popular minimally invasive approach. More recently, in accordance with the rule of courses and historical claims, the experimental works performed in the laboratories at Stanford University and New York University have refocused the attention to right thoracotomy leading to the development of minithoracotomic video-assisted or video-guided port-access approach [4].

This new technique exploited a peripheral perfusion and a balloon catheter for aortic occlusion allowing a less invasive

procedure through a mini thoracotomy (4–6 cm) approach [5–7], with undoubted advantages related to an overall reduction in surgical trauma, an effective improvement in patient comfort, lower morbidities, and shorter in-hospital stay, besides the remarkable cosmetic advantages especially in women [8–10].

Due to materials and instrumentation improvements different cannulation and aortic clamping strategies are nowadays available:

(i) Full extra-thoracic CPB with external transthoracic aortic clamping.

(ii) Full extra-thoracic CPB with endoaortic clamping.

(iii) Central arterial cannulation with external transthoracic aortic clamping.

(iv) Central arterial cannulation with endoaortic clamping.

Intraluminal aortic clamping has been achieved until now by means of a sophisticated device consisting of a three-lumen catheter named Endoclamp, which allows at the same time occlusion of the aorta, antegrade delivering of cardioplegia, and venting through the aortic root. This tool has shown important advantages allowing aortic occlusion and perfusate delivering without a direct contact with ascending aorta reducing meanwhile the risk of traumatic and/or iatrogenic injuries. Therefore, other than in less invasive surgery procedures, the Endoclamp can be successfully adopted in systematic surgery especially in presence of extensive calcification of ascending aorta and in redo procedures allowing safer cross-clamping without requirement for dissection and manipulation of the ascending aorta and aortic root. Recently, a new device (Intraclude catheter) with the same characteristics and properties has been proposed and introduced in clinical practice. The aim of this paper is to analyze the differences between Endoclamp and Intraclude catheters and to analyze the advantages advocated by this new device for intraluminal aortic occlusion since it is noticeable as these new technological tools are gaining more and more attractiveness due to their appraised clinical efficacy.

2. Surgical Technique Using Endoclamp

Under general anesthesia, the patient is positioned in the supine position, with slight elevation (30°) of the right hemithorax. All candidates for minithoracotomic video-assisted or video-guided port-access approach are ventilated with a double-lumen endotracheal tube in order to exclude, when needed, right lung ventilation. Monitoring includes double side arterial lines and use of TEE (Transesophageal Echocardiography). A small right mini thoracotomy (working port) and two additional ports are performed as previously described [4, 11–13]. Venous drainage is generally achieved with double venous cannulation with a 14–20 Fr cannula placed percutaneously, under transesophageal echocardiographic guidance, through the internal jugular vein into the superior vena cava and a cannula into the inferior vena (Fr) cava through the femoral vein using Seldinger technique.

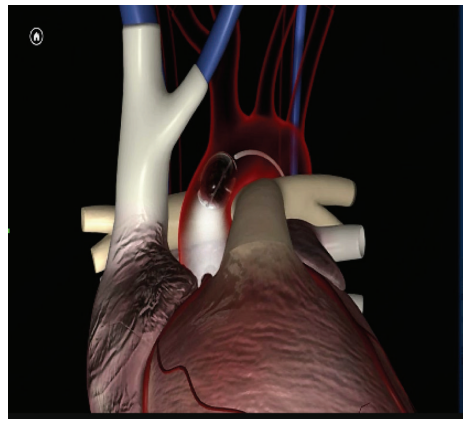

Figure 1: Endoaortic balloon correct placement.

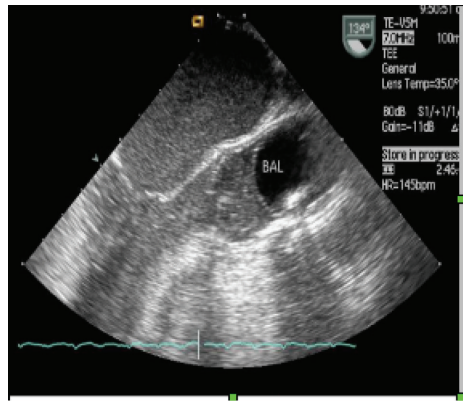

Figure 2: TEE monitoring of balloon position.

Arterial cannulation is performed with placement under direct vision into the femoral artery of a dedicated 21–23 Fr cannula (Endoreturn), a Y-shaped device with a side branch that allows the introduction of the occlusion balloon. The Endoclamp endoaortic balloon is at this time placed from the Endoreturn cannula side branch, under TEE guidance, in the ascending aorta just above the sinotubular junction (Figures 1 and 2).

The Endoclamp is a 10.5 Fr, 100 cm long, three-lumen catheter with an elastomeric balloon near its tip customized for endoluminal occlusion of the ascending aorta in order to separate the aortic root from arterial circulation. The surface contact of the balloon with the aortic wall is limited to 10 mm in length to avoid coronary occlusion during cardioplegia delivery. The large central lumen of the catheter attends two functions: delivery of cardioplegic solution during occlusion and venting from the left cardiac chambers both through the aortic root. The two remaining lumens are designed to serve as conduits for balloon inflation and aortic root pressure monitoring throughout the cardiac arrest. After proper position of the device, CPB is instituted and, under TEE monitoring to avoid balloon migration, the endoaortic balloon is progressively inflated with careful attention to its position at the level of the sinotubular junction [14]. Once its correct position is ascertained the cardioplegic

solution is administered via an antegrade route. Using this technique blood pressure through the arterial line should be continuously monitored in order to promptly detect possible modifications that might be suggestive of partial or transient occlusion of the arterial arch vessels. In our experience the balloon should initially be inflated using an amount of saline solution proportional to the diameter of the sinotubular junction (1 : 1) to avoid unnecessary overexpansion. The balloon pressure is continuously monitored to reach and maintain a target pressure of around 350/400 mmHg. It is important to remember that during the cardiac arrest the Endoclamp pressure can progressively decrease by 10–20% due to the variation of temperature and the reduced stiffness of the aortic wall. In this case, no additional volume of intraballoon saline is needed if the heart is asystolic and the field is dry. The adhesion of the balloon with the aortic wall is crucial for steadiness of the device. Usually a balance is achieved because the balloon is pushed downstream by arterial flow from femoral arterial cannula and upstream by the pressure originated inside the root by cardioplegia delivery or by the systolic ejection from the heart before complete cardiac arrest. In presence of trivial aortic regurgitation and/or inadequate drainage of the left ventricle with unsatisfactory cardiac arrest during the antegrade cardioplegic induction, adenosine injection directly in the aortic root can be used to facilitate heart arrest and therefore facilitate the correct endoclamping function. In addition, to optimize left ventricular drainage, an Endopulmonary Vent Catheter, previously inserted by anesthesiologist through central vein access, can be used when needed. During surgery continuous TEE monitoring is recommended, to provide an optimal monitoring of venous cannula position, deairing maneuvers, and, certainly, an assessment of valve function after the operation [15, 16]. Other indirect monitoring tools include NIRS (INVOS) or transcranial Doppler [17] both able to detect any functional impairment of cerebral blood flow [18] caused by balloon migration. Once the surgical procedure has been completed, the balloon is deflated and partially withdrawn. At this time aortic venting is achieved through the same sideline of the catheter. After weaning from CPB, the device is fully removed through the side branch of the Endoreturn arterial cannula. A major concern of this technique with adoption of this device is the possible reduction of the arterial cannula lumen after introduction of the endoluminal balloon. Although this phenomenon is unusual with a 23 Fr cannula it has been described with the 21 Fr size with possible negative impact on systemic perfusion or in safety of CPB management. The reduction of the arterial cannula lumen related to the steric hindrance of the Endoclamp catheter can, in fact, result in an elevated pressure on the line of arterial perfusion (>250 mmHg), especially in cases of small and elastic femoral arteries, like in young women with small body surface area or in patients with severe atherosclerotic disease of the iliac-femoral tree. In our experience, with pressure >300 mmHg during full flow CPB, a contralateral femoral arterial cannulation, even with a small (18-19 Fr) cannula, is advisable by means of a double Y line perfusion to avoid malperfusion or complication on CPB lines or oxygenator.

3. Pitfalls

Although most of studies showed the feasibility and safety of minimally invasive mitral valve surgery using the endoaortic clamping technique [19–21], several specific issues emerged from data reported in literature. Particularly in first series, multiple severe complications have been described, such as aortic dissection or iliac artery injury [22], probably due to first generation stiffer catheters, worse monitoring techniques, and learning curve of the operator. In fact, originally Endoclamp position monitoring was performed using fluoroscopy only during the positioning of the device without any further control during the surgical procedure and, moreover, surgeons were not enough skilled to manage catheters and guide-wires. Nowadays severe vascular complications are very rare and cannot be considered a specific burden of endoluminal clamping technique itself [23, 24].

Concerning generic complications some authors and especially the ISMICS (International Society for Minimally Invasive Cardiothoracic Surgery) summit claimed an augmented risk for cerebrovascular events, hypothetically due to greater use of femoral arterial cannulation for CPB. Plaque embolization during catheter introduction into the femoral artery or related to retrograde perfusion as well as traumatic injuries with consequent artery dissection or pseudoaneurysms formation and epiaortic vessel obstruction caused by balloon migration are well known complications [25–27] limited in more recent practice by the adoption of a more careful monitoring and dedicated catheter and devices.

Furthermore, another pitfall can be related to insertion of the endoluminal balloon into the arterial cannula that in some cases can induce increased resistance in the arterial line requiring a double arterial cannulation [28].

Other possible complications, described particularly in the first "era," are directly related to the endoluminal balloon device. Retrograde aortic dissection, balloon migration, balloon caught by suture for proximal anastomosis in coronary surgery, and balloon perforation during mitral valve procedure have been described and reported in literature [29]. Other authors, despite the overlapping results between classical sternotomy technique and port-access technique using Endoclamp, described cases that required switching to external cross-clamping to solve the unexpected problems arisen with endoluminal balloon [30].

4. Intraclude Improvements

In this milieu a novel device, the Intraclude catheter, has been designed and approved for clinical use to overcome and solve these issues. Intraclude is a three-lumen catheter designed as an evolution with the same purposes of the Endoclamp. Innovations of this device compared to the Endoclamp are related particularly to the catheter size. New developed technology permitted, in fact, reducing the size of the device from 10.5 Fr to 9.5 Fr leading to the attainment of decreased resistances through the arterial cannula and, therefore, allowing a major blood flow at minor pressures, with consequent limitation of the stress and the so called "sand blast effect" related to the high-pressure blood jet.

Some authors reported occasionally experiences of catheter "kinking," probably due to the minor caliber and softer material with respect to Endoclamp. This phenomenon is not frequent since the reduced caliber of the tip of Intraclude compared to the proximal part (hub) that maintains a diameter of 10.5 Fr is specifically designed to avoid the risk of kinking or twisting of the catheter and consequently to prevent the hazard of high pressure during cardioplegia delivering. Femoral artery injury or pseudoaneurysm formation leading to limb ischemia could be complications of femoral cannulation itself, regardless of the endoaortic balloon use, and prevention strategies are described elsewhere [27, 31]. Concerning balloon migration, Intraclude has a different shaped balloon with wider cylindrical-shape compared to the spherical balloon of the Endoclamp with advantages in terms of surface contact that is increased from 10 mm to 18 mm. This change is supposed to ameliorate the stability of the endoluminal balloon with a better "fitting" into the aortic lumen and improved adhesion to the aortic wall allowing a more reliable sealing after inflation and therefore reducing the incidence of dislocation and/or blood leak into the ascending aorta.

To further ameliorate safety and performance of the endoluminal clamping, the Intraclude shaft is curve-shaped, allowing a better adhesion to the aortic arch and a perfect tip orientation towards the aortic valve for the cardioplegia delivery. Moreover, this curvature is supposed to avoid the "slack effect" experienced with the straight Endoclamp shaft limiting the possible migration of the balloon toward the aortic valve due to the tension generated by the catheter bended into the aortic arch.

The different balloon shape also allows the availability of a wider range of calibers, ranging from 20 to 40 mm, rather than the Endoclamp limited to a range of 20–38 mm. Dealing with balloon disruption or perforation, continuous TEE monitoring is essential to avoid malposition and prevent accidental perforation of the balloon during mitral valve surgery as well described in literature [14]. To prevent spontaneous ruptures, the inflation volume has been reduced to 35 mL (from 40 of Endoclamp), preventing in this way either possible clamp failure, or balloon migration. In this regard, it is useful to keep in mind that pressure variations throughout the surgical procedure can lead to migration of the device as well as that migration itself can determine pressure change with vicious cycle mechanism.

In a recent analysis conducted by several European surgeons [32], the routine adoption of Intraclude device has been considered as one of the key factors leading to a significant reduction of morbidity and complications with particular emphasis concerning the stroke rate incidence.

5. Discussion

Minimally invasive surgical treatment of valvular heart disease has steadily increased over the last several years becoming an established technique with high successful outcome in many specialized centers. The vast majority of larger clinical studies demonstrate that minimally invasive valve surgery using the port-access approach after an initial learning curve [19, 20, 33–36] is a safe and effective approach in terms of short- and long-term results, mainly for redo operations and even for elderly patients with moderately elevated perioperative risk. Furthermore this technique has shown a low morbidity and mortality achieving functional and echocardiographic outcomes comparable to those obtained with conventional surgery. Measurable patient benefits from case-matched control trials [37–39] include less pain, less blood transfusions, fewer wound infections and pulmonary complications, and faster recovery as well as a better cosmetic result. Moreover recent improvements in Thru-Port systems offer excellent visualization of cardiac structures (Figure 3) through a virtually bloodless, unobstructed operative field without any increase of operative difficulty, procedure, and pump times, thus consenting to adopt successfully the same well established surgical techniques through the smallest incision possible.

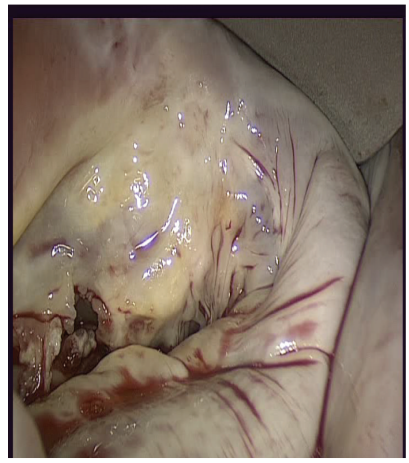

Figure 3: Excellent visualization of the mitral valve by Thru-Port System.

In this setting the new device Intraclude undoubtedly improved safety and properness of intraluminal aortic occlusion during minimally invasive mitral surgery. The preshaped curved silhouette, the reduced diameter, and the cylindrical balloon profile have shown unquestionable advances allowing an easier and more reliable endoartic clamping with positive impact in terms of reduced stroke incidence suggesting a spreader use of this device other than in minimally invasive surgery. New catheter Intraclude has been introduced in the European market in 2012, replacing the old catheter Endoclamp. Since then, more than 2500 catheters have been used in Europe for ascending aorta occlusion and cardioplegia delivering during minimally invasive mitral valve surgery.

In our experience, we used the catheter Endoclamp from 2000 in our patients in more than 600 operations. We moved to use the new device, Intraclude, from the beginning, in our patients and we performed more than 60 cases with this device. At the beginning of the experience, we had some concerns regarding the extreme softness of the catheter with some risk of twisting and kinking. Nevertheless, we immediately appreciated, with respect to Endoclamp, the

fact that its reduced size allowed a minor increase in the arterial line pressure during perfusion. Using Endoclamp we experienced at least five cases of migration of the balloon toward aortic valve and left ventricle. We did not have similar cases with Intraclude probably due to wider adhesion of this balloon to the aortic wall. The preshaped curvature of Intraclude makes its position easier in contact with the aortic arch reducing the need of additional maneuvers to avoid slack of catheter in thoracic aorta. From our experience, during introduction of the device it is mandatory to avoid any twisting of the curvature before the tip of the catheter reaches the root of ascending aorta. We had four cases of spontaneous rupture of Endoclamp but none using Intraclude. This could be related to the different shape of the balloon and/or to the material of this new device that seems to be helpful in terms of strength and fitting of the balloon with irregularities of the aortic wall. In conclusion we firmly believe all these technological developments and tools applied to minimally invasive procedure have gained, over time, more and more attractiveness due to their appraised clinical efficacy, and the acquired clinical experience in thousands of patients worldwide has led to a global improvement and to an implementation of this promising and ground-breaking surgical approach. In brief we are facing a gradual process where something changes into a different and usually more complex or better form, which simply means evolution.

Conflict of Interests

Professor Ernesto Greco has consulted for Edwards Lifesciences on minimally invasive valve surgery and holds a patent concerning minimally invasive access (no. US D701,305 S). The other authors declare that they have no competing interests.

Authors' Contribution

Ernesto Greco and Antonino G. M. Marullo conceived the paper. All authors have been involved in drafting the paper or revising it critically for important intellectual content and have given final approval of the version to be published. All authors read and approved the final paper.

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Review Article

Evidential Value That Exercise Improves BMI z-Score in Overweight and Obese Children and Adolescents

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Background. Given the cardiovascular disease (CVD) related importance of understanding the true effects of exercise on adiposity in overweight and obese children and adolescents, this study examined whether there is evidential value to rule out excessive and inappropriate reporting of statistically significant results, a major problem in the published literature, with respect to exercise-induced improvements in BMI z-score among overweight and obese children and adolescents. *Methods.* Using data from a previous meta-analysis of 10 published studies that included 835 overweight and obese children and adolescents, a novel, recently developed approach (p-curve) was used to test for evidential value and rule out selective reporting of findings. Chi-squared tests (χ^2) were used to test for statistical significance with alpha (p) values <0.05 considered statistically significant. *Results.* Six of 10 findings (60%) were statistically significant. Statistically significant right-skew to rule out selective reporting was found ($\chi^2 = 38.8$, $p = 0.0001$). Conversely, studies neither lacked evidential value ($\chi^2 = 6.8$, $p = 0.87$) nor lacked evidential value and were intensely p-hacked ($\chi^2 = 4.3$, $p = 0.98$). *Conclusion.* Evidential value results confirm that exercise reduces BMI z-score in overweight and obese children and adolescents, an important therapeutic strategy for treating and preventing CVD.

1. Introduction

Cardiovascular disease (CVD) is the leading cause of mortality from noncommunicable diseases worldwide, estimated in 2012 at 17.5 million, or 46.2%, of all noncommunicable disease deaths [1]. By 2030, it is estimated that mortality from CVD will increase to 23.3 million [2]. Two of the major risk factors for CVD are overweight and obesity [3, 4], a global and increasing problem among children and adolescents in both developed and developing countries. To illustrate, between 1980 and 2013, the worldwide prevalence of overweight and obesity among children and adolescents in developed countries increased from 16.9% to 23.8% for boys and from 16.2% to 22.6% for girls [5]. For children and adolescents in developing countries, increases ranged from 8.1% to 12.9% for boys and 8.4% to 13.4% for girls [5]. The deleterious effects of overweight and obesity during the childhood and adolescent periods are both immediate and long term. For example, in a population-based sample of United States (US) children and adolescents 5 to 17 years of age, approximately 70% of obese youth had at least one CVD risk factor [6]. From a long-term perspective, overweight and obesity during childhood and adolescence have been shown to track into adulthood [7], thereby placing this population at an increased risk for CVD and the mortality associated with such [3, 4]. The tracking of obesity into adulthood is especially noteworthy given that up to 80% of obese adolescents are at risk of becoming obese adults [8].

Therapeutic strategies aimed at reducing the prevalence of overweight and obesity among children and adolescents are important for reducing the lifetime risk of CVD. One such strategy is exercise, a low-cost, nonpharmacological approach that is available to the vast majority of overweight and obese children and adolescents. In a recent meta-analysis that included 835 overweight and obese children and adolescents, a statistically significant, exercise-induced reduction in BMI z-score was reported [9]. While these results are encouraging, all of the included studies were published in journals. This is problematic because studies published in journals suffer from an excess of statistically significant

findings [10]. Consequently, such findings may not represent the true truth. This excess in statistically significant findings has been shown to be the result of factors that include, but are not necessarily limited to, selective reporting by investigators [11–17]. At all levels of use (research, practice, and policy), it is critically important to understand the true effects of exercise in overweight and obese children and adolescents. However, while guidelines for the assessment of selective reporting and related biases in meta-analysis exist, all have notable limitations and no adjustment methods are recommended [18]. Recently, however, *p*-curve, a new and novel method that can rule out selective reporting and does not require access to nonsignificant findings, has been developed and validated [19, 20]. Thus, given the CVD-related importance of understanding the true effects of exercise on adiposity in overweight and obese children and adolescents, the purpose of this study was to examine whether there is evidential value that exercise improves BMI *z*-score in overweight and obese children and adolescents.

2. Methods

2.1. Data Source. Data for the current study were derived from a recently published aggregate data meta-analysis that has previously been described in detail elsewhere [9]. Briefly, studies were included if they were randomized controlled trials examining the effects of exercise (aerobic, strength training, or both) on BMI *z*-score in overweight and obese children and adolescents [9]. A total of 10 studies representing 835 overweight and obese children and adolescents (456 exercise, 379 control) were included [21–30]. BMI *z*-score was chosen as the primary outcome based on previous research suggesting its greater validity over other types of BMI-related measures [31]. The focus was on a BMI-related measure over other measures of adiposity, for example, fat mass, given that BMI-related measures not only are the most common method for assessing adiposity, but also are used to define overweight and obesity in children, adolescents, and adults. BMI *z*-scores from each study were calculated by subtracting the change outcome difference in the exercise group from the change outcome difference in the control group and weighting by the inverse of the pooled variance. Overall results for BMI *z*-score from each included study were pooled using a random-effects model that incorporates heterogeneity into the analysis. Heterogeneity was assessed using Cochran's Q statistic and I^2 [32–34].

2.2. Determination of Evidential Value. To determine whether evidential value exists with respect to exercise improving BMI *z*-score in overweight and obese children and adolescents, *p*-curve, a recent and novel approach, was used [19, 20]. The objective of *p*-curve is to test for evidential value in order to eliminate selective reporting as a reason for statistically significant findings. Statistical inference includes (1) studies that contain evidential value (right-skew), (2) studies that lack evidential value (flatter than 33% power), and (3) studies that lack evidential value and were intensely *p*-hacked (left skew). It consists of the distribution of statistically significant p values <0.05 for a group of studies, with nonsignificant p values >0.05 not included in the analysis. Right-skewed p values are indicative of true effects and thus evidential value because they include a greater number of low (ps = 0.01) versus high (ps = 0.04) statistically significant alpha values. Probability values that are not right-skewed suggest a lack of evidential value while those that are left-skewed are suggestive of *p*-hacking, that is, investigator-suppression of subsets of nonsignificant results.

Testing for evidential value consisted of two steps. First, for each statistically significant p value <0.05, the probability of observing a significant p value at least as extreme as if the null were true was calculated. This is known as the *pp* value (p value of the p value) and was calculated by dividing statistically significant probability values from each study by 0.05. For this study, the probability values were derived from *z*-values calculated from the exercise minus control group differences in BMI *z*-score for each study. In order to maintain independence, the results from one study that included more than one intervention group were collapsed so that only one probability value was included for that study [22]. The second step consisted of pooling the *pp* values using Fisher's method [35]. This yields an overall χ^2 test for skew with degrees of freedom equal to twice the number of p values. Thus, a statistically significant χ^2 test is indicative of a significant right-skewed *p*-curve and thus evidential value that exercise improves BMI *z*-score in overweight and obese children and adolescents. The absence of a statistically significant right-skewed p value suggests either a lack of information to make inferences about evidential value or a lack of evidential value. To test for a lack of information, that is, power, the same approach as for right-skew was used except that *pp* values were recalculated for expected *p*-curves using a power of 33% and the study's sample size, accomplished via the use of noncentral distributions. To test for a lack of evidential value suggestive of intense *p*-hacking (left skew), the same approach was used as for testing for evidential value of a real effect, that is, right-skew, except that the *pp* values for left skew were calculated as 1 minus the right-skew *pp* value. All calculations were robust to outliers, with *pp* values winsorized at 0.01 and 0.99. Chi-squared probability values ≤0.05 were considered statistically significant. Data were analyzed using *p*-curve (version 2.0), a free online statistical program available at http://www.p-curve.com/app2/, version 3.0 of Comprehensive Meta-Analysis [36], and Microsoft Excel 2010 [37].

3. Results

3.1. Changes in BMI z-Score. Figure 1 shows a forest plot that depicts the overall results for changes in BMI *z*-score, details of which have been previously described [9]. As can be seen, a statistically significant reduction in BMI *z*-score in favor of exercise was observed as well as nonoverlapping 95% confidence intervals. Heterogeneity was statistically significant (Q = 21.5, p = 0.01) but moderate (I^2 = 58.2%, 95% confidence interval = 15.7% to 79.2%). Changes in BMI *z*-score ranged from −0.29 to 0 while overall results were equivalent to a relative reduction of approximately 2%. Six of 10 results (60%) were statistically significant ($p < 0.05$).

Study name	Subgroup within study	Point estimate	Lower limit	Upper limit	Z-value	p value
Daley et al., 2006	None	−0.010	−0.052	0.032	−0.472	0.63714
Davis et al., 2012	Combined	−0.063	−0.095	−0.032	−3.981	0.00007
Farpour-Lambert et al., 2009	None	−0.100	−0.159	−0.041	−3.311	0.00093
Hagströmer et al., 2009	None	−0.090	−0.160	−0.020	−2.507	0.01218
Kelly et al., 2004	None	0.000	−0.052	0.052	0.000	1.00000
Maddison et al., 2011	None	−0.060	−0.090	−0.030	−3.922	0.00009
Meyer et al., 2006	None	−0.290	−0.449	−0.131	−3.576	0.00035
Murphy et al., 2009	None	−0.063	−0.160	0.034	−1.269	0.20455
Shaibi et al., 2006	None	−0.021	−0.129	0.087	−0.380	0.70362
Weintraub et al., 2008	None	−0.080	−0.157	−0.003	−2.036	0.04179
		−0.059	−0.087	−0.031	−4.146	0.00003

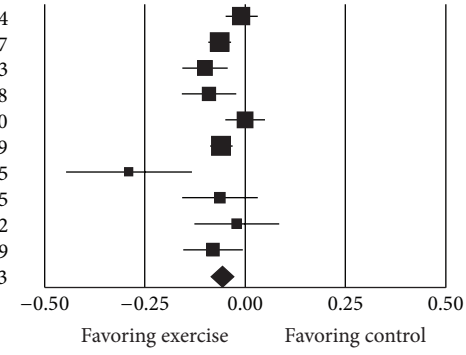

Figure 1: Forest plot for study-level changes in BMI z-score. The black squares represent the mean difference while the left and right extremes of the squares represent the corresponding 95% confidence intervals. The middle of the black diamond represents the overall mean difference while the left and right extremes of the diamond represent the corresponding 95%confidence intervals.

Table 1: Evidential values for changes in BMI z-score.

Statistical inference	χ^2	df	p
Studies contain evidential value (right-skewed)	38.8	12	0.0001*
Studies lack evidential value (flatter than 33% power)	6.82	12	0.87
Studies lack evidential value and were intensely p-hacked (left-skewed)	4.27	12	0.98

Notes: χ^2, chi-squared tests; df, degrees of freedom (2 × the number of statistically significant values); p, probability value; *statistically significant ($p < 0.05$); calculations robust to outliers with pp values winsorized at 0.01 and 0.99.

3.2. p-Curve Results. Results for evidential value are shown in Table 1 and Figure 2. As can be seen, there was statistically significant right-skew and thus evidential value that exercise reduces BMI z-score in overweight and obese children and adolescents. Consistent with this finding are the nonsignificant results for power and left skew.

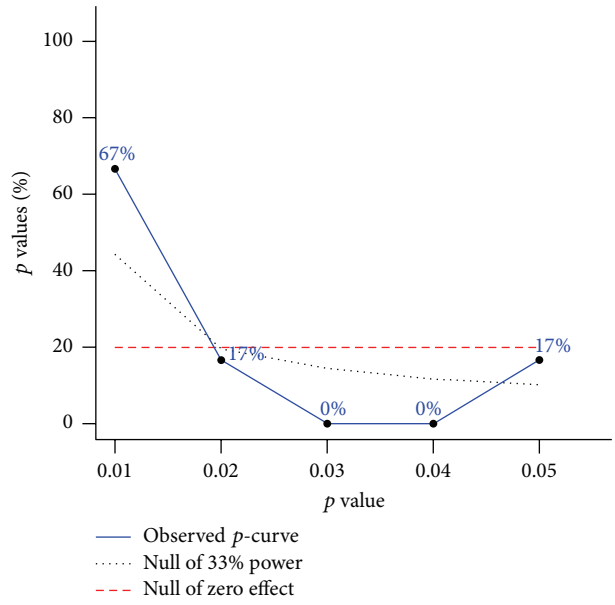

Figure 2: p-curve results. p-curve results for evidential value. Results are significantly right-skewed ($p = 0.001$), suggesting that evidential value exists that exercise improves BMI z-score in overweight and obese children and adolescents. The graphed results include six statistically significant p values <0.05. Four additional results were entered but excluded from the analysis because of nonsignificance ($p \geq 0.05$). All graphed calculations were adjusted for outliers by winsorizing pp values at 0.01 and 0.99.

4. Discussion

4.1. Overall Findings. The purpose of this study was to determine whether there is evidential value that exercise improves BMI z-score in overweight and obese children and adolescents. The findings indicate that the included studies contain evidential value that exercise improves BMI z-score in overweight and obese children and adolescents and provide much-needed reinforcement to previous work on this topic [9]. These results are important given that (1) overweight and obesity are two of the major risk factors for CVD [3, 4], (2) the worldwide prevalence of overweight and obesity in children and adolescents is high [5], and (3) a need exists to develop therapeutic strategies aimed at reducing CVD. The former notwithstanding, the results could be questioned given that the magnitude of improvement in BMI z-score was approximately 2% and p-curve does not directly assess for such. However, as previously reported, gross estimates suggest that approximately one million overweight and obese children and adolescents worldwide could reduce their BMI z-score by exercising regularly [9]. From the investigators' perspective, these findings are important.

4.2. Implications for Research and Practice. The results of the current study provide overall results in relation to the effects of exercise on BMI z-score in overweight and obese children and adolescents. However, the dose-response effects of exercise were not examined and when previously examined [9] did not glean any substantive findings. Given the former and as previously suggested [9], a need exists to examine the dose-response effects of exercise in a representative sample of this population. Until that time, adherence to the World Health Organization (WHO) recommendation of 60 minutes per day of physical activity for children and adolescents appears appropriate and is in concordance with the WHO year 2025 goals of reducing the global prevalence of physical inactivity across all age groups by 10% as well as halting the rise in obesity [38]. Increased participation in exercise among overweight and obese children and adolescents will also likely contribute to the WHO year 2025 goal of reducing mortality from cardiovascular diseases, cancer, diabetes, or chronic respiratory diseases by 25% [38].

4.3. Strengths and Potential Limitations. The major strength of the current study is the use of a recent and novel approach to address selective reporting of results [19, 20] and thus provide more convincing evidence regarding the true effects of exercise on BMI z-score in overweight and obese children and adolescents. This is critically important given the prevalence of self-report bias and subsequent overestimation of treatment effects in the published literature [11–17]. In contrast, one potential limitation is the fact that the current findings were based on 835 overweight and obese children and adolescents nested within 10 studies [21–30]. Consequently, there may have been a lack of precision given that the larger the number of studies as well as number of subjects nested within each study, the greater the precision of p-curve results [19]. Another potential limitation is that p-curve can fail to detect studies that lack evidential value because it is significantly right-skewed [20]. Furthermore, p-curve does not include p values >0.05, including those close to 0.05. As a result, p values suggestive of no effect, while extremely infrequent in the presence of a genuine effect, are excluded [20].

5. Conclusions

The results of the current study provide evidential value that exercise reduces BMI z-score in overweight and obese children and adolescents. Given its CVD-related importance, exercise should be recommended as a therapeutic strategy for reducing BMI z-score in overweight and obese children adolescents.

Disclaimer

The content is solely the responsibility of the authors and does not necessarily represent the official views of the American Heart Association or National Institutes of Health.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Kristi S. Kelley is the coauthor.

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Review Article

Are Endothelial Progenitor Cells the Real Solution for Cardiovascular Diseases? Focus on Controversies and Perspectives

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Advanced knowledge in the field of stem cell biology and their ability to provide a cue for counteracting several diseases are leading numerous researchers to focus their attention on "regenerative medicine" as possible solutions for cardiovascular diseases (CVDs). However, the lack of consistent evidence in this arena has hampered the clinical application. The same condition affects the research on endothelial progenitor cells (EPCs), creating more confusion than comprehension. In this review, this aspect is discussed with particular emphasis. In particular, we describe biology and physiology of EPCs, outline their clinical relevance as both new predictive, diagnostic, and prognostic CVD biomarkers and therapeutic agents, discuss advantages, disadvantages, and conflicting data about their use as possible solutions for vascular impairment and clinical applications, and finally underline a very crucial aspect of EPCs "characterization and definition," which seems to be the real cause of large heterogeneity existing in literature data on this topic.

1. Introduction

The most important determinant of cardiovascular health is person's age [1]. By 2030, approximately 20% of the population will be aged 65 or older [2]. In this age group, cardiovascular diseases (CVDs) will result in 40% of all deaths and rank as the leading cause [2]. Furthermore, the cost to treat CVDs will triple in that time [3]. Of consequence, urgent interventions both in preventive measures and biomedicine research are imperative. In the last years, some progresses have been realized. For example, primordial prevention based on healthful lifestyle (i.e., Mediterranean diet, lifestyle, and physical activity) has been proposed as preferred preventive's method to lower cardiovascular risk [4]. Advances have been achieved through percutaneous coronary intervention and coronary artery bypass grafting in management of coronary artery diseases, having higher prevalence and incidence in the world [5, 6]. Despite these efforts, there are no effective solutions until now. In addition, numerous gaps still remain between knowledge of precise CVD cellular and molecular mechanisms and identification of disease pathways to use as appropriate biomarkers and targets for new and more efficient therapeutic treatments, that is, personalized therapies.

Biomedical community is pursuing new ways in trying to face this imposing challenge. In particular, the latest discoveries and advanced knowledge in the fields of stem cell biology and their ability to provide a cue for counteracting several diseases are leading numerous researchers to focus their attention on "*regenerative medicine*" as possible solutions for CVDs [7]. However, the lack of consistent evidence in this arena has hampered the clinical application [8]. The same condition affects the research on endothelial progenitor cells (EPCs), creating more confusion than comprehension. In this review, this aspect is discussed with particular emphasis. In

particular, we describe biology and physiology of EPCs, outline their clinical relevance as both new predictive, diagnostic and prognostic CVD biomarkers, and therapeutic agents, discuss advantages, disadvantages, and conflicting data about their use as possible solutions for vascular impairment and clinical applications, and finally underline a very crucial aspect of EPCs "*characterization and definition,*" which seems to be the real cause of large heterogeneity existing in literature data on this topic.

2. Recent Efforts of Biomedical Research in Cardiovascular Repair: EPC Cells as Promising Candidates

Actually, the principal purpose of scientific community is to improve life quality and reduce and/or retard CVD onset and progression, even if it appears to be very ambitious. Its realization seems to be difficult for different reasons. Firstly, CVDs have a very complex pathophysiology orchestrated by mechanisms not completely clear and articulated in multistep clinical events. Another limiting factor is CVD progression generally assumed as irreversible and one-directional [9]. However, a small reverse probability has been recently suggested for each step. Accordingly, some individuals, even in the presence of potent risk factors, remain sheltered from consequences of cardiovascular alterations. The potential reason has been attributed to substantial ability to have an efficient cardiovascular self-repair, which appears to be prevalently modulated by genetic background and environmental factors [9]. As result, the interest on cardiovascular repair is increasing. It has led to evidence that three major processes drive it: (i) replacement (tissue transplant), (ii) rejuvenation or restoration (activation of resident or not stem and progenitor cells via autocrine, paracrine, or endocrine mechanisms; modulation of apoptosis, inflammation, angiogenesis, or metabolism), and (iii) regeneration (progenitor or stem cell engraftment forming differentiated cardiovascular cells) [10]. The three different entities may singularly function or be interlinked [10]. However, their mechanisms remain to be determined. Furthermore, in the regeneration, hematopoietic stem and progenitor cells (HSCs and HPCs) seem to have a crucial role. HSCs and HPCs are, indeed, becoming the potential therapy's agents for improving reparatory mechanisms in the heart and vascular system. Many studies have investigated their role in different CVDs, such as acute coronary syndromes, stroke, limb ischemia, and cardiac nonischemic injury. Discordant results have been obtained [11]. Thus, their real contribution is until now uncertain. However, it has been observed that cardiovascular risk factors induce impairment in their circulating levels and function. In contrast, physical exercise and statins mediate their improvement [11]. Of note, it also is their contribution in physiological endothelial and cardiac renewal, as observed in healthy subjects [11]. However, the weight of these observations is remarkably influenced by an essential limitation. HSCs and HPCs have been identified only as $CD34^+$ cells. Thus, the validity of these results needs to be confirmed.

Among the HSCs and HPCs, EPCs are the most widely studied adult human progenitor cell subpopulation up to now. Here, we report a summary of literature data on biological features of EPC cells.

3. Biological EPC Features

3.1. EPC Origins and Sources. EPC's discovery occurred in 1997 by Asahara and colleagues, which questioned the paradigm of angiogenesis and vasculogenesis in adult, by identifying H-precursor cells, defined as EPC cells able to differentiate into an endothelial phenotype *ex vivo* [12]. From then, a plethora of evidence supports EPC existence, origins, and contribution in new blood vessel formation [13]. EPCs have, indeed, capacity to proliferate, migrate, and differentiate into mature endothelial cells (ECs). In 2004, Urbich and Dimmeler defined EPCs using three biological parameters: (1) to be nonendothelial cell, but having capacity to give rise to ECs and (2) to show clonal ability to multiply, (3) and stemness characteristics [14].

Concerning their origin and sources, they have been object of a strong debate for different years. Actually, EPCs can be divided into two categories: H-EPCs and non-H-EPCs [13, 15, 16]. Here, we try to clarify this relevant and delicate aspect. We also point EPC origin from cord blood, as another relevant source.

3.1.1. H-EPCs. HSCs (expressing the classical CD34 marker or more immature CD133 marker) are the principal EPC source (see Table 1). They are maintained within bone marrow (BM) stem cell niches and released upon induced mobilization (see below), as firstly demonstrated by Asahara and colleagues [12]. This initial discovery has led to define EPCs as $CD34^+$ or $CD133^+$ cells. HSC contribution to neovascularization has been initially evaluated in animal models [16]. The promising results obtained have led to several clinical studies on progenitor cell therapy (in humans, see below) [13, 15, 16].

However, other BM-stem cells can generate EPCs, including BM-myeloid cells and BM-mesenchymal stem cells (MSC) (see Table 1). BM-myeloid cells are also mobilized from BM and derive from HSCs. Schmeisser and colleagues evidenced that $CD14^+/CD34^-$ myeloid cells can coexpress endothelial markers and form tubelike structure *ex vivo* [17]. Thus, BM-myeloid cells within peripheral blood can differentiate into endothelial lineage with a lower proliferative capacity than HSCs or cord blood derived EPCs [13]. Certainly, additional studies are necessary to determine differences in incorporation and particularly to clear the long-fate of HSCs versus monocyte derived cells [13, 15, 16].

BM also contains MSCs, which are stromal cells having ability to self-renew and also exhibit multilineage differentiation into both mesenchymal and nonmesenchymal lineages. BM-MSCs can differentiate into ECs and improve neovascularization, as demonstrated by *in vitro* studies. In addition, BM-MSCs have been also isolated from peripheral blood. This has opened the question on possibility of their mobilization in case of ischemia and their contribution to endogenous cardiovascular repair [13, 15, 16]. Further studies are, certainly, necessary for clarifying this question.

Table 1: Origins and sources of EPCs cells.

Stem and progenitor cells	Features and functions	References
	Hematopoietic stem or progenitor cells	
Hematopoietic stem cells (HSCs)	Limited differentiation capacity compared to embryonic stem cells Commonly identified by the expression of $CD34^+$ and CD133 cell surface antigens Clinically used for bone marrow transplantation in a variety of hematologic disorders Potentiality to differentiate into cardiac myocytes A subset of HSCs assume an endothelial phenotype promoting neovascularization by secreting proangiogenic growth factors and stimulating reendothelialization. These cells were named "endothelial progenitor cells" (EPC) The pattern of EPC surface markers includes CD133, VEGFR-2, CD34, Tie-1, Tie-2, CD146, c-Kit, and CXCR-4	[13, 15, 16]
H-myeloid cells	Mobilized from bone marrow $CD14^+/CD34^+$ myeloid cells coexpress endothelial markers, form tubelike structures *ex vivo,* and differentiate in endothelial cells incorporated in newly formed blood vessels Show a lower proliferation capacity than cord-blood-derived EPCs but have similar capacity to augment neovascularization in experimental models	[13, 15, 16]
H-mesenchymal stem cells (MSCs)	Limited differentiation capacity compared to embryonic stem cells Located in bone marrow and adipose tissues Transdifferentiate into functional cardiomyocytes and a variety of other cells Modulate immune responses	[13, 15, 16]
	Nonhematopoietic stem and progenitors cells (non-HSCs)	
Fat tissue	Can be obtained in large quantities under local anesthesia with minimal discomfort Adipose tissue-derived stromal vascular cells lack both CD31 and CD34 markers and differentiate into ECs promoting angiogenesis	[13, 15–20]
Liver and intestine	Progenitor cells derived from transplanted liver and intestine contribute to neovascularization after hind limb ischemia it is still debated whether these incorporated progenitors are derived from vessel wall in the organ or they are tissue-resident progenitor cells of nonvascular origin	
Spleen	Can differentiate to give an "EPC phenotype" and modulate endothelial function or vascular remodelling	
Kidney	$Pax\text{-}2^+$ cells displaying mesenchymal markers $CD133^+$ cells derived from human renal carcinoma are able to differentiate into ECs and were found to be directly incorporated into neovessels	
Skeletal myoblasts	First cells to be injected into the ischemic myocardium as part of a cell-based strategy Determine improvements in left ventricular function but little evidence shows transdifferentiation into cardiomyocytes	
Blood vessel wall	MSCs cells also called pericytes or adventitial cells	
	Cord blood stem cells	
	Greater plasticity than adult cells due to their prenatal origin Lacking evidence of pluripotency after *in vitro* expansion Cord blood contains a number of progenitor cell populations, including HSCs and MSCs Having not yet been investigated in a clinical setting	[13, 15, 16]

3.1.2. Non-H-EPCs. Other cell populations from other sources (i.e., adipose tissue, blood vessel wall, liver, intestine, spleen, and kidney) can give rise to EPCs [13, 15, 16] (see Table 1).

Adipose tissue represents an alternative source of autologous adult stem cells, which can be obtained in large quantities under local anaesthesia and with minimal discomfort. Human lipoaspirate contains stem cells able to differentiate into several lineages. Furthermore, it has been also observed that isolated-tissue-derived, cultured, and stromal-vascular $CD34^{-}CD31^{-}$ cell fractions can differentiate into ECs and promote angiogenesis [13, 15, 16].

Furthermore, MSCs, originally identified in BM, have been also detected in many other tissues, such as adipose tissue. They are able to differentiate into EC mature cells in an appropriate microenvironmental. In addition, they show ability to modulate immune responses. This leads to consider them as more attractive candidates for regenerative medicine. Allogeneic transplant of these cells is feasible without a substantial risk of immune rejection. MSCs secrete various immunomodulatory molecules which provide a regenerative microenvironment for a variety of injured tissues or organs to limit the damage and to increase self-regulated tissue regeneration. Autologous/allogeneic MSCs delivered via the bloodstream augment the titers of MSCs that are drawn to sites of tissue injury and can accelerate the tissue repair process [17, 18]. Recently, it has been also discovered that MSCs also derive from a perivascular location, where they reside as pericytes or adventitial cells. This finding has generated some momentum in the field of adult stem cell research and provided some insights into the developmental origins of these much exploited but little understood cells. It is now evident that the perivasculature represents MSC niche *in vivo*, where local cues coordinate the transition to progenitor and mature cell phenotypes. Here, MSCs can stabilize blood vessels and contribute to tissue and immune system homeostasis under physiological conditions and assume a more active role in tissue repair in response to injury. The establishment of a perivascular compartment as the MSC niche provides a basis for the rational design of additional *in vivo* therapeutic approaches [19, 20].

3.1.3. Cord Blood EPCs. A rich EPC source also is cord blood (see Table 1). Cord blood contains higher numbers of $CD133^{+}$ and $CD34^{+}$ cells compared with peripheral blood from adults $CD133^{+}/CD34^{+}$ cells [13, 15, 16]. In addition, a higher proliferation capacity and high levels of telomerase have been evidenced in cord blood derived EPCs [13, 15, 16]. These characteristics are typical of stem cells and very low or absent in other progenitor cell populations.

3.2. EPC Recruitment and Mobilization from BM, Their Migration, and Adhesion to Injured Vessel Wall. The EPC related formation of new blood vessels includes multiple steps comprising mobilization, migration, adhesion, and differentiation [21]. The mobilization of EPCs from BM into the peripheral circulation is the crucial step for these cells to participate in postnatal vasculogenesis. The precise mechanism of EPC mobilization is not entirely elucidated and it is still under investigation. It has been demonstrated that these cells are quiescent and tethered by integrins to stromal cells in a microenvironment within the BM. They can be converted into functional cells and released from the stem cell niche in response to various special cytokines and factors [21]. Mature ECs represent the crucial players in initiating H-EPC mediated vasculogenesis, by releasing attracting EPC factors under shear stress and hypoxia [13, 15, 16, 21, 22]. In the case of vascular occlusion, it has been observed that ECs seem to sense altered (low or oscillatory) shear stress and consequently improve prooxidant enzyme expression, mediated principally by the most crucial transcription factor, Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-$\kappa\beta$) [13, 15, 16, 21, 22]. In case of hypoxia, several signaling pathways on ECs are stimulated. They particularly induce activation of hypoxia-inducible transcription factor (HIF) [22]. As result, different growth factors, cytokines, and chemokines are released mediating H-EPC mobilization. Another crucial and specific factor associated with EPC mobilization from BM is nitric oxide (NO), as demonstrated in endothelial NO synthase $(eNOs)^{-/-}$ mice [23] (see below) (Table 2).

Furthermore, several types of chemokines are involved in EPC mobilization, such as stromal cell derived factor-1 (SDF-1), angiopoietin (Ang-1), and, probably the most important of all, vascular endothelial growth factor (VEGF) [21]. VEGF seems to determine a rapid EPC and HSC mobilization, as evidenced by Fox and colleagues in burned patients [21]. This last aspect has also consented to detect EPCs in peripheral blood as $VEGFR2^{+}$ or KDR^{+} (see below and Table 5). After their homing, EPCs can release VEGF themselves and create a local angiogenetic environment. Recently, Li and colleagues reported that SDF-1 and VEGF mediate EPC mobilization, through their interaction with their respective receptors (C-X-C chemokine receptor type 4 (CXCR4) and VEGFR2). This interaction determines the production of NO through the activation of eNOs. NO can stimulate metalloproteinase-9, which results in the release of sKitL from the stromal cell membrane-bound kit ligand (mKitL). Protooncogene c-kit (c-kit) expressed by EPCs contributes to the retention of EPCs within the BM niches. C-Kit is also the receptor for sKitL and can be released from BM in response to binding to sKitL, resulting in mobilization of $c\text{-}Kit^{+}$ EPCs from the cell niche into circulation [21].

Other factors as erythropoietin (EPO) can mobilize EPCs [24]. Ang-1 seems to have a delayed and inhibitory effect, as evidenced in a unique study performed in 1999, where EPCs were defined as $Tie^{+}/Flk\text{-}1^{+}/CD31^{+}$ cells [25].

Adhesion of EPC cells to injured vessel wall involves the interaction between glycoprotein ligand-1 (PSGL-1) expressed on EPCs and P-selectin expressed on platelets, as suggested by Li and colleagues [21]. Within minutes after vessel injury, platelets, indeed, aggregate on the exposed subendothelium. Adherent platelets express P-selectin on the surface and secrete high levels of SDF-1. In this process, circulating EPCs also upregulates PGSL-1 via the stimulation of SDF-1, which interact with their ligand P-selectin, thereby leading to EPC adhesion. Subsequently (within the next hours and days after endothelial disruption), apoptotic

Table 2: Key players involved in modulating circulating EPC levels and functions.

Factors	Effects	References
	Factors and conditions associated with altered circulating EPCs levels	
Aging	It determines a decrease in progenitor cells activity and mobilization	[26, 27]
Inflammation	Restricted acute inflammatory response stimulates EPCs mobilization while persistent chronic inflammatory stimuli have deleterious effects and result in decreased number of circulating mature and functional EPCs C-reactive protein (CRP) exerts direct inhibitory effects on EPC differentiation and survival. Proinflammatory TNF-α reduces EPCs number	[28, 29]
Oxidative stress	It reduces EPCs number, induces apoptosis, and reduces EPCs capacity of mobilization, migrating, and incorporating into vasculature	[28, 29]
Hypothyroidism	It decreases $CD34^+/CD133^+/KDR^+$ EPCs	[31, 32]
Cardiovascular risk factors (smoking, diabetes, hypertension, lipid disorders, abdominal obesity, metabolic syndrome, etc.)	They influence the circulating levels of EPCs; precisely they reduce their levels	[30]
Hyperparathyroidism	It increases circulating EPCs levels	[31, 32]
	Physiological factors involved in EPCs mobilization	
Gender	It upregulates VEGF and SDF-1 It modulates EPCs levels and cardiovascular risk profile, due to the beneficial effects of estrogens particularly in women The increase seems to be related to oestrogens levels	[33]
Pregnancy	It increases EPCs-derived colonies	[34]
	Drug therapies modulating circulating EPCs levels	
Antihypertensive drugs		[35]
Calcium channel blockers (CCBs) nifedipine and barnidipine	They enhance EPC number and function	
Angiotensin II receptor blocker (ARB) telmisartan	It enhances EPC number and function	
Angiotensin converting enzyme (ACE) inhibitors	They improves clonogenic capacity	[35]
Cholesterol lowering medications		
Statines (atorvastatin, rosuvastatin)	They increase mobilization of EPCs and $CD34^+/CD117^+$, $CD34^+/CXCR4^+$	[35]
Antidiabetic medications		
Oral dipeptidyl peptidase-4 inhibitor (sitagliptin)	It increases number of circulating EPCs in patients with diabetes	[35]
Thiazolidinedione/metformin	They improve EPCs number and function	
Other drugs		
Estradiol	It improves capacity of neovascularization	[26]
PPAR-γ agonist	It increases EPCs migration	[26]
CXCR4 agonists	They stimulate SC mobilization	
AMD3100 (plerixafor)	It increases $CD34^+$, $CD117^+$, and $CD133^+$ cells	[26]
POL6326		
Erythropoietin	It increases EPC and HSC levels	[24]
Nitroglycerin (chronic use)	It increases apoptosis and decreases phenotypic differentiation and migration	[26]
Granulocytes colony stimulating factor (G-CSF)	It induces SC mobilization by interruption of CXCR4/CXCL12, c-Kit/SCF, and VLA-4/VCAM-1 axis	[26]
Growth hormone	It reduces apoptosis It improves EPCs migratory capacity	[26]

Table 2: Continued.

Factors	Effects	References
	Lifestyle modification and nutritional interventions	
Red wine resveratrol, salvianolic acids, Gingko Biloba, ginsenoside, berberine, and puerarine.	They exert anti-inflammatory and antioxidant effects They enhance EPCs activity	[26]
Diet	It affects the number of circulating EPCs	[36, 37]
Dietary cocoa-derived flavonoids	They increase number of functional circulating angiogenic cells	[26]
Red ginseng extracts	They increase EPCs number	[26]
Physical exercise	It improves circulating EPCs levels. Prolonged 4-week exercise program improves EPCs functions. Maximal and endurance exercise influence the number of both EPCs and hematopoietic stem cells.	[26]

smooth muscle cells mainly contribute to SDF-1 release, which is required to sustain the process of vascular remodeling and repair [21].

3.3. Circulating EPC Levels and Their Alterations: Effects Mediated by Different Factors. Augmented or reduced circulating EPC levels, as well as their function, have been observed in a large number of studies. Several factors have been identified as possible causes (see Table 2 and Figure 1). Here, we describe them and their effects on EPC number and function.

3.3.1. Unfavourable Factors Modulating Circulating EPC Levels. Different endogenous factors can also influence EPC levels (Table 2 and Figure 1). In particular, ageing has been associated with an altered EPC function and viability, by determining a decreased potentiality of endothelial repair [26, 27]. Recently, it has been suggested that age-related inflammation and oxidative stress modulate EPC bioactivity and determine dysfunction [28, 29]. In particular, increasing evidence indicates EPC mobilization in case of transient restricted inflammatory response. On the contrary, persistent or excessive inflammatory stimuli may have deleterious effects, by decreasing EPC circulating numbers [28, 29]. Functional EPC activity is significantly impaired in case of high inflammatory stimulation, as in heart failure. Mechanisms regulating this effect are still unclear. However, convincing evidence leads to suppose that prolonged exposure of BM to increased proinflammatory stimulation may determine EPC pool exhaustion. In this condition, a small EPC number, prevalently immature or dysfunctional, might be released. However, existing clinical evidence on association of inflammation with reduced EPC levels is largely circumstantial and observational [28, 29]. Thus, further clinical studies are required.

As mentioned above, oxidative stress may also play a crucial role in EPC mobilization from BM and functional bioactivity. ROS exert a direct cytotoxic effect on the vascular endothelium. Increased superoxide generation reduces EPC levels and impairs EPC function, as demonstrated by increased apoptosis and reduced EPC number after incubating with high levels of hydrogen peroxide (H_2O_2) [28, 29] (see Table 2).

An increasing body of evidence also suggests that cardiovascular risk factors (smoking, diabetes, hypertension, lipid disorders, abdominal obesity, metabolic syndrome, etc.) affect EPC number and proprieties [30] (see Table 2 and Figure 1).

Endocrine disorders, such as hyperparathyroidism and hypothyroidism, may also alter EPC levels (see Table 2) [31, 32].

3.3.2. Physiological Factors Involved in Raising Circulating EPC Levels. An increased number of studies have demonstrated that physiological factors influence EPC circulating levels and function. Among physiological factors, gender appears to modulate EPC levels, as demonstrated by Fadini and colleagues [33]. Women have high EPC levels than men and oestrogens are the physiological factors significantly associated with these useful effects [31] (see Table 2 and Figure 1). In addition, pregnancy represents the physiological condition characterized by high EPC circulating levels [34].

3.3.3. Drug Therapies, Nutrition Interventions, and Lifestyle Modifications as Strategies to Improve Circulating EPC Levels. Drug therapies can also influence EPC levels and function in a positive manner. They prevalently operate as anti-inflammatory and antioxidant factors. In 2014, Lee and Poh stressed the significant interaction between cardiovascular pharmacotherapies and improvement of EPC number and functions. In particular, they reported the effects observed in clinical studies on EPC number and function from patients with different CVDs and treated with different medications, including antihypertensive, cholesterol lowering, and antidiabetic medications [35] (see Table 2 and Figure 1).

Recently, a growing number of studies are also evidencing an improvement of EPC number and function related to nutrition interventions and lifestyle modifications (see Table 2 and Figure 1). In particular, some research groups are reporting that Mediterranean diet determines an increase in circulating EPC levels and function [36, 37]. Similarly, physical exercise seems to induce an improvement of circulating EPC levels (see Table 2 and Figure 1) [26]. This has been evidenced in both healthy subjects and patients affected by CVDs. Thus, even in patients, with diffuse atherosclerosis and multiple risk factors, reparative capacity dependent on circulating BM-derived EPC is retained and can be enhanced in a most physiological way [26].

4. EPC in Vascular Impairment and Their Clinical Relevance

The important role of ECs in maintaining of the entire vessel wall (of arteries or veins) homeostasis, as well as their recognized finite lifespan and continuous response to different triggers responsible of endothelium dysfunction and injury, is well recognized (see Figure 2) [38–40]. This has led to identify a system able to replace these cells. This system has been conventionally established and identified in mature ECs adjacent to regions of injury [38–40]. It has been speculated that, under influence of paracrine mediators released from the injured segments and/or loss of contact inhibition, ECs migrate and proliferate [38–40]. Today, it is recognized that mature ECs possess limited regenerative capacity [41–44]. The discovery of EPCs has opened this question. EPCs seem to be a real source of ECs in maintaining vascular homeostasis. Thus, they constitute a very reservoir of circulating cells, which could home to sites of injury, restore endothelium integrity, and consent a normal function. The contribution of EPCs to vascularization has been demonstrated in animal models and in humans (see below). Their crucial role in this process has cotemporally led to hypothesize that a reduction in EPC circulating number and/or alterations in their functions associated with different factors (as the above discussed) might have a remarkable impact on endothelium function and CVD onset and complications and consequently in the survival of CVD affected individuals [41–44]. Accordingly, growing evidence is underling the clinical relevance of EPCs

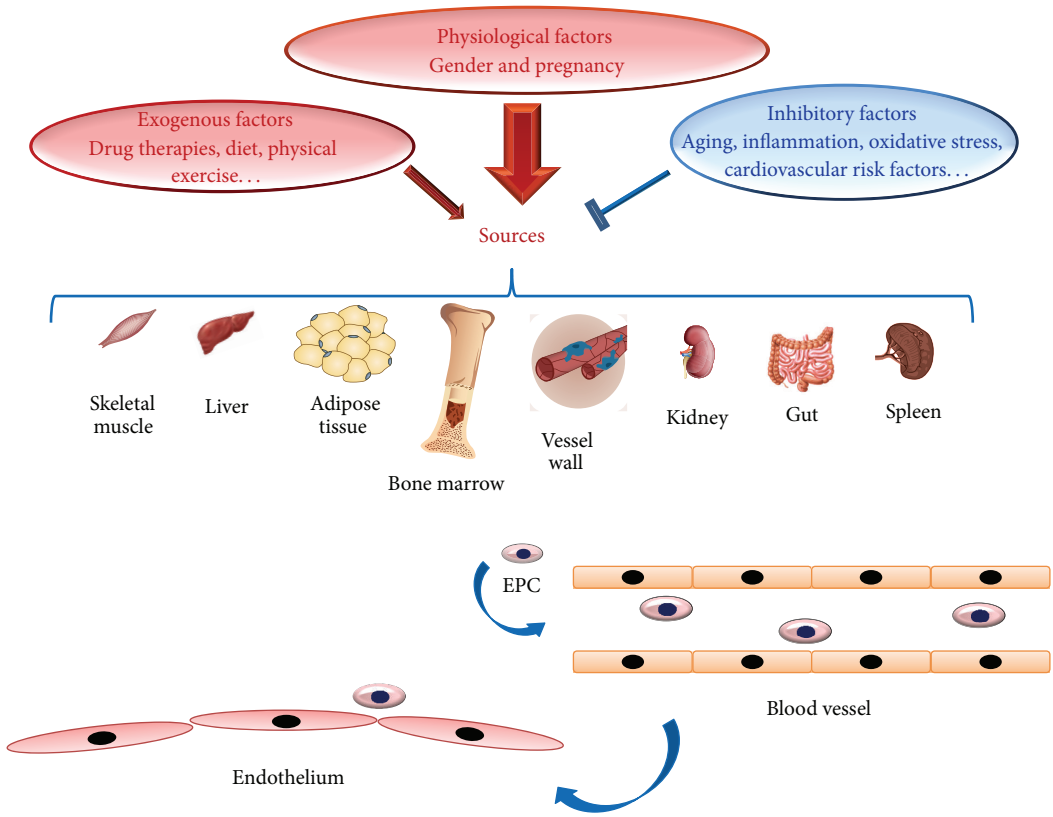

FIGURE 1: The several origins and sources of EPC cells.

as biomarkers of vascular function and cardiovascular risk in healthy individuals, as well as diagnostic and prognostic CVD biomarkers. In addition, several studies report how EPC can be used as therapeutic agents.

We below report data existing in literature about the possible clinical applications of these cells.

4.1. EPCs as Predictive CVD Biomarkers. The relationship between EPC circulating levels and cardiovascular risk might be of clinical relevance, and possible new recommendations and preventive CVD measures might be applied. Accordingly, in 2003, Hill and colleagues showed that the number of circulating EPCs represents a better predictor of vascular reactivity than conventional cardiovascular risk factors [45]. In addition, a significant correlation between *in vitro* EPC senescence and CVD risk profile has been also reported in donors. Thus, EPCs might be considered as an optimal biomarker for vascular function and cardiovascular risk. Certainly, ulterior studies are needed.

4.2. EPCs as Diagnostic and Prognostic CVD Biomarkers. Abnormalities in circulating EPC levels and function have been observed in a large number of studies on different CVDs. As result, EPCs have been suggested as diagnostic and prognostic CVD biomarkers [35, 40]. We report a summary of literature data in Table 1S (see Supplementary Materials available online at http://dx.doi.org/10.1155/2015/835934).

4.3. EPCs as Therapeutic Agents. Since the successful isolation of EPCs in 1997 [12], encouraging data have demonstrated EPC presence in the sites of vascular injury and ischemia. This has led to perform several preclinical studies in animal models (see Table 3). Promising findings have been obtained. In particular, a favorable improvement in left ventricular (LV) function in a rat model of myocardial infarction (MI) after intravenous injection of *ex vivo* expanded human $CD34^+$ cells has been reported [46]. Furthermore, another study examined the effect of catheter-based intramyocardial transplantation in a swine model of MI, providing encouraging outcomes in favoring the application of EPCs as a potential cell therapy in clinical trials [47, 48]. In 2005, Naruse and colleagues carried out a study related to the therapeutic treatment of diabetic neuropathy by *in vivo* expanded human EPCs, using streptozocin-induced diabetic Nude rats [49]. They developed augmented conduction velocity and ameliorated blood flow of sciatic nerve. An increased number of microvessels were also observed on the site of EPC injection [49]. These results led to use this treatment for cerebrovascular disease [50]. An improvement of neurological functions was reported in chronic cerebral ischemic rats injected with $CD34^+$ HSC cells, including EPCs [50] (see Table 3).

The ability of EPCs to expand in cultures under *in vitro* conditions raises another hesitant vision for their therapeutic use. Genetically modified and *ex vivo* expanded

Table 3: EPCs therapeutic applications.

	Preclinical studies in animal models	References
	Therapeutic approaches and effects	
Animal models		
Rat model of myocardial infarction	Intravenous injection of *exvivo* expanded human $CD34^+$ cells improves left ventricular function	[46]
Swine model of myocardial infarction	Catheter-based intramyocardial transplantation of EPCs leads to encouraging outcome	[47, 48]
Rat model of diabetes	Infusion of *invivo* expanded human EPCs augments conduction velocity and blood flow in sciatic nerve	[49]
Rat model of chronic cerebral ischemia	Injection of $CD34^+$ HSC cells (including EPCs) improves neurological functions	[50]
Macacus rhesus	Skin autograft of $CD34^+$ cells transfected with recombinant nonreplicative Herpes virus vector results in vector-gene expression and determines an increase in local angiogenesis	[51]
	Clinical trials in humans	
Pathological CVD conditions		
Myocardial infarction	Endovenous administration of G-CSF as mobilizing factor for BM-derived progenitor cells improves left ventricular function	[52]
	Intracoronary infusion of BM-derived progenitor cells improves left ventricular function (TOPCARE-AMI and BOOST trials)	[53–55]
Diffuse coronary heart disease and angina pectoris	Transepicardial and transendocardial injection of unfractioned BM cells improve left ventricular function and physical capacity	[59–62]
Chronic limb ischemia	Direct administration of EPCs determines a reduced rate of limb's amputation at three-year follow-up	[63, 64]
	Autologous EPCs application	
Pathological CVD conditions		
Chronic limb ischemia	Intramuscular injection of autologous BM-derived mononuclear cells containing 1% of $CD34^+$ cells determines a local increase in endothelial markers (CD133 and VE-cadherin)	[65, 66]
Symptomatic coronary atherosclerosis	Administration of autologous EPCs expanded for four days in culture improves endothelial function and wall motion abnormalities, showing a benefic effect on the metabolism in the target area	[67]
Chronic limb ischemia	Intramuscular injection of autologous BM mononuclear cells improves local neovascularization (TACT study) and significantly lowers amputation rate at 3-year follow-up	[64]

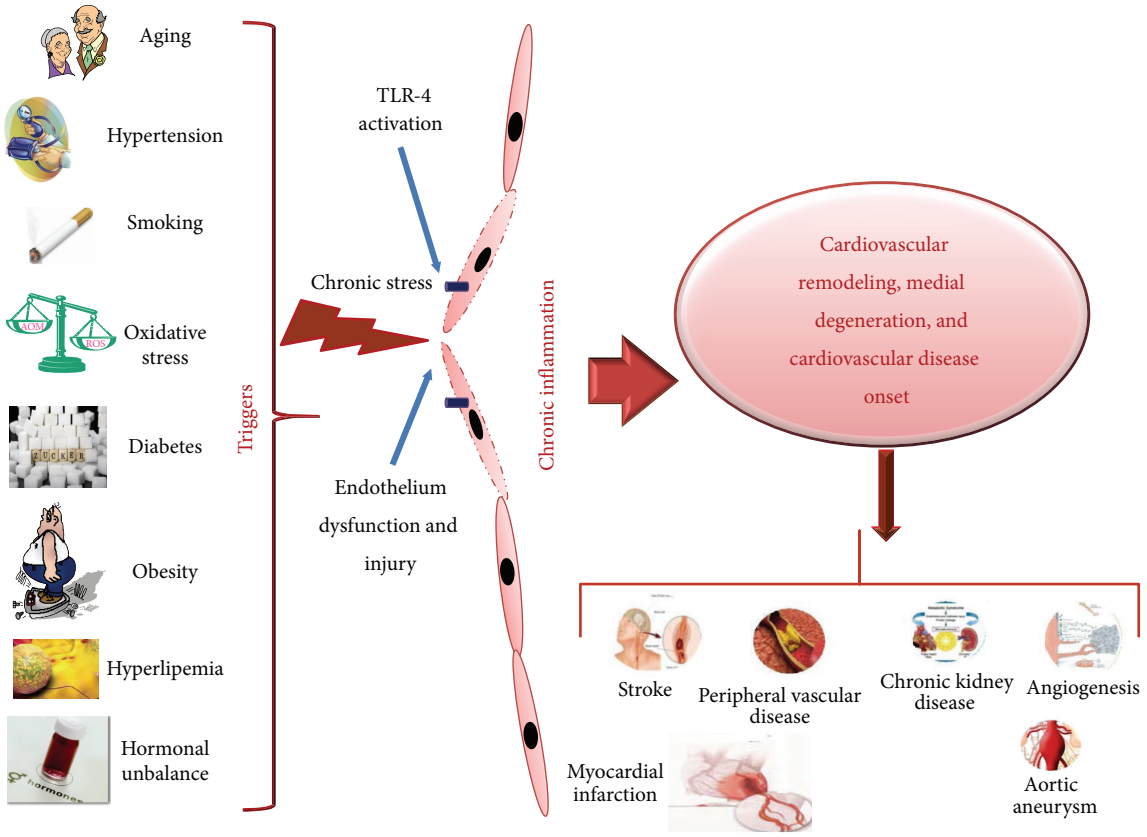

Figure 2: Endothelium dysfunction, injury, cardiovascular remodeling and onset of CVD diseases. Several factors (ageing, hypertension, oxidative stress, diabetes, hyperlipemia, obesity, and unbalance of hormones) by acting as triggers determine a chronic stress on endothelium of vascular wall and evocation of a chronic inflammatory response which cause endothelium dysfunction, injury, and cardiovascular remodeling and the onset of several CVDs.

Table 4: Methods and ways for the administration of EPCs cells.

First strategy: intravenous administration	BM-MSCs are transfused into the left ventricular cavity. Stem cells mainly reach the lungs, with significantly smaller amounts in the liver, heart, and spleen.	[52]
Second strategy: intracoronary infusion	Patients are infused with BM-progenitor cells using a balloon catheter after restoration of arterial patency	[53–55]
Third strategy: Transepicardial administration	Direct transepicardial injection of BMSCs can be performed, using a surgical thoracotomy into the border zone of the infarct	[59]
Third strategy: transendocardial administration	Catheter-based transendocardial injection of SCs using electromechanical voltage mapping to define tissue viability	[59]

EPCs may become new promising agents, which will be able to appropriately rescue impaired neovascularization process under disease conditions. In Rhesus model, *ex vivo* $CD34^+$ cell transfection with recombinant nonreplicative herpes virus vector and subsequent cell transplantation resulted in the expression of vector genes in angiogenic areas of skin autografts of rhesus macaques. Since $CD34^+$ cells possess a natural angiogenic tropism to injured endothelium, they may serve as ideal candidates for the delivery of genes into areas of angiogenesis [51] (see Table 3).

These encouraging data have led to perform clinical trials in order to detect whether EPCs increase endothelial integrity and vascularisation at ischemia sites in patients with CVDs. Three different strategies have been principally used, as reported in Table 4. The first strategy consists in the *administration of granulocyte-colony stimulating factor (G-CSF)* in the order to determine the recruitment of the patient's own BM resident progenitors. Using this treatment, two preliminary studies demonstrated an increased LV function [52]. This certainly requires a confirmation in large studies. The second is *the intracoronary infusion of BM progenitor cells in patients with MI*. It demonstrated positive effects on LV function in three smaller studies [53–55]. Subsequently, two prospective large trials assessed significant LV function after 4–6 months of administration of BM progenitor cells. Other ten recent and large trials confirmed the successfulness and the safety of this procedure with a follow-up over 1.5 years [56, 57]. In addition, the intramyocardial and intracoronary

Table 5: Surface markers used in EPC identifying.

Molecules	Biological features and relevance in EPC detection
CD34	105- to 120-kD transmembrane cell surface glycoprotein, selectively expressed (within human and murine hematopoietic systems) on stem and progenitor cells, and initially used by Asahara and colleagues for EPC identifying. It is not specific and expressed by mature endothelial cells as well as HSCs [76].
VEGFR2	A kinase insert domain receptor (KDR) or Flk-1, or CD309, suggested as further marker for identifying circulating EPC cells. It is expressed mainly on EC cells, and besides EPC cells, in low number, on osteoblasts, pancreatic duct cells, neuronal cells, and lung epithelial cells, even if the biological role in nonendothelial cells remains unclear. VEGFR2 has been shown to be a vital promoter of pathological neovascularization, including cancer and diabetic retinopathy, by making it a potential target in therapy of these diseases. However, neither of these markers is specific for EPCs, either alone or together. Vascular endothelial cells, expressing CD34 and VEGFR2, are not considered to be EPCs [76].
CD133	Also known as AC133. It is a marker of immature stem cells, proposed as the third marker for EPCs. Thus, EPCs have been identified as VEGFR-2^+/$CD133^+$/$CD34^+$ cells. However, more than 99% of $CD34^+$/KDR^+/$CD133^+$ triple positive cells also express CD45, which is a pan leukocyte marker, even if these cells are not able to give rise to EPCs capable of highly differentiating in endothelial cells. As such, CD45 expression on putative EPCs became a bone of contention [76].
CD31	Platelet endothelial cell adhesion molecule-1, also defined as PECAM [76].
CD146	S-endo, P1H12 antigen [76].
VWF	Von Willebrand factor [76].
eNos	Endothelial nitric oxide synthase [76].
E-selectin	Also known as CD62 antigen-like family member E (CD62E). Endothelial-leukocyte adhesion molecule-1 (ELAM-1), or leukocyte-endothelial cell adhesion molecule 2 (LECAM2), is a cell adhesion molecule expressed only on endothelial cells activated by cytokines [76].
C-kit	The protooncogene c-kit is a 145,000 Dalton transmembrane glycoprotein designed as CD117. This receptor tyrosine kinase and its ligand stem cell factor (SDF) mediate pleiotropic functions, including cell survival, differentiation, homing, migration, and proliferation as well as functional activation. It is present on the surface of cells of the mast cell and erythroid lineage as well as on multipotent stem and progenitor cells and megakaryocytes [76].
CXCR4	Also known as fusion or leukocyte-derived seven transmembrane-domain receptor (LESTR). It represents the receptor of SDF-1, highly expressed on the surface of CD34 positive cells [76].
UEA-I	Ulex europaeus lectin [76].

administration has been recently suggested as a suitable strategy for treatment of patients with refractory angina [58]. The third strategy is more invasive and consists in *the direct injection of cells into target tissues* [59]. This treatment (and precisely *transepicardial or transendocardial injection of unfractioned BM cells*) has been performed in patients with diffuse coronary artery disease and intractable angina with no option of recanalisation. Ventricular function and physical capacity have been observed to increase, but the small sample size of these studies requires to be confirmed in larger studies [60–62] (see Table 3).

The treatment with *direct administration of EPCs* has been also effectuated in patients with chronic limb ischemia, demonstrating a reduced rate of limb's amputation at 3 years of follow-up [63, 64] (see Table 3).

Of special interest are the studies with *autologous cell therapy*. In line with this, the Yamamoto group performed an intramuscular injection of autologous BM-derived mononuclear cells containing 1% of $CD34^+$ cells in patients with chronic limb ischemia [65]. They quantitatively evaluated the expression of EPCs and endothelial markers (i.e., CD133 and VE-cadherin) before the experiment and after the injection. Before investigation, the transcription of these molecules was undetectable. Autologous injection caused an elevation of EPC marker transcription. Thus, they concluded that autologous BM cells may be used in the therapy of patients with arterial diseases. A replication of these results was obtained by Lenk and colleagues [66]. Erbs and colleagues used this autologous treatment in patients who underwent recanalisation of chronic coronary total occlusion [67]. The autologous treatment with EPCs, expanded four days in endothelium growth medium, improved coronary endothelium function and wall motion abnormalities and had a benefit effect on the metabolism in the target area in patients with symptomatic coronary atherosclerosis [67] (see Table 3).

Despite of these promising data, EPC clinical application as exogenous or autologous cell therapy remains still unclear because of different reasons. We below discuss the limitations on EPC clinical applications.

5. Focus on Controversies and Perspectives about the EPC Clinical Use as Possible Solutions for Vascular Impairment and CVDs

Since their discovery, EPCs have been object of an intensive investigation and a plethora of clinical applications has been opened, as reported above. As result, EPCs have been suggested as potential predictive, diagnostic, and prognostic CVD biomarkers, as well as therapeutic agents. These efforts have encouraged the researchers in the vision to modulate the vasculogenesis process and consequently potentiate cardiovascular self-repair. However, the enthusiasm is actually

dampened by a large number of critical viewpoints [68–72]. In particular, insights into EPC biology are leading several research groups to discuss on critical EPC aspects and to evidence the limitations. Thus, these perspectives reduce the large relevance and potentiality of these cells and cotemporally underline urgent necessity to move versus standardized and common criteria of research for EPC cells. This might reduce the heterogeneity of EPC literature data.

Here, we summarize the aspects of EPC cells principally discussed by scientific community (see Figure 3).

5.1. Real Capacity of EPCs Cells to Improve In Vivo Neovascularization. In healthy adults, EPC cells (as $CD34^+CD133^+VEGFR2^+$ EPC cells) represent only 0.0001%–0.01% of peripheral blood mononuclear cells (PBMCs) [73]. These low percentages lead to question on their impact in pathological or physiological processes. Current evidence reports changes in EPC number and function in several CVDs (see Table 1S). However, different factors may influence levels and viability of EPC cells, including methodological approaches (i.e., the timing and ways of taking samples) [74], detection methods and their protocols, panel of antibodies used for their phenotypical evaluation, age of patients and their clinical conditions, and ethnicity of populations studied (see below).

5.2. EPCs as Vascular Healthy and CVD Biomarkers. It is current opinion that number and/or functionality of EPCs do not adequately describe CVD risk. This perplexity is due to inconsistent EPC definitions, different number of CVD risk factors in different patient populations studied, and the interaction of EPCs cells with other HPCs, inflammatory cells, and platelets.

5.3. EPCs as Therapeutic Agents. Available clinical studies of EPCs as therapeutic agents show beneficial results (as described above). However, their validity is limited by different factors: (a) the small number of patients enrolled in the major number of studies, their randomization not blinded, the involvement of few centres, (b) the exact phenotypic profile of cells used for the treatments which is always not indicated or missing, (c) the different administration ways and methods used, and (d) the safety and feasibility of the treatments not proved by long-term follow-up results. Teratoma formation, immunoreactivity, or arrhythmias may represent the adverse effects of these treatments. In addition, there are other limitations in the large-scale clinical use of EPCs. As the above mentioned, EPCs are relatively rare cells, and expansion of sufficient numbers of subpopulations from peripheral blood is hardly possible. Furthermore, *in vitro* enumeration of progenitor cells for a quantity sufficient for a therapeutic treatment is associated with changes in phenotype and differentiation and risk of cell senescence and it may require artificial cell preactivation or stimulation. The *in vitro* cultures consent the production of two subpopulations from $CD133^+/CD34^+/CD309^+$ BM-hemangioblasts according to Hristov and Weber's schema [75], the early EPCs (eEPCs) and late EPCs (outgrowth endothelial cells, OECs), having different features (see Figure 4).

5.4. Lack of Standardized Criteria and Consensus for Defining, Characterizing, and Identifying EPCs with Well Established Surface Markers, Protocols, and Methods. EPCs cells have been largely described as $CD34^+CD133^+VEGFR2^+$ cells [69]. However, other progenitor populations have been recently considered in EPC studies, that is, circulating angiogenic cells (CACs), circulating endothelial cells (CECs), circulating H-progenitor cells (CPCs), and circulating endothelial progenitors (CEPs), playing important roles in tissue neovascularization, but having diverse features [76]. CAC and CEP cells represent variable proportions of $CD14^+$ monocyte cells having different angiogenic properties. Despite their lower *in vitro* proliferation than HSCs or cord stem cells, they seem to have a similar ability to increase neovascularization, as reported in experimental models [76]. This leads to suppose that EPCs might be essentially H-monocyte-derived $CD14^+$ cells with variable expression of CD34, CD133, CD45, and KDR and angiogenesis capacity, as evidenced by Sieveking and colleagues [77]. Given the heterogeneous presence of EPC subpopulations in peripheral blood and absence of standardized criteria, we suggest considering the EPCs as "*putative cells.*" Their identification might be performed with a combination of several surface antigens. Other markers have been, indeed, detected, including platelet endothelial cell adhesion molecule-1 (CD31), CD146, von Willebrand factor (vWF), eNos, and E-selectin, C-kit, and CXCR4 (see Table 5) [13, 76]. Concerning methods for isolating and quantitatively or qualitatively evaluating these putative EPC cells, a large number of methodologies are disposable until now. However, immunohistochemistry or immunocytochemistry is principally used for quantifying EPCs in tissue samples [7, 76]. For circulating EPC evaluation, four different methods are available after their isolation from PBMCs: (1) cell culture of colony forming cells to reveal EPC features, that is, high proliferative potential, expression of endothelial markers, endothelial morphology, and formation of blood vessels in coculture experiments [7, 76]; (2) phenotypic EPC identification and enumeration by flow-cytometry analysis according to Duda protocol published in 2007 [78]; (3) quantitative real time PCR, which permits detecting and quantifying EPC specific markers in preenriched PBMC cell population [7, 76]; and (4) MCA method which includes magnetic (M) isolation of $CD34^+$ cells from PBMCs, followed by a $CD133^+$ immunocytochemical (CA) staining [7, 76]. To date, flow-cytometry and CFU assays are the two most used methods for EPC enumeration.

6. Conclusions and Recommendations: Standardized Criteria on EPC Investigations Are Imperative

The observations described above about the critical aspects on EPC cells point out the following considerations: (1) results of earlier studies on EPCs have to be reexamined; (2) the impact of these subpopulations has to be evaluated and considered only when future studies will be performed; (3) precise biological role or roles of several EPCs have to be clarified before their clinical application as both biomarkers

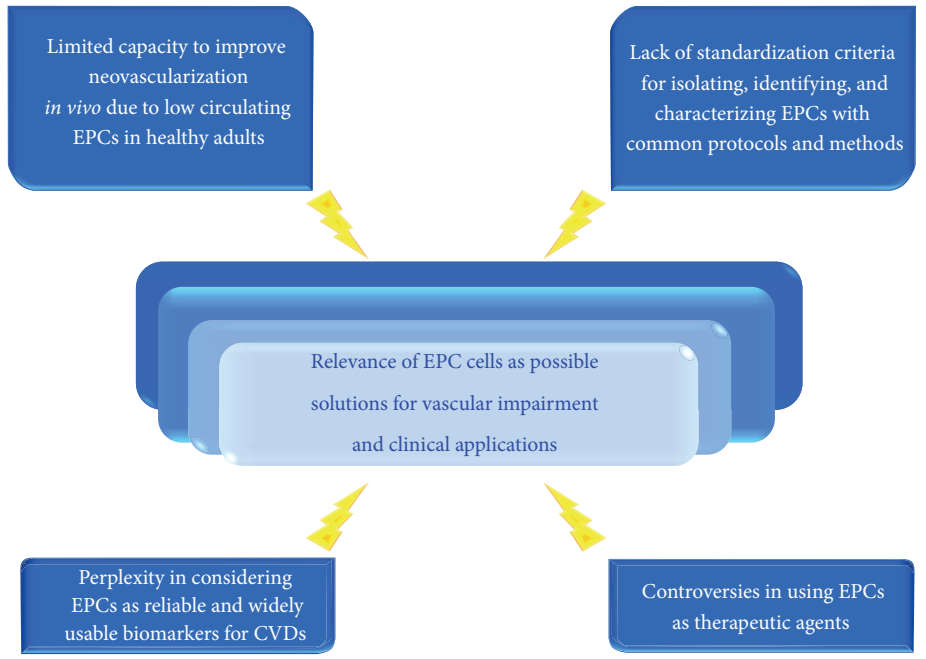

Figure 3: Critical aspects of the EPC relevance as possible solutions for vascular impairment and clinical applications. As reported in the figure and text (see Section 5), four critical aspects reduce the EPC potentiality as potential actors of endothelium repair, optimal CVD biomarkers, and therapy agents.

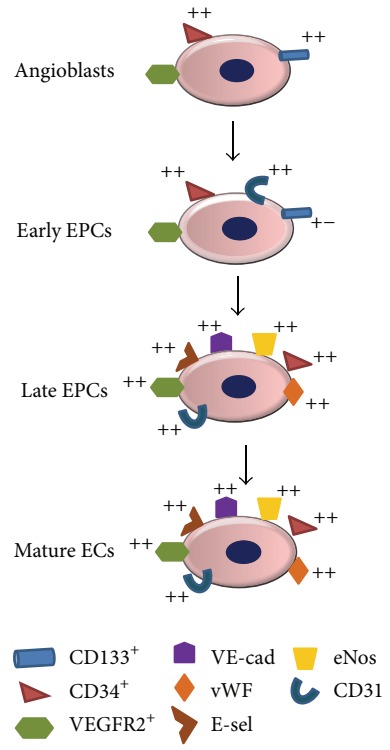

Figure 4: Angioblast differentiation into mature endothelial cells according to the schema proposed by Hristov and Weber, 2004 [75]. As illustrated in the figure, $CD133^+$, $CD34^+$, and $VEGFR2^+$ ($CD309^+$) angioblasts give rise to early EPCs expressing high intensity CD31, CD34, and CD309 markers which differentiate in late outgrowth endothelial cells (OEC)s, having high expression not only of CD34, CD309, and CD31 but also of vWF, E-selectin, VE-cadherin, and eNOs.

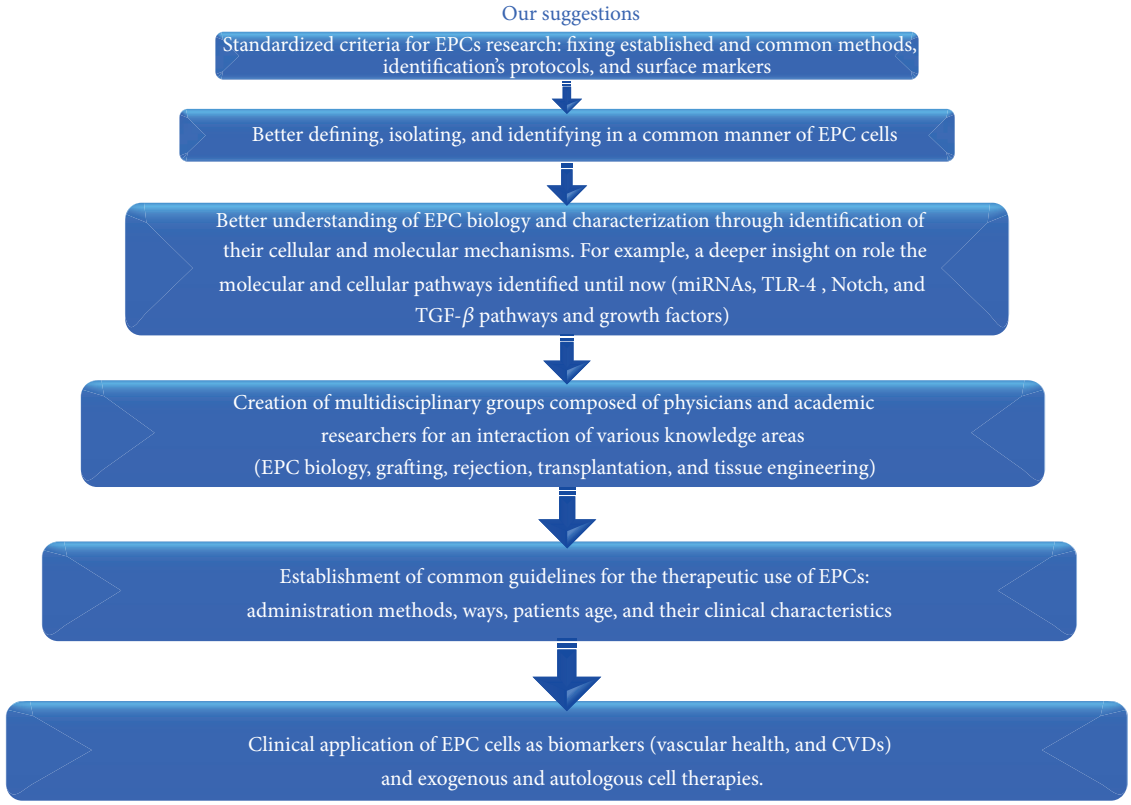

Figure 5: Our working hypothesis on the possible steps to perform to overcome the critical limitations and problems of EPC research and to develop real therapeutic applications.

to test cardiovascular health or candidates for cardiovascular cell therapy; (4) EPC definition using surface markers has to be reevaluated considering their heterogeneous origin and nature and probably performing not only flow-cytometry analysis but preferably a combination of other biomolecular assays.

In line with these considerations, recent advances in molecular EPC mechanisms highlight involvement of different growth factors and signaling pathways (i.e., VEGF, TGF-β, ROS, Wnt, Notch, and TLR-4 pathways) and microRNA in EPCs mobilization and differentiation into mature ECs [79, 80]. Future and intensive studies on the role of these molecules in EPC biology will be needed for improving or inducing vascular neoformation and angiogenesis in different CVD conditions. The common hope is in early overcoming various EPC problems and developing their real clinical applications, as biomarkers and regenerative cell agents. This might likely permit inducing and improving vascular regeneration under ischemic or other CVD events or provide a good substrate for vascular grafting, that is, bypass surgery and vascular reconstruction following aneurisms or traumatic injuries.

In order to achieve this gold purpose, we suggest the following working hypothesis, as reported in Figure 5. Firstly, we underline that it is imperative to move versus a deep EPC characterization and precise definition, by performing future and further studies and establishing standardized criteria for EPC identification protocol and methods. This might really consent EPC defining and specifying functions. Probably, a combined and standardized analysis based on cytometric, transcriptomic, proteomic, and metabolomic evaluations might preferentially be needed for a definitive and true characterization of these cells, fixing standardized criteria. In addition, the development of an ideal EPC therapy and its clinical applications as CVD biomarkers might require the creation of interdisciplinary teams for fixing precise clinical elements of design and standardization. They might derive an intersection of investigations on EPC biology, tissue engineering, transplantation, grafting, rejection biology, clinical cardiovascular medicine, and device technology.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Dr Carmela R. Balistreri was involved in conception and design of the paper. Dr Calogera Pisano researched PubMed evidence on the role of EPC cells in CVDs and data were reported on Table 1S (see online Supplementary Materials). Dr Silvio Buffa performed a research on literature data (on PubMed) about methods used for evaluating EPC cells (see Section 5). Dr Carmela R. Balistreri was involved in drafting the paper, its critical revision, and supervision. Dr Carmela

R. Balistreri gave the final approval of the version to be published. All authors read and approved the final paper.

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Research Article

Biological Niches within Human Calcified Aortic Valves: Towards Understanding of the Pathological Biomineralization Process

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Despite recent advances, mineralization site, its microarchitecture, and composition in calcific heart valve remain poorly understood. A multiscale investigation, using scanning electron microscopy (SEM), transmission electron microscopy (TEM), and energy dispersive X-ray spectrometry (EDS), from micrometre up to nanometre, was conducted on human severely calcified aortic and mitral valves, to provide new insights into calcification process. Our aim was to evaluate the spatial relationship existing between bioapatite crystals, their local growing microenvironment, and the presence of a hierarchical architecture. Here we detected the presence of bioapatite crystals in two different mineralization sites that suggest the action of two different growth processes: a pathological crystallization process that occurs in biological niches and is ascribed to a purely physicochemical process and a matrix-mediated mineralized process in which the extracellular matrix acts as the template for a site-directed nanocrystals nucleation. Different shapes of bioapatite crystallization were observed at micrometer scale in each microenvironment but at the nanoscale level crystals appear to be made up by the same subunits.

1. Introduction

Calcific aortic valve stenosis (CAVS) is an important public health problem and represents the most common form of valvular heart disease in the industrialized countries [1]. It is strictly associated with the formation of ectopic calcifications within aortic valve leaflets that interfere with cusps opening and lead to ventricular outflow obstruction [2] causing important clinical consequences in terms of mortality and morbidity [3]. To date there is no proven medical therapy to halt CAVS course progression, and surgical or percutaneous valve replacement is the only possible treatment of severe CAVS. The degenerative valve calcification process, however, not only is limited to native heart valves but also affects bioprosthetic implants [4]. Despite much effort devoted to unveil the molecular mechanisms leading to valve calcification, comprehension of the exact process remains uncertain.

Chemically, the calcific deposit within human valve tissue is constituted by a nonstoichiometric apatite, containing high carbonate (CO_3^{2-}) content, from 5% to 10% in weight, and AB-type substitutions in apatite lattice [5, 6], as we previously reported; it is often indicated as "carbonate apatite" or more

in general "bioapatite" even if both names are not accepted by the International Mineralogical Association (IMA) Commission on New Minerals, Nomenclature, and Classification (CNMNC) [7]. In this paper, the term "bioapatite" will be used to define the calcium phosphate phase growing in a biological system and forming the ectopic calcification within human heart valve tissue. From a strictly mineralogical point of view such ectopic calcification represents the final product of complex biomineralization processes [8] mediated by biological and physicochemical parameters, as well as the normal mineralized tissues (enamel, dentine, and bone), and falls in the field of calcium phosphate biominerals [9]. Crystal-chemistry features of bioapatite crystals are strongly linked with apatite crystallographic structure [10] and are strictly dependent on the characteristics of the medium in which they form, such as pH, temperature, supersaturation degree, and solution composition [11–13]. More generally a specific microenvironment is required to let biominerals crystallize [8]. This must be a localized zone able to achieve and maintain a sufficient supersaturation degree for crystals nucleation and growth. Therefore the concept of local microenvironment or native biological niche plays an important role in the formation process of biominerals and appears to be the fundamental requirement leading to mineral deposition.

The aim of this paper was to investigate the formation of hierarchical architectures and to determine the spatial relationship existing between crystals and their growth environment. Different experimental techniques, namely, polarized light microscopy, electron microprobe, scanning and transmission electron microscopy, energy dispersive spectrometry, powder X-ray diffraction, Fourier transform infrared spectroscopy, and Raman spectroscopy, were applied on the aortic valve samples to study the calcification process in all its components [5, 6]. Here we discuss the scanning and transmission electron microscopy findings in severely calcified human aortic valves. As biomineral phases are soft materials, subjected to deterioration, amorphization processes, and artifacts formation [14], different preparation methods were used for electron microscopy analyses to monitor the reliability of the experimental results.

2. Materials and Methods

2.1. Study Subjects. Severely calcified aortic (tricuspid type, $n = 29$; bicuspid type, $n = 3$) and mitral valves ($n = 4$) were obtained from European patients of both sexes (males = 25) and different ages (mean age 72 ± 10, range 41–90 years old). Samples were collected as surgical waste from patients undergoing valve replacement due to severe aortic and mitral valve stenosis without any sign of endocarditis or inflammation. Surgical interventions were performed at the Division of Cardiac Surgery, San Bortolo Hospital, Vicenza, Italy. The institutional committee approved the study and the patients gave written informed consent. Patients' characteristics and clinical data are presented in Table 1; peak and mean gradient were obtained by echocardiographic evaluation [15, 16]. Immediately after surgical excision, part of the heart valve leaflets underwent sample treatment 1; the rest underwent sample treatment 2 to evaluate the same specimen under different methodologies and to reduce hypothetic artifacts due to a single treatment.

2.2. Scanning Electron Microscopy (SEM) and Energy Dispersive X-Ray Spectrometry (EDS) Analyses: Sample Treatment 1. Immediately after surgical excision, heart valves were preserved and dehydrated in absolute alcohol. To sterilize the biological material, valve samples were exposed to UV radiation for almost 72 hours at room temperature, which has been proved not to induce any further calcification. A dual beam Zeiss Auriga 405 HR-FESEM with resolution of 1 nm equipped with a Bruker QUANTAX energy dispersive system was used. Investigations were conducted on both uncoated and coated samples. The latter were chromium-coated (5 ÷ 10 nm in thickness) using a Q150T ES turbomolecular-pumped sputtering coater. Low accelerating voltage (<15 kV) was used to obtain information about biomineral/organic structure interface.

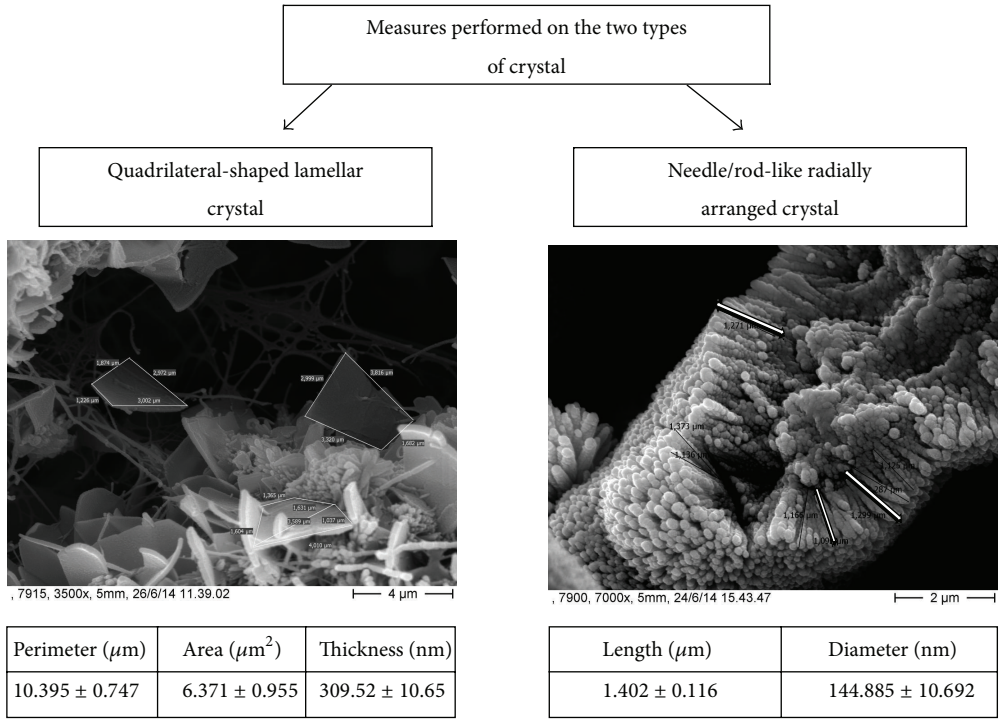
2.3. Scanning Electron Microscopy (SEM) and Energy Dispersive X-Ray Spectrometry (EDS) Analyses: Sample Treatment 2. Immediately after surgical excision, heart valves were fixed in glutaraldehyde 2,5% in PBS 0.1 M pH 7,4 immediately after recovery and stored at 4°C for at least 4 days. The parts of the valves in which calcifications had larger dimensions were excised and then postfixed in osmium tetroxide 1% in PBS for 2 hours. Samples were then washed in PBS 0.1 M pH 7,4 and then dehydrated in ascending series of alcohol solutions (30-50-70-95-100%); after this treatment samples were dried in an Emitech K850, "critical point drying" apparatus (Emitech Ltd., Ashford, Kent, England). The dried samples were mounted onto aluminium stubs and then sputter-coated with platinum (~45 nm in thickness) using an Emitech K 550 sputter coater (Emitech Ltd., Ashford, Kent, England). Samples were observed with two different electron microscopes: a Hitachi S400 field emission scanning electron microscope (Hitachi Ltd., Japan) operating at 10 kV and interfaced with a DISS 5 point electronic imaging and analysis system and a ZEISS scanning electron microscope equipped with energy dispersive X-ray spectrometer (SEM-EDS) Zeiss DSM 940A, LEO Elektronenmikroskopie GMbH, Oberkochen, Germany. On needle/rod-like radially arranged crystals, length and diameter were measured. Quadrilateral-shaped lamellar crystal thickness and surface area were measured. Surface area was measured on crystals frontally visible; thickness was measured on crystal laterally placed. Surface area was calculated according to Brahmagupta formula, $s = \sqrt{(p-a)\times(p-b)\times(p-c)\times(p-d)}$, where p is the half perimeter and a, b, c, and d are the values in micrometers of the four quadrilateral sides (Figure 1). Measures were performed by the digital image processing system DIPS 5 (point electronic GmbH).

2.4. Transmission Electron Microscopy (TEM) and EDS Analyses. A JEOL JEM 2010 TEM operating at 200 KV with LaB_6 source, nominal point resolution of 1.9 Å, and spherical aberration of 0.5 mm was used. An Oxford LINK energy dispersive X-ray spectrometer with a Si (Li) detector and ultra-thin window was used for qualitative chemical analyses. EDS spectra were collected using an acquisition time of

Table 1: Patients characteristics.

Characteristic	Overall $N = 36$	Tricuspid aortic valve $N = 29$	Bicuspid aortic valve $N = 3$	Mitral valve $N = 4$
Age, y	72.4 ± 10	74.5 ± 7.8	55 ± 15	69 ± 9
Males, *n*	25 (69.4%)	20 (69%)	3 (100%)	2 (50%)
BMI, Kg/cm^2	25.1 ± 5.5	25.5 ± 6	22.2 ± 3.7	22.4 ± 2
BSA, m^2	1.8 ± 0.2	1.8 ± 0.2	1.7 ± 0.1	1.7 ± 0.2
Peak gradient, mmHg	71.2 ± 20.5	70.5 ± 23	88.4 ± 18	9 ± 3.5
Mean gradient, mmHg	55.8 ± 16.2	53.4 ± 18.3	58.8 ± 14	6 ± 3.4
Associated CAD	13 (36%)	13 (44.8%)	0 (0%)	0 (0%)
Dialysis	0 (0%)	0 (%)	0 (0%)	0 (0%)
Osteoporosis	22 (61%)	21 (72%)	0 (0%)	1 (25%)

BMI: body mass index; BSA: body surface area; CAD: coronary artery disease.



Perimeter (μm)	Area (μm^2)	Thickness (nm)
10.395 ± 0.747	6.371 ± 0.955	309.52 ± 10.65

Length (μm)	Diameter (nm)
1.402 ± 0.116	144.885 ± 10.692

Figure 1: Scanning electron microscope (SEM) analysis on needle/rod-like and quadrangular-shaped crystals and measured considering each type of crystals.

60 s. A single tilt specimen holder was used, and images and diffraction patterns were recorded on Kodak film. The samples were manually grinded in an agate mortar and pestle to a very fine powder. The powdered samples were then subject to an enzymatic attack, with trypsin, in basic pH conditions. After the treatment the powders were exposed to UV radiation for 32–96 hours and then sifted using sieves smaller than 50 μm. This specific treatment allowed us to work with samples with a strongly reduced quantity of collagen. The powder was ultrasonically dispersed in ethanol and subsequently small drops of the slurry were deposited onto a 3 mm diameter Ni-Cu grid coated by a Holey carbon film. The Anticontamination Device (ACD) was used to prevent the contamination of the column due to decomposition of the residual collagen and to minimize the effect of irradiation.

Bright field (BF) images were acquired to define the morphology of the pathological crystals. Selected Area Electron Diffraction (SAED) patterns of large areas, nano-beam electron diffraction (NBD) patterns, and lattice fringes were acquired to determine bioapatite crystal structure. Experimental diffraction patterns after indexing were compared with simulated electron diffraction patterns starting from known crystallographic structures. Simulations were performed with the Java electron microscopy software (JEMS) package (Stadelmann 1999–2008).

2.5. Statistical Analysis. Continuous variables are reported as mean ± standard deviation and categorical variables as n (%). Computations were performed with SPSS 19 (IBM, Armonk, NY, USA). Comparative analyses could not be performed due to the differences in sample size among groups.

3. Results

The electron microscopy images, applied either at the biological specimen or at the inorganic phase, can give clues about the process of growth of pathological bioapatite crystals, from the micrometre up to the nanometre scale, from ex vivo calcified heart valves. The mineralogical features of bioapatite crystals, their spatial relationship with the extracellular matrix, and the formation of hierarchical structures, were determined by the analysis of electron microscopy images as well.

3.1. Scanning Electron Microscopy. Starting from a micrometer-scale and using SEM for biological specimen techniques [14, 17] we highlighted the presence of biological niches within the calcified extracellular matrix, very similar to vugs, small, unfilled cavities inside rock that may be formed through a variety of processes. Within the niches we observed bioapatite deposits in different crystallization shapes (Figure 2). Crystals appear as semispherical deposits covering the pocket walls (Figure 2(a)), as well as lamellar crystals (Figure 2(b)) and spherical particles (Figure 2(c)). The normal architecture of the extracellular matrix fibers is also altered, as shown in Figure 3; fibers are arranged in a loose network or disorganized bundles that can acquire different shapes.

Images at higher magnification (Figure 4) showed that semispherical deposits, lamellar crystals, and spherical particles are made up by submicrometer particles such as granular units. Therefore the different crystal shapes observed within the niches appear to be due to different spatial arrangement of submicrometer particles.

Needle/rod-like radially arranged crystals showed a mean length of 1.402 ± 0.116 μm, with a diameter of 145±10.692 nm, while quadrangular-shaped lamellar crystals showed a perimeter of 10.395 ± 0.747 μm, an area of 6.371 ± 0.955 μm^2, and a thickness of 309.52±10.65 nm (see Figure 1).

3.2. High Resolution Field Emission Scanning Electron Microscopy. Through investigations at nanometer-scale carried out by HR-FESEM, we observed the presence of needle/rod-like bioapatite nanocrystals belonging to different sets of crystals. We observed crystals in the range of 300–680 nm in length and 100 nm in width and smaller ones in the range of 170–200 nm in length and 25–40 nm in width; the first ones appear to be randomly orientated and always localized in small cavities within the organic tissue (Figure 5); despite the similar morphology of these crystals, some differences in shape and size between crystals belonging to different calcified deposits were detected but also within the same mineral deposit have been observed. Otherwise the smaller bioapatite crystals appear to be directly formed onto the organic interface and appear to be oriented with respect to the matrix, indicating a strong interaction between nanocrystals and the organic substrate (Figure 5(c)). High-magnification images also showed the presence of flower-like aggregates of about 300–400 nm in diameter constituted by radiating nanocrystals. At lower magnification degree these aggregate appear as micrometric spheres.

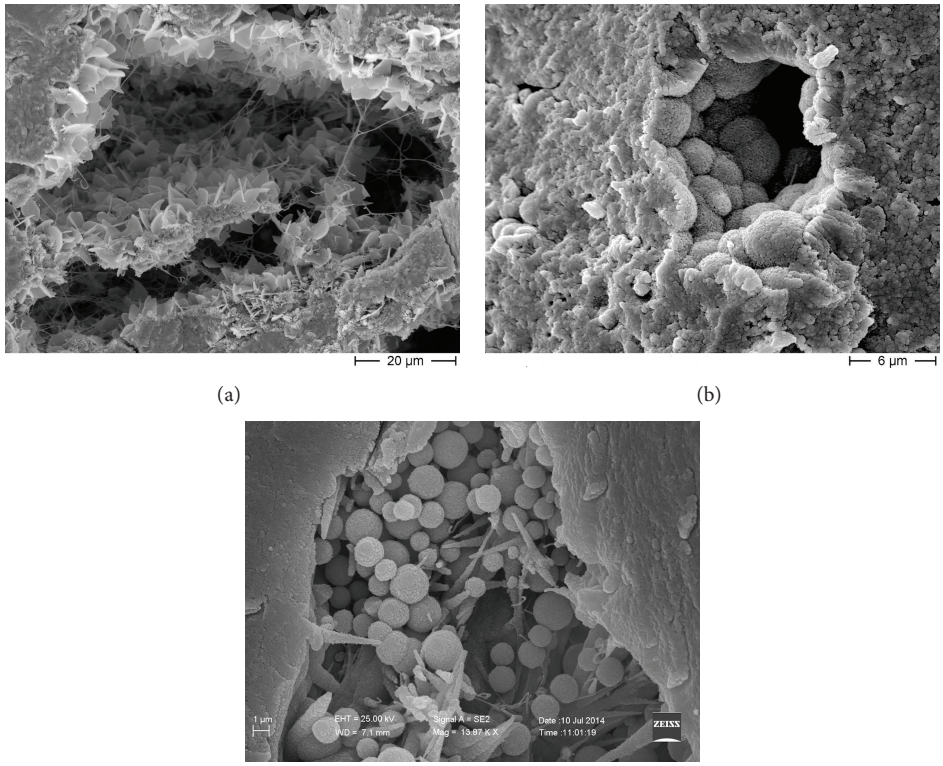
3.3. Transmission Electron Microscopy. For a complete characterization, pathological crystals were investigated using TEM images that confirmed the needle/rod-like morphology and the wide range of crystallite size of bioapatite (Figure 6(a)). EDS analyses defined the chemical composition of bioapatite (Figure 6(b)). TEM analyses also confirmed the crystalline character of the pathological phase investigated and allowed us to ascribe it to bioapatite, the hexagonal crystal structure similar to hydroxylapatite (Figure 7).

4. Discussion

From our experimental results we hypothesize that the different bioapatite crystallization shapes observed at micrometer- and nanometer-scale are strictly linked to the physicochemical parameters of their native growth niche and to the local condition of the extracellular matrix that represents the framework in which pathological crystals take place. Recent studies have highlighted the essential role of microenvironments in biological systems, indicating the extracellular matrix (ECM) as a local and dynamic niche able to promote the formation of pathological microenvironments [18, 19].

We hypothesize that the spatial organization of collagen fibrils can assume an important role for the delineation of the native growth niches. Studies on bioprosthetic heart valve [20, 21] indicate that calcific deposits are often located within the leaflet tissue, in particular in areas of leaflet higher stress. In these areas it is possible to observe tissue deterioration, distortion in extracellular matrix structure, and small voids as final stage of progressive mechanical tissue damage. These small voids can represent biological niches within the organic matrix, in which subsequently the formation of high-concentrated extracellular fluids takes place, leading to the mineral precipitation and to the activation of the pathological process. Crystals can grow with a regular shape only in a void. In literature, different proposed theories, nonmutually exclusive for vascular calcification, are proposed [22] as loss of inhibition, induction of bone formation, circulating nucleational complexes released from actively remodeling bone, and cell death leading to release of apoptotic bodies and/or necrotic debris.

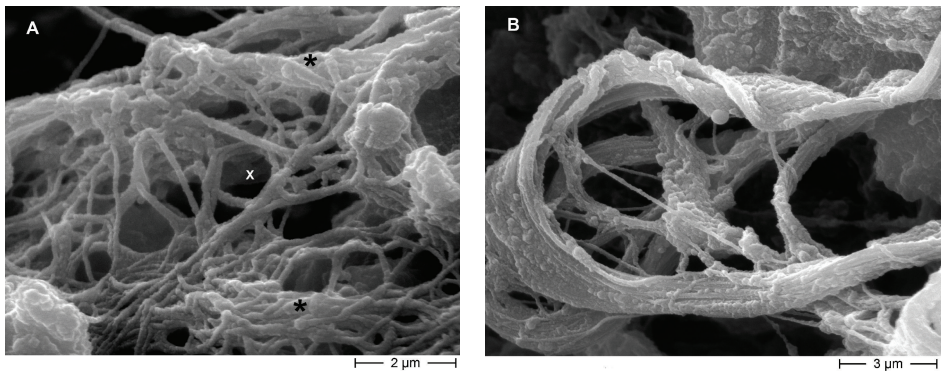
The differences in shape and size of needle/rod-like nanocrystals observed within the same sample and among different samples might be due to specific conditions of the mineralization niche such as the different degree of supersaturation, the different nucleation frequency, the physicochemical parameters of the starting solution, and the stage of the calcification process. Probably patients' clinical history influences these conditions, but its impact is far from being understood. Taken together, these observations highlight the important role of a purely physicochemical process in the formation of pathological bioapatite crystals within the niches.



(a) (b)

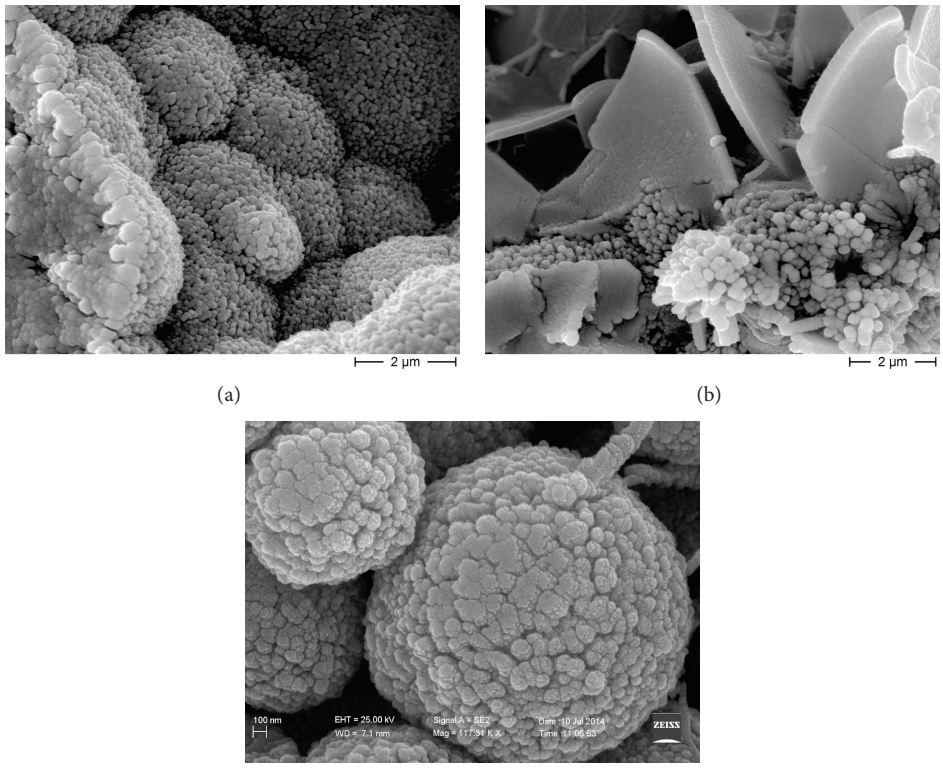
(c)

Figure 2: Scanning electron microscopy (SEM) micrographs of niches within human heart valve tissue. (a) Starting from a micrometer scale, within the niches we observed bioapatite deposits in different crystallization shapes. Calcifications appear as lamellar crystals covering the pocket walls. (b) Semispherical deposits containing Ca and P (see EDS spectrum in Figure 6(b)) localized on the walls of the micro-cavity (niches) observed within the organic matrix where Ca and P are below detection limit. Images at higher magnification showed that spherical and semispherical deposits together with lamellar crystals were in turn formed by submicrometer globular structures. (c) Local pocket filled by spherical particles variable in size. Therefore, different crystal shapes observed within the niches appear to be due to different spatial arrangement of submicrometer particles. Scale bars are placed in different ways in the pictures because images were obtained by two different scanning electron microscopes: (a) and (b) are from Hitachi S4000 and (c) is from Zeiss DSM 940.



(a) (b)

Figure 3: Scanning electron microscopy (SEM) micrographs of extracellular matrix fibers altered architecture. (a) Extracellular matrix fibers are arranged in a loose network (∗) or in compact but disorganized bundles (×). Filaments are thicker than normal and show a rough appearance due to the presence of mineral deposits. This is evidence of alteration in normal extracellular matrix deposition process. The three-dimensional mesh creates a microenvironment in which extracellular fluid stagnates, and physicochemical processes of calcium deposition may have taken place. (b) In the foreground, a horseshoe-shaped collagen bundle is visible. In the background wavy, twisted, and bent collagen bundles are present. The space among bundles is crossed by single collagen fibers. This unusual arrangement indicates modification in normal extracellular matrix deposition, and consequently the existence of areas in valve tissue with different stress resistance. Filaments are also thicker due to mineral deposit presence.



(a) (b) (c)

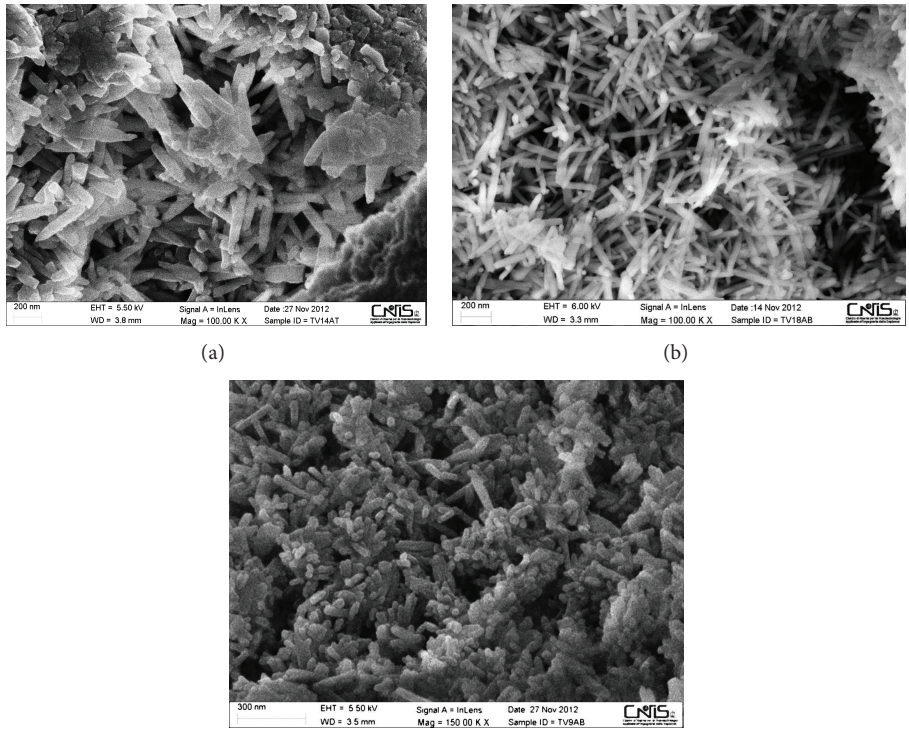
Figure 4: Scanning electron microscopy (SEM) micrographs of submicrometer granular units forming different crystalline morphologies. (a) Higher magnification of semispherical deposits shown in Figure 2(b). Each semispherical deposit is formed by rod-shaped structures with a radial arrangement. Each rod-shaped structure is formed by submicrometer granular units, stacked on each other. (b) Lamellar crystals formed by submicrometer granular units. (c) Higher magnification of micrometric spherical particles shown in Figure 2(c). Smaller units are visible. Scale bar is 100 nm. Scale bars are placed in different ways because images were obtained by two different scanning electron microscopes: (a) and (b) are from Hitachi S4000 and (c) is from Zeiss DSM 940.

Indeed these crystals show the typical features of hydroxylapatite crystals precipitated in aqueous solutions [23–26], and their tendency to form radiated aggregates can be the result of a surface energy minimization [27]. The further growth of those elements may follow the Ostwald ripening, with an additional surface energy minimization. However the presence of spherically assembled structures could also be controlled by the local concentration and 3D spatial organization of the fibrillar collagen [28]. Crystals similar to those found in our ex vivo samples were observed in vitro by Tavafoghi Jahromi et al. [29] and Leopold [3]. In particular the similarity of crystals with those observed by Kumon et al. [30] indicates a possible involvement of oxidized lipids in the formation of ectopic calcification within heart valve tissues.

In the matrix-mediated microenvironment, nanocrystals were observed spread through the thickness of the valve tissue and orientated in various ways with respect to the substrate. This can suggest an active role of the ECM in inducing bioapatite nucleation due to its physical properties (rigidity, porosity, insolubility, spatial arrangement, and orientation). The formation of this set of crystals might be also related to a heterogeneous nucleation, probably due to a surface-mineralization process. This mechanism might be mediated by the presence of negatively charged functional groups of the amino acids constituting the proteins of the extracellular matrix [31–33].

The presence of submicrometer units assembled into larger units to form microstructures is typical of biomineralization processes. Biomineralization can develop with a solid phase forming from a solution and then proceeds with the formation of hierarchical structures whose dimensions vary from Ångstroms to millimetres [34–36]. The smallest units aggregate into larger-scaled once producing structures with unusual morphologies. Based on our results, we suggest that the formation of the ectopic biomineralization within the human heart valve follows the same steps (and the same hierarchical organization) of the biomineralization that normally happens in physiologically calcifying tissues [36–38].

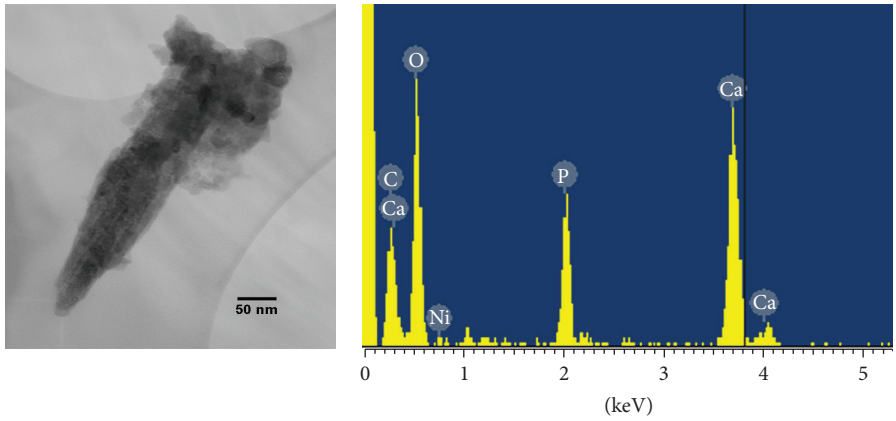
The spherical particles (matrix vesicles), similar to those reported by Bertazzo et al. [39], were always observed in strict association with organic filaments. Energy dispersive spectroscopy (EDS) analyses acquired on these particles confirmed that they are made up of calcium phosphate, sulphur (S), and azote (N). This means that in the vesicles there are also proteins that act on the growth of these mineralized phases [40], morphologically different from bioapatite nanocrystals. This is confirmed from previous studies on



(a) (b)

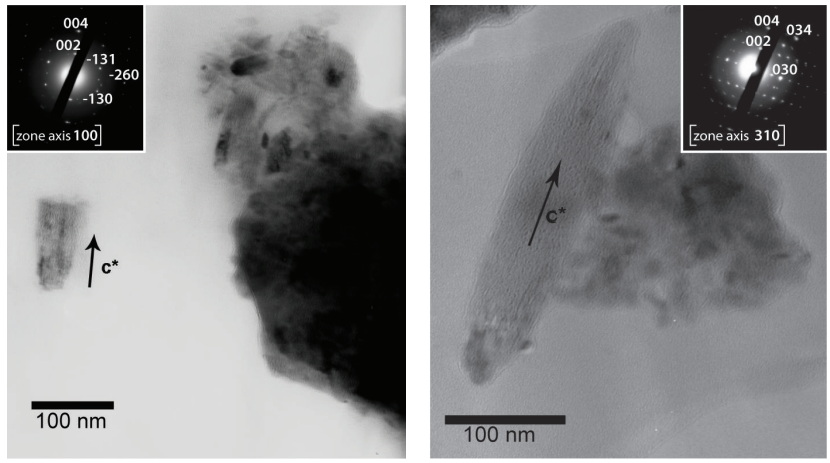
(c)

Figure 5: High resolution field emission scanning electron microscopy (FESEM) micrographs of pathological bioapatite nanocrystals. (a) Needle/rod-like nanocrystals distributed as randomly aggregate are visible within a small cavity of the organic tissue of a tricuspid aortic valve. (b) Very thin needle/rod-like nanocrystals grown as randomly aggregate in a small cavity of a bicuspid aortic valve. (c) Needle/rod-like nanocrystals grown onto the organic substrate. Their orientated growth over the organic interface is visible.



(a) (b)

Figure 6: Transmission electron microscopy (TEM) analyses of needle/rod-like pathological crystals. (a) Bright field (BF) image of a needle/rod-like nanocrystal collected from a calcified bicuspid aortic valve. In the bright field (BF) mode of the TEM, an aperture is placed in the back focal plane of the objective lens, which allows only the direct beam to pass. In this case, the image results from a weakening of the direct beam by its interaction with the sample. Therefore, mass thickness and diffraction contrast contribute to image formation: thick areas, in which heavy atoms are enriched, and crystalline areas appear with dark contrast. (b) EDS spectrum corresponding to the nanocrystal showed in panel (a). The spectrum clearly demonstrated the calcium phosphate nature of the nanocrystal. Ni and Cu belong to the 3 mm diameter Ni-Cu grid.



(a) (b)

Figure 7: Transmission electron microscopy (TEM) analyses of needle/rod-like pathological crystals. (a, b) Bright field (BF, see Figure 6 for explanation) images and nano-beam electron diffraction (NBD) patterns (in the insets) collected from a calcified bicuspid aortic valve. NBD is a technique that allows determining strain in crystalline materials with a high spatial resolution. A parallel beam with small diameter (~5 nm) is formed in the TEM and scanned across the sample, and diffraction patterns (at a zone axis) are recorded and analyzed for each location. The indices of the diffraction spots indicated in NBD patterns in panels (a) and (b) correspond to interplanar spacings of hydroxylapatite with hexagonal structure ($P6_3/m$). The interplanar spacings 002 and 004 are parallel to the elongated form, indicating that nanocrystals elongate parallel to their c-axis.

the involvement of specific proteins (glycosaminoglycans, GAG) and lipoproteins in controlling the size and shape of hydroxylapatite [41–43].

Our nanoscale observations indicate that the formation of pathological bioapatite nanocrystals within heart valves is related to the presence of a highly heterogeneous mineral deposit. Different growth processes may occur in different microenvironments (each one with its own physicochemical characteristics) influencing the shape of the biomineralization. The presence of compartmental niches within the extracellular matrix assumes a relevant role in the formation of ectopic biomineralization in human heart valves as well as the action of the organic substrate on the crystals features.

We suppose bioapatite nanocrystals to be the first mineralized product within the organic framework and we consider their appearance as the pivotal step in disease initiation and progression in agreement with the study of Ewence et al. [44] that have demonstrated the bioactivity of calcium phosphate crystals as function of their crystal size. Bioapatite nanocrystals in the soft tissues of the human heart valve may activate an inflammatory process that increases and strengthens, within the endocardial valve layer, leading to irreversible pathological conditions such as valve structure disorganization, excessive GAG accumulation, elastic fiber fragmentation, cell activation, and cell death. This pathological condition once activated may aggravate the inflammatory response started by inorganically formed bioapatite nanocrystals and lead to a severe calcification process. In addition, autophagy, a critical mechanism for the aging process, involved in the regulation of cardiac homeostasis and response to stress, is deeply implicated in this process, as hypoxia, nutrient deprivation, and ischemia are a strong stimulus for autophagy [45]. If autophagia becomes upregulated, the digestion of damaged proteins and organelles can create the perfect conditions for the niche, as a newly created cavity, resulting from cell death that can host calcification process.

4.1. Limitations. We are aware that several limitations exist, but to our knowledge this is the first report to describe the two different growth processes linked to the different microenvironments. Due to the small sample size and the prevalence of tricuspid aortic valves, we could not compare results of the three different types of valves, to highlight significant differences in their biomineralization. As we already reported a significant difference in the content of calcium and phosphorous [6] between tricuspid and bicuspid aortic valves, we are convinced that architectural differences in collagen fibers and nanocrystal orientation exist and will be the object of a future study. A larger sample size would allow us also to correlate clinical data to the different forms of biomineralization and to better investigate the multifactorial pathogenesis of this complex disease and this study is actually ongoing. This is not an in vivo study to investigate biominerals crystal growth in a follow-up over time, but in its nature of ex vivo observational study we are convinced that different stages of growing are represented in a whole heart valve, as we analyzed heavy calcified samples but also areas with no appearance of visible macrocalcification that were indeed present as microcalcification at a submicrometer scale.

5. Conclusions

Our findings on pathological bioapatite nanocrystals growth processes may be helpful for understanding the biomineralization that affects human heart valve tissues and we stress on the importance that the mineralogical approach

joined to the medical and biological fields can have to resolve this multidisciplinary phenomenon. To understand the formation of biomineralized deposits in the human heart tissues it is important to focus the attention on the nucleation of the bioapatite crystals, because all crystalline material, including bioapatite, can grow only after a nucleus is formed. In particular, biominerals growth in preferential sites for nucleation within complex crystal composites and the generation of crystallites with specific crystallographic orientations can be explained with a potential active control during the nucleation stage, operated by lipoproteins or other molecules, still not extensively investigated yet.

Therefore mineralogical and biological electron microscopy analyses on pathological bioapatite crystals may be extremely important to gain information about their crystallization pathway and on factors involved in the heart valve calcification process.

Conflict of Interests

The authors declare no competing financial interests.

Acknowledgments

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Review Article

Molecular Characterization of Reactive Oxygen Species in Myocardial Ischemia-Reperfusion Injury

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Myocardial ischemia-reperfusion (I/R) injury is experienced by individuals suffering from cardiovascular diseases such as coronary heart diseases and subsequently undergoing reperfusion treatments in order to manage the conditions. The occlusion of blood flow to the tissue, termed ischemia, can be especially detrimental to the heart due to its high energy demand. Several cellular alterations have been observed upon the onset of ischemia. The danger created by cardiac ischemia is somewhat paradoxical in that a return of blood to the tissue can result in further damage. Reactive oxygen species (ROS) have been studied intensively to reveal their role in myocardial I/R injury. Under normal conditions, ROS function as a mediator in many cell signaling pathways. However, stressful environments significantly induce the generation of ROS which causes the level to exceed body's antioxidant defense system. Such altered redox homeostasis is implicated in myocardial I/R injury. Despite the detrimental effects from ROS, low levels of ROS have been shown to exert a protective effect in the ischemic preconditioning. In this review, we will summarize the detrimental role of ROS in myocardial I/R injury, the protective mechanism induced by ROS, and potential treatments for ROS-related myocardial injury.

1. Introduction

Myocardial ischemia-reperfusion (I/R) injury occurs when the blood flow to the myocardium is obstructed, followed by the restoration of blood to the ischemic heart [1, 2]. Ischemia to a specific region of the body can provoke tissue damage due to lack of oxygen and nutrients; heart is particularly vulnerable since it demands high energy to function [1, 3]. It is reasonable to consider that the rapid and early restoration of blood flow to the ischemic regions prevents further damage. However, numerous studies have observed the reduced cardiac function and even the acceleration of myocardial injury after reperfusion [1, 3, 4]. Ischemic injury is a recognizable consequence of cardiovascular diseases including myocardial infarction, stroke, and coronary heart diseases. Moreover, some patients who suffer from severe coronary heart disease choose to undergo a coronary artery bypass grafting to promote cardiac function, yet they experience myocardial injury postoperatively [3, 5].

In response to a sudden ischemia, coronary vessels dilate to compensate for the low oxygen supply, allowing for maximal oxygen return/recirculation [6]. However, the continuous deficiency of oxygen during ischemia shifts cardiac metabolism toward anaerobic glycolysis, disrupts ATP generation in the mitochondrial oxidative phosphorylation (accounting for 95% of ATP production in the heart), and thus reduces overall ATP availability [3]. The ATP-dependent ion pumps, such as sodium potassium (Na^+/K^+) ATPase and calcium (Ca^{2+}) ATPase, are disturbed largely by the ATP depletion. Secondary channels, including Na^+/H^+ exchanger, and decreased intracellular pH, lead to intracellular Na^+ and Ca^{2+} overload [3, 6, 7]. Subsequently, the altered ion homeostasis and metabolism reduce cardiac contractility and structural organization, and initiate cell death via necrosis and apoptosis [3, 6]. Replenishment of blood during reperfusion rapidly restores cellular balance in the myocardium and therefore prevents further ischemic injury.

Perhaps somewhat counterintuitively, such normalization concurrently causes injury [6]. The molecular mechanisms regarding I/R injury are multifactorial, and various hypotheses have been proposed to describe the pathogenesis of myocardial I/R [7, 8]. Reperfusion treatments, including thrombolysis and percutaneous coronary interventions, serve to manage the ischemic conditions but unexpectedly induce additional damage [9, 10]. Indeed, the reestablishment of blood flow may trigger apoptosis and necrosis in the myocardium [8].

A growing number of studies have investigated how reactive oxygen species (ROS) play an intriguing role in I/R, ranging from beneficial to inimical [7, 11]. At the basal level, ROS function as a mediator for multiple cellular signaling cascades including cell growth and stress adaptation [9]. Conversely, excess ROS can damage tissues by oxidizing important cellular components such as proteins, lipids, and DNA, as well as activating proteolytic enzymes such as matrix metalloproteinases [7, 12]. Furthermore, the induction of protective mechanisms during ischemic preconditioning (IPC) can be associated with low level of ROS [7, 11]. The production of ROS has been implicated in both myocardial ischemia and reperfusion injury. During the past decade, studies continued to explore the mechanisms as well as develop clinical applications centered on ROS-related therapies to alleviate I/R injuries. More recently, novel concepts and treatments such as postconditioning and mesenchymal stem cells-based interventions have been thoroughly updated focusing on ROS-centered interventions in myocardial I/R diseases [13–16]. By highlighting these innovative findings, the current paper provides a timely review of the latest understanding of I/R and associated protective mechanisms linked to the unique role of ROS.

2. Involvement of ROS during Myocardial Ischemia

2.1. Physiological Relevance of ROS in the Cardiovascular System. ROS are essential in mediating physiological responses [12, 17]. Upon the exposure to environmental stresses such as hypoxia, ROS production can be significantly elevated to a level that overwhelms the endogenous antioxidant system and engenders tissue damage [7, 12, 17]. Typically, a small amount of superoxide ($O_2^{\bullet-}$, a type of ROS) is generated through electron leakage in mitochondrial electron transport chain, which can further lead to the formation of other ROS such as hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$) [3, 9, 18]. These byproducts of respiration may exert beneficial effects on cardiovascular functions and can be safely metabolized by the antioxidants under normal conditions [19, 20]. Cardiac mitochondria have been recognized as an important source of ROS in the myocardium, considering that a large number of mitochondria reside in the cardiomyocytes to meet a high energy demand [3, 20]. NADPH oxidases (Nox) also contribute to the major production of $O_2^{\bullet-}$ and H_2O_2 in cardiovascular cell types. Particularly, highly expressed Nox2 and Nox4 isoforms in the heart play an essential role in regulating the development of cardiomyocytes [21–23]. Other $O_2^{\bullet-}$ generating systems include lipoxygenase and xanthine oxidase (XO) [9, 18]. Moreover, the predominant expression of XO under stress has been shown to contribute to ROS generation in the perfused ischemic tissue [9].

2.2. ROS Formation during Ischemia. Although reperfusion is responsible for generating a ROS burst during reintroduction of molecular oxygen (O_2) to the ischemic environment, accumulating evidence has suggested that oxidant stress commences before reperfusion [11, 17]. Indeed, Zhu and Zuo observed a rapid increase in $O_2^{\bullet-}$ production within three minutes of ischemia [17]. Interestingly, the study identified oxymyoglobin as a novel source of $O_2^{\bullet-}$ generation in the rodent heart model during early ischemia, through an interaction between the iron ion in the heme group and reduced oxygen tension [17]. Myoglobin can also serve to reservoir O_2 but is quickly exhausted [3].

Studies have noted that the presence of residual O_2 is a critical element for ROS generation during ischemia, in which an impaired mitochondrial electron transport chain is believed to facilitate the conversion of residual O_2 to $O_2^{\bullet-}$ due to increased electron leakage (Figure 1) [11, 20]. Levraut et al. detected an irreversible decline in mitochondrial membrane potential in ischemic chicken cardiomyocytes. ROS-induced mitochondrial depolarization during ischemia is associated with myocyte death in I/R; specifically, the severity of depolarization is related to the extent of cell death in perfused tissues [11, 24]. The decline in membrane potential may be attributed to the opening of mitochondrial permeability transition pore (mPTP). However, the inhibition of mPTP activation does not significantly prevent depolarization, whereas the application of antioxidants restores membrane potential and prevents subsequent cell death [11]. In fact, mPTP opening is limited during ischemia due to low pH environment, but mPTP does play a crucial role in reperfusion injury [25]. At reperfusion, mPTP is activated, which exacerbates injury via ROS-induced ROS release cycle and initiates cell death signaling pathways [7]. Collectively, Levraut et al. proposed a putative scheme of ischemic injury involving the disruption of mitochondrial inner membrane by ROS-induced lipid peroxidation that contributes to the repolarization failure in mitochondria later during reperfusion [11]. Moreover, the impediment of mitochondrial depolarization and Ca^{2+} dysregulation by sarcolemma stabilizer suppress apoptotic and necrotic pathways, despite the presence of oxidative stress (OS) and an altered redox state [24].

In an attempt to detoxify the oxidative insult, a significant rise in the antioxidant defense system, such as glutathione increment, has been observed during ischemia [4]. In addition, the formation of $O_2^{\bullet-}$ at the early period of ischemia is likely to be involved in facilitating protections towards ischemic tissues, which is the so-called IPC [15, 17]. A detailed discussion of this will be provided later in the review.

3. ROS Mechanism in Myocardial I/R

3.1. A Burst of Oxidants during Reperfusion. It is true that a timely reperfusion is essential to ease ischemic injury and

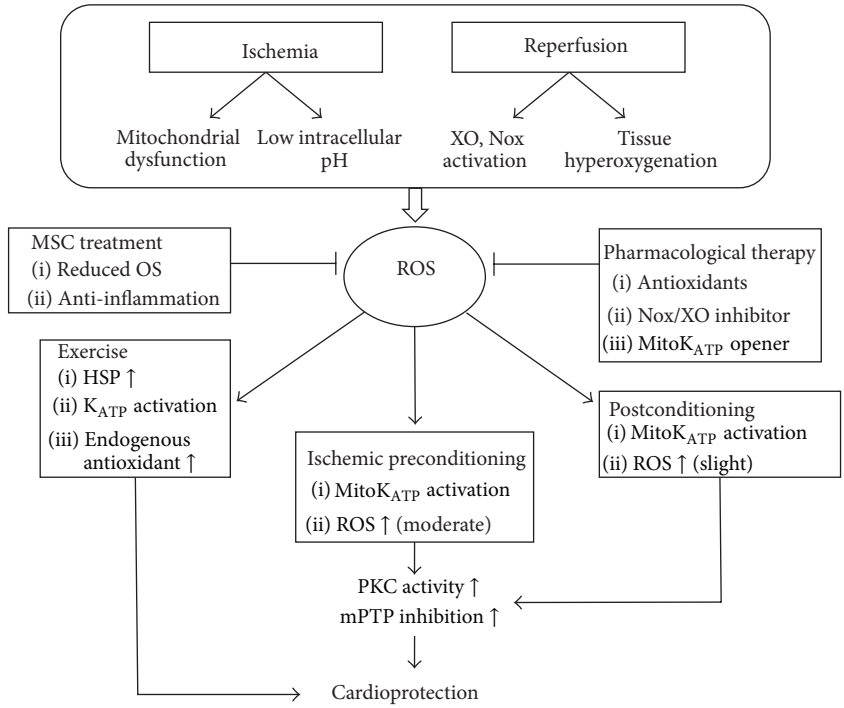

Figure 1: This schematic summarizes the role of reactive oxygen species in myocardial ischemia-reperfusion and related treatments. Reactive oxygen species generated during myocardial ischemia-reperfusion are involved in multiple cellular pathways that eventually lead to cardioprotection. HSP: heat shock protein; K_{ATP}: ATP-sensitive K^+ channel; mitoK$_{ATP}$: mitochondrial ATP-sensitive K^+ channel; mPTP: mitochondrial permeability transition pore; MSC: mesenchymal stem cell; Nox: NADPH oxidase; OS: oxidative stress; PKC: protein kinase C; ROS: reactive oxygen species; and XO: xanthine oxidase.

salvage viable myocardium, yet it can promote cardiomyocyte damage [26]. During reperfusion, the reactivation of aerobic metabolism induces an increase in ROS, particularly $O_2^{\bullet-}$, which exceeds the endogenous antioxidant capacity [3, 6]. The shift towards a more reduced redox state during ischemia may also contribute to the production of ROS upon reoxygenation [4]. The reduced form of iron ion mediates the Fenton chemistry to generate highly reactive hydroxyl ions [27]. Of various cellular alterations in the perfused ischemic myocardium, distorted redox state is widely recognized in the initiation of I/R cellular damage, although the precise role of ROS in I/R remains elusive [24].

The overproduction of $O_2^{\bullet-}$ derives further ROS generation in the mitochondria, forming a vicious cycle of OS [20, 27]. For instance, the reaction between aconitase (a mitochondrial protein) and $O_2^{\bullet-}$ leads to the formation of $^{\bullet}OH$ [20]. The aconitase inactivation can be therefore regarded as an indicator for $O_2^{\bullet-}$ toxicity [27]. An activation of XO during reperfusion is attributed not only to oxygen influx, but also to excess hypoxanthine (a substrate of XO) that is produced during ischemia as a result of ATP degradation [3, 10, 17]. In addition, ROS mediate the infiltration of neutrophils, which contribute to further $O_2^{\bullet-}$ generation via Nox activation (Figure 1) [26]. Increased OS during I/R results in the uncoupling of nitric oxide synthase (NOS), which reduces NO production and enhances NOS-derived $O_2^{\bullet-}$ level [28]. Those studies attributing the myocardial injuries to the decreased NO production during reperfusion are primarily based on the fact that, by applying NO or NO donors prior to I/R, the related injuries can be remarkably attenuated [29–33]. For instance, Liu et al. have proposed that the reperfusion-induced endothelial dysfunction can be caused by the decrease of NO formation, resulting in the diapedesis and neutrophil adherence in the ischemic region, thereby exacerbating I/R injuries [34]. Moreover, NO can react with excessive $O_2^{\bullet-}$ to form peroxynitrite ($ONOO^-$), an oxidant that suppresses the mitochondrial respiration by modulating the nitration of complexes I and IV [4, 35]. Since the respiratory oxygen consumption is interfered, additional ROS may be generated due to tissue hyperoxygenation (Figure 1) [4, 36].

3.2. Detrimental Effects Induced by ROS. As discussed earlier, overexuberant ROS that overwhelm the antioxidant defenses can induce protein denaturation and cause oxidative damage directly to DNA, especially mitochondrial DNA due to its proximity to the dysfunctional electron transport chain [26, 37]. The destabilization of mitochondrial and sarcolemmal membranes caused by lipid peroxidation enables the influx of nonspecific, small molecules across the membrane, resulting in mitochondrial matrix swelling and apoptosis [6, 11, 24]. ROS trigger inflammatory cascades and expressions of adhesive molecules, leading to leukocyte/capillary plugging and

endothelial swelling that interfere with capillary flow. Matrix metalloproteinases and other proteases are also activated by ROS, further deteriorating the functions of multiple proteins such as glycolytic and antioxidant enzymes [7, 38]. Impaired ROS and Ca^{2+} regulation can propagate to spread the injury through gap junctions [6]. Furthermore, the molecular alterations at the onset of reperfusion, such as ROS burst, recovery of pH, and Ca^{2+} overload, all facilitate the abrupt opening of mPTP, which is critical to reperfusion injury [7, 25, 39]. Although transient mPTP opening is involved in cardioprotection, prolonged opening results in irreversible changes within cellular bioenergetics and cell death due to the release of proapoptotic factors (e.g., cytochrome *c*) [7, 25]. Mitochondrial cardiolipin peroxidation also liberates cytochrome *c* and enhances electron leakage at complexes I–III [3, 7]. The administration of mPTP inhibitor has been reported to reduce myocardial infarct size in animal models, serving as promising therapeutic to avoid lethal myocardial reperfusion injury [26]. Despite the numerous detrimental outcomes related to ROS, repair processes including vascular remodeling and angiogenesis may occur at the later stage of reperfusion when ROS production returns to lower levels and resumes their roles as signaling molecules [7, 40].

3.3. Clinical Outcomes of Reperfusion Injury. Myocardial stunning is one of the clinical consequences associated with reperfusion injury. Although cellular homeostasis has been reestablished after reperfusion, the reversible contractile dysfunction may remain persistent and it is likely attributed to multiple mechanisms such as decreased ATP resynthesis [10, 26]. The sudden changes in ion concentration after reperfusion in the effort to restore balances induce reperfusion arrhythmias, a condition frequently experienced by patients undergoing surgical revascularization [10]. Reperfusion arrhythmia, such as ventricular fibrillation and ventricular tachycardia, is primarily responsible for the sudden cause of death after blood flow restoration [10, 41]. Moreover, microvascular obstruction is observed after reperfusion in which the blood cannot completely reperfuse to the ischemic region after the release of occlusion [10, 41]. Based on the prior discussions, it is likely that ROS play an important role in these events and further investigation is necessary.

4. Protective Role of ROS in IPC

Although excessive ROS production has been indicated as a primary contributor of I/R injury, ROS-targeted therapies including antioxidant treatments have yielded mixed results in attenuating I/R-induced damage [19]. The accumulation of such evidence suggests the potential role of ROS in the protection of cardiomyocytes. Indeed, studies have shown that the application of antioxidants impedes preconditioning protection. ROS formation during both ischemia and reperfusion is likely to participate in the protective mechanisms induced by preconditioning. Particularly, mitochondrial ROS are paramount in signaling IPC as detailed below [19].

4.1. Myocardial Preconditioning. Murry et al. first described the protective effect of preconditioning on myocardium in 1986 and observed a slower ATP depletion rate and a smaller infarct size in the heart treated with brief episodes of I/R cycles before prolonged occlusion followed by reperfusion [42, 43]. Later research recognized several types of preconditioning protocols including IPC, exercise preconditioning, and pharmacological preconditioning [2, 44, 45]. Preconditioning provides a beneficial "warm-up" that primes the tissue to subsequent injuries when it is subjected to prolonged stresses such as ischemia and hypoxia [2, 46]. Specifically, IPC is marked by transient exposures of sublethal I/R that induces myocardial protection against later I/R injury. Small amounts of ROS generated during short periods/cycles of I/R are indeed highly associated with the protective effect exerted by preconditioning [2]. In particular, ROS originating from the mitochondria play a pivotal role in mediating cardioprotection via mechanisms involving the activation of survival programs [2, 7]. Moreover, lower OS has been observed in I/R preconditioned cardiac muscle during prolonged I/R, which is attributed to the reduced ROS generation in mitochondria [2]. One of the well-established IPC mechanisms involves the opening of mitochondrial ATP-sensitive K^+ (mitoK_{ATP}) channel (Figure 1). MitoK_{ATP} channel is activated upon the exposure to preconditioning stimuli while the subsequent influx of K^+ leads to depolarization and matrix alkalization, which consequently induces a moderate increase in ROS and the activation of downstream survival signaling events [7, 47]. Notably, these preconditioning-induced ROS may mediate protein kinase C (PKC) activity and, via multiple steps, inhibit the opening of mPTP (Figure 1) [2, 7]. As discussed earlier, mPTP is a major regulator of necrosis and apoptosis [48]; such inhibition of mPTP opening is therefore essential to the cardioprotection [2]. In addition, the opening of the mitoK_{ATP} channel can generate mild matrix swelling which can improve ATP synthesis and fatty acid oxidation, leading to cardioprotective effects [2]. The application of mitoK_{ATP} openers mimics IPC whereas K_{ATP} blockers, such as 5-hydroxydecanoate, attenuate cardioprotection, further suggesting the importance of mitoK_{ATP} in IPC protection [47]. Besides the exclusive role of mitochondrial ROS in signaling IPC [7], the initial burst of ROS is correlated with IPC efficacy, and it serves as essential preconditioning stimulus to the activation of mitoK_{ATP} as well as sarcolemmal K_{ATP} (sarcK_{ATP}) [2, 49].

Cardioprotection can also be provided by preconditioning of regular or mild exercise [2]. Exercise has long been known for its potential to prevent cardiovascular diseases by modulating related risk factors such as obesity and hypertension [45]. Termed exercise preconditioning, exercise-induced protection involves the interplay of several protective mediators including endogenous antioxidants that eventually leads to myocardial-specific biochemical adaptations [45, 50]. For instance, exercise stimulates the upregulation of heat shock proteins (HSPs), a group of proteins that is overexpressed spontaneously under stressful events (Figure 1) [50]. HSPs are equipped by the cell as a defense mechanism in order to maintain cellular homeostasis. Specifically, HSPs are responsible for assisting proper folding of proteins and facilitating

degradation of damaged proteins [50]. The increased HSP activity may certainly be beneficial in the protection against I/R injury [51]. Despite the cardioprotection exerted by HSPs, studies have shown that the application of antioxidants on exercised animal can suppress HSP72 expression, yet the cardioprotective effects remain unaffected [52]. However, several studies have reported that the cardioprotective effect exerted by exercise against reperfusion injuries can be abolished when antioxidants are administrated during exercise, indicating the crucial role of ROS signaling in exercise-induced preconditioning pathways [53–55]. Similar to IPC, the activation of mitoK_{ATP} and sarcK_{ATP} channels in the exercised heart, where elevated PKC level is evidenced, also contributes significantly to cardioprotection (Figure 1). However, the exact role of K_{ATP} channels in exercise preconditioning remains ambiguous [45, 56]. Most importantly, exercise enhances endogenous antioxidant systems and improves ATP synthesis and mitochondria functions, which all serve to strengthen and increase tolerance of heart [51].

4.2. Postconditioning. Ischemic postconditioning, first put up by Zhao et al. in 2003, demonstrates a cardioprotection that is tantamount to IPC in a nonpretreated heart after I/R [13]. It is later defined as "brief periods of ischemia alternating with brief periods of reflow applied at the onset of reperfusion following sustained ischemia" [13, 57]. Since reperfusion injuries occur within several minutes of blood reflow, postconditioning must be introduced as soon as the reperfusion is initiated [57]. Basically, postconditioning and preconditioning follow similar protocol in which the myocardium is exposed to cycles of ischemia and reperfusion; however, the timing of the treatment varies. In a rat I/R model established by Kin et al., three postconditioning cycles were performed at the onset of reperfusion. Each cycle consisted of 10 s reperfusion followed by 10 s reocclusion. This postconditioning protocol decreased I/R-induced damage but demonstrated less cardioprotection as compared to preconditioning, which consisted of 5 min ischemia/10 min reperfusion cycles before the initiation of occlusion [58]. Although the extent of postconditioning in attenuating reperfusion injury remains elusive [59], it is clear that the duration of reperfusion-ischemia cycles and the number of cycles greatly influence the degree of the protective effect [57]. For instance, in a 30 min occlusion model, rats that were treated with three cycles of 30 s reperfusion and ischemia had less infarct size. However, detrimental effects occurred when the duration of treatment in each cycle was lowered to 5 or 15 s [60]. The protective effect of postconditioning has demonstrated a similar mechanism to preconditioning, in which ROS are readily involved. By applying mitoK_{ATP} channel blockers, PKC inhibitors, or ROS scavengers during reperfusion, Penna et al. discovered a unique PKC-oriented redox pathway involved in postconditioning protections [61]. ROS generation during early reperfusion was found to play an essential role in initiating the protective cascade, possibly via the activation of mitoK_{ATP}. The mitoK_{ATP} opening raises the level of H_2O_2, which ultimately leads to mPTP inhibition and thus prevents cell apoptosis (Figure 1) [57]. It has also been revealed by Hausenloy et al. that the mK_{ATP}/ROS/PKC pathways involved in IPC are required to be activated during early reperfusion in order to achieve the protective effect [62]. This is consistent with Downey's finding that IPC exerts protection by triggering the ROS signaling pathway during the initial stage of reperfusion [63]. The emphasis of the redox signaling that occurred at the onset of reperfusion implies a potentially parallel mechanism underlying both IPC and postconditioning. Currently, the effect of postconditioning against I/R has yielded variable results and further research is necessary to evaluate its protection on the heart.

5. Pharmacological Strategies in Myocardial I/R Injury

5.1. Antioxidants Treatment. Despite the beneficial role of ROS in preconditioning [19], excess ROS have been implicated in the pathogenesis of I/R injury and studies have presented substantial interests in developing therapies to prevent ROS accumulation [7]. In particular, site-targeted treatments such as inhibiting ROS generation at mitochondrial or Nox source may improve the protective effect on the stressed myocardium [19, 64]. Furthermore, the combination of different antioxidants has been found effective in resisting I/R injuries (Figure 1) [19]. Gao et al. have reported that glutathione provides better cardioprotection than ascorbic acid when treated at the beginning of reperfusion in a rat heart model. Moreover, the coadministration of both antioxidants enhances the protective effect as compared to individual treatment [65]. One study examined the effect of VitaePro, a mixed antioxidant compound, and vitamin E in a 21-day oral treatment on rats before the induction of I/R. The results showed that both VitaePro and vitamin E exert cardioprotective effects during I/R, while VitaePro demonstrated a much stronger effect. The work suggests potential prospects of antioxidant drugs in resisting I/R injury [66]. However, other studies on the effectiveness of antioxidant treatments in attenuating I/R-induced damage have yielded varied results [17]. For instance, Flaherty et al. administrated human superoxide dismutase (h-SOD) intravenously to 61 patients prior to coronary angioplasty surgery. Such treatments have failed to demonstrate any improvement on heart function as compared to control group [67]. In fact, several clinical studies have been performed to investigate the role of antioxidant in ROS-mediated reperfusion injuries by administrating antioxidant drugs either before percutaneous coronary intervention or after thrombolysis. Unfortunately, results are not optimistic from the antioxidant interventions in terms of reducing infarct size or enhancing heart function [68].

Apart from antioxidant treatments that scavenge excess ROS, targeting inhibition of ROS production at their own sources may be more favorable. With the sole function of generating ROS in both physiology and disease states [40], Nox has been considered as a therapeutic target in ROS-related injury. Discussed earlier, Nox contributes to a portion of ROS production during reperfusion [26]. In response to I/R injury, both Nox2 and Nox4 isoforms are upregulated in the heart [23]. Although it is reasonable to inhibit Nox activities in order to lessen the ROS activation during I/R injury,

a complete inhibition of Nox family is not favorable since Nox is also responsible for the physiological production of ROS. Indeed, a minimal amount of ROS is essential to prevent I/R injury via metabolic adaptations involving hypoxia-inducible factor-1α (HIF-1α) and peroxisome proliferator-activated receptor-α- (PPARα-) dependent mechanisms [23]. Therefore, selective blockage of Nox is highly desirable [23, 40]. Unfortunately, commonly known Nox inhibitors such as apocynin (a Nox2 inhibitor) and diphenyleneiodonium have failed to achieve sufficient specificity [23]. In addition to Nox, accumulated evidence also suggests the involvement of XO in myocardial I/R oxidative injury [69]. Studies evaluating allopurinol, a potent XO inhibitor, have generated positive results on reducing ROS generation and inhibiting cardiomyocyte apoptosis in myocardial infarction models (Figure 1) [70, 71].

Other pharmacologic agents can be used to stimulate conditioning pathways, in order to be as effective as preconditioning treatment. For instance, adenosine reduces myocardial infarct size by activating cardiomyocyte receptors and subsequent PKC pathways that are involved in preconditioning-induced cardioprotection [72]. Cyclosporin administration before or at the onset of reperfusion also significantly ameliorates I/R injury by inhibiting mPTP opening and reserving mitochondrial function [26, 72]. Furthermore, drugs targeting the activation of K_{ATP} channels, such as nicorandil and pioglitazone, have been shown to demonstrate prominent cardioprotection against I/R-induced injuries (Figure 1) [73]. Therefore, activating specific conditioning cascade sites by corresponding drugs highlighted potential treatments to achieve similar protective effect as preconditioning.

5.2. Mesenchymal Stem Cell- (MSC-) Based Treatment. Recently, studies have attempted to excavate novel possibilities for treating I/R injuries. Arslan et al. identified that I/R-induced cellular damage may be attributed to the damage of functional proteins, which are essential for fatty acid oxidation, tricarboxylic acid (TCA) cycles, and glycolysis [38]. MSC-derived exosomes can function as reservoirs of functional proteins and alter key biomedical markers of reperfusion injury via paracrine signaling, such as OS, ATP/NADH, and cell death in a positive manner. MSC engraftment also provides reparative effects on tissues that are irreversibly damaged by I/R [38, 74]. Indeed, the injection of MSCs into infarcted myocardium of rats one week after I/R has demonstrated significant improvement in heart conditions including decreased infarct size, indicating the potential therapeutic approach for I/R-induced injury via tissue regeneration [75]. Other research has noticed the potential role of MSCs in suppressing OS and inflammation (Figure 1). Chen et al. have found that I/R-induced ROS production can be limited by injecting adipose-derived MSCs into the rats prior to I/R treatment, which is accompanied by a lower expression of inflammatory and apoptotic biomarkers [14]. All those findings suggest the multiple potentials of MSCs in developing therapeutic interventions to reduce myocardial I/R injuries.

6. Conclusions

The current review updated the positive and negative role of ROS in myocardial I/R injury. ROS are responsible for the myocardial damage during both ischemia and reperfusion. On the other hand, a small amount of ROS is essential for exerting cardioprotective effects in preconditioning. Besides IPC, potential treatments such as postconditioning and exercise preconditioning have shown promising results in reducing I/R injury. In addition, specific pharmacological strategies including antioxidants, Nox and XO inhibitors, and MSCs-based treatments are encouraging therapeutics targeting ROS-related I/R injury. Although various therapies have been proposed to prevent or reduce I/R injuries, further research is required to determine their applications in the clinical setting.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Tingyang Zhou and Chia-Chen Chuang contributed to the work equally.

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Review Article

A Review of Computational Methods to Predict the Risk of Rupture of Abdominal Aortic Aneurysms

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Computational methods have played an important role in health care in recent years, as determining parameters that affect a certain medical condition is not possible in experimental conditions in many cases. Computational fluid dynamics (CFD) methods have been used to accurately determine the nature of blood flow in the cardiovascular and nervous systems and air flow in the respiratory system, thereby giving the surgeon a diagnostic tool to plan treatment accordingly. Machine learning or data mining (MLD) methods are currently used to develop models that learn from retrospective data to make a prediction regarding factors affecting the progression of a disease. These models have also been successful in incorporating factors such as patient history and occupation. MLD models can be used as a predictive tool to determine rupture potential in patients with abdominal aortic aneurysms (AAA) along with CFD-based prediction of parameters like wall shear stress and pressure distributions. A combination of these computer methods can be pivotal in bridging the gap between translational and outcomes research in medicine. This paper reviews the use of computational methods in the diagnosis and treatment of AAA.

1. Introduction

Rapid improvements in computational power coupled with better understanding of hemodynamics have spawned the interdisciplinary science of computational medicine. A multidisciplinary effort with clinicians, radiologists, and biologists on the one hand and engineers and computer scientists on the other hand has greatly increased the ability to diagnose medical conditions and has improved delivery of healthcare. Computational methods have played a very important role in this, as experimentally determining parameters that affect a certain medical condition is not possible in many cases. CFD methods can be used to accurately determine the nature of blood flow in the cardiovascular system [1] and the nervous system [2] and air flow in the respiratory system [3]. Machine learning/data mining methods have also been used to develop models that learn from retrospective data to make a prediction on factors affecting progression of disease [4–6]. These models have also been successful in incorporating factors such as patient history and occupation. Some of these variables (patient history, occupation, and family history) are difficult to quantify directly and so, using methods such as machine learning, they can be incorporated into predictive models.

AAA is a condition affecting the aorta usually in its infrarenal segment and involves the abnormal dilatation of this artery (Figure 1). The infrarenal aorta is a site predisposed to aneurysmal widening. The cyclic stress caused by the pulse wave in conjunction with factors which decrease the strength of the wall may lead to dilatation and ultimately to rupture [7]. It is the 13th most common cause of death in the United States [8]. The condition occurs mainly in patients over 65 years of age and affects approximately 2% of the elderly population [9]. There are several risk factors that have been known to affect the genesis, growth, and rupture of AAA: advanced age, greater height, coronary artery disease, atherosclerosis, high cholesterol levels, hypertension [10], and smoking [11, 12]. AAAs are known to have an incidence that is approximately

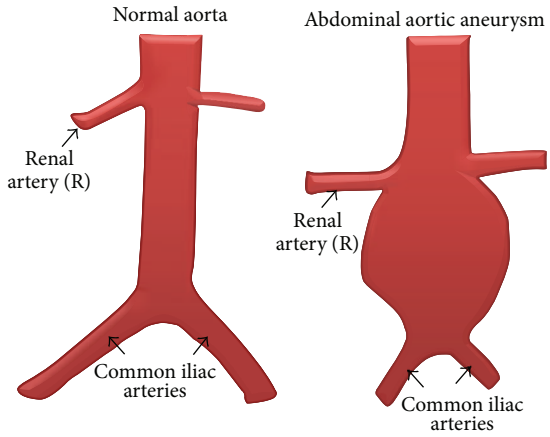

Figure 1: Abdominal aortic aneurysm.

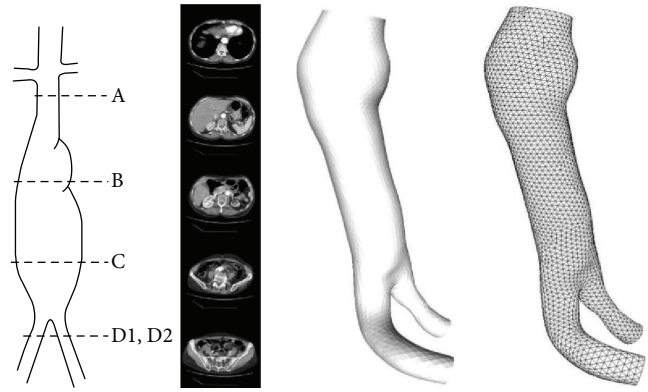

Figure 2: AAA geometry from CT images [19].

four to six times higher in men than in women. However, the incidence in women also rises with age, although it starts later in life than in men [13].

Surgical repair of AAA can be performed in two ways, by traditional open surgery or endovascular aortic aneurysm repair (EVAR). Initially, open surgery was the norm but since the introduction of EVAR [14], there has been a widespread acceptance of the procedure. This is a less invasive procedure with a decrease in 30-day mortality in patients when compared to open surgical repair. In addition, the current generation of devices approved has smaller delivery system profiles, tracks better, and can be used to treat difficult AAA morphology more easily [15]. EVAR has rapidly become the preferred method of AAA repair amongst vascular surgeons. Vascular surgeons have used lumen diameter as a metric to guide surgical intervention for some time now. A cutoff diameter of about 5–5.5 cm is used as a base to recommend surgery for patients with AAA [16]. Some studies like The UK Small Aneurysm Trial Participants, 1998 [17], have reinforced this value as a reasonable measure of the risk to benefit ratio between the risks of aneurysm rupture and those of surgical intervention.

As a criterion, the use of the maximum diameter metric is a crude way to estimate the critical state of an AAA [18]. Vorp et al. [18] argued that, from a biomechanical perspective, the use of wall stress in the lumen can more accurately predict the rupture of AAA. They defined the critical state of an AAA as that at which the mechanical stress within the aneurysmal wall exceeds the tensile strength of the tissue. Other parameters such as wall tensile strength, length of aneurysm, and patient-specific pulsatile velocity and pressure boundary conditions also play an important role in the progress to rupture of the AAA. Biomechanics of rupture in AAA is affected due to variations in a combination of these parameters. Hence, to account for the contributions of several parameters in what is essentially a patient-specific problem, computational methods could play a crucial role. This paper provides a summary of the different computational methods that can be used, a review of the literature that has incorporated computational methods to account for several parameters that lead to rupture in AAA, and suggestions on the direction that future research using these methods should take in order to improve our understanding of the rupture of AAA. Computational methods in imaging are left out of this review as the focus is on the biomechanical analysis of AAA.

2. Computational Methods

2.1. CFD Methods. Biomechanical analysis of an AAA system involves a series of steps to assess the factors that may influence the risk of rupture. Firstly, preoperative computed tomography (CT)/magnetic resonance imaging (MRI) images of the diseased aorta are obtained. These are then put through a process of image segmentation to convert what are 2D slices into a 3D geometric model, suitable for use in a CFD code (Figure 2). The pulsatile velocity and pressure boundary conditions for use in the CFD model are obtained during surgery. These are measured at the inlet and outlet of the aneurysm and at any other suitable point of interest for the researcher. CFD codes are then used to obtain the wall shear stress, pressure distributions, and the flow physics of blood in the aneurysm.

Computational methods in biomechanics incorporate the parameters mentioned in the previous section. Models are based on constitutive laws of continuum mechanics, such as the Law of Laplace that had been originally used in the analysis of bursting of cylindrical shells, the laws of rheology to describe the properties of blood, fluid mechanics in the analysis of vortex formation in the lumen, and material properties of stent grafts in a postsurgical aorta. Most CFD solvers are based on the Navier-Stokes (N-S) equations (1) which form the basis for describing the flow of fluids. They describe the momentum field of the flow under investigation and need to be used in conjunction with the continuity equation that describes mass transport

$$\frac{\partial u_i}{\partial x_i} = 0,$$
$$\rho\left(\frac{\partial u_i}{\partial t} + u_j\frac{\partial u_i}{\partial x_j}\right) = -\frac{\partial P}{\partial x_j}\delta_{ij} + \mu\frac{\partial^2 u_i}{\partial x_j \partial x_j} + f_i, \quad (1)$$

where u is the velocity, P is the pressure and ρ is the density of the fluid and f is the body force acting on the fluid. The N-S equations are discretized using two methods: the finite volume method (FVM) and the finite element method (FEM). FVM is generally seen to approximate the solution accurately to a large extent. But FVM may not be the best way to understand the deformation of a tissue due to blood flow. FEM has been used to solve fluid mechanics based problems [20, 21] and specifically for AAA simulations as well [22, 23]. It has the capability to incorporate the displacement of the biological tissue in a medical simulation problem more accurately than a FVM-based solver where the fluid becomes the most important component in the solution unlike in FEM where the solid displacement is more important.

The most accurate solutions to a biomechanics problem would incorporate the material properties of the blood vessel/tissue in question. This would allow a fluid-structure interaction (FSI) model to be embedded in the resulting physics. As the blood vessel/tissue is normally not rigid, it is imperative that this aspect is accounted for in the computed solution. FSI methods [24, 25] have been demonstrated to be effective in the description of flow physics in aneurysms. The coupling of the aneurysmal wall motion and blood flow is commonly made by using the Arbitrary Lagrangian Eulerian (ALE) method. The incompressible continuity and N-S equations in ALE form can be expressed as

$$\begin{gathered} \nabla \cdot \mathbf{u} = 0 \\ \rho_f \left(\frac{\partial \mathbf{u}}{\partial t} + \left(\left(\mathbf{u} - \mathbf{u}_g \right) \cdot \nabla \right) \mathbf{u} \right) = -\nabla \mathbf{p} + \mu \nabla^2 \mathbf{u}, \end{gathered} \tag{2}$$

where ρ_f, $\mathbf{p}$, $\mathbf{u}$, and $\mathbf{u}_g$ are the fluid density, the pressure, the fluid velocity, and the moving coordinate velocity, respectively. In ALE formulation, the term $(\mathbf{u} - \mathbf{u}_g)$, which is the relative velocity of the fluid with respect to the moving coordinate velocity, is added to the conventional Navier-Stokes equation to account for the movement of the grid [26]. From a computational point of view, we have to incorporate the moving interface between the fluid and the rigid body. The ALE method has been successfully applied to such moving boundary problems, which arise in free surface problems and fluid-structure interaction problems.

The ALE method has been employed because

(i) it is convenient to describe the fluid motion on the moving interface by the Lagrangian description in order to treat the compatibility conditions and the equilibrium conditions between the fluid and the rigid body;

(ii) it is apparently impossible to employ the Lagrangian description for the entire fluid domain because of severe mesh distortion due to vortex shedding or flows through outer boundaries.

Therefore it is natural to employ the mixed viewpoint of the Lagrangian and Eulerian descriptions [27]. Whilst the above method has been used for a rigid tissue, the ALE has been extended to the FSI problem as well [28]. Hirt et al. provide further detail of the ALE method [29] wherein they describe the applications of ALE to problems involving different flow speeds using the finite element method.

2.2. Machine Learning Methods. Significant experience over many years in the management of AAA amongst hospitals and clinicians has led to the availability of a large volume of data on patients who have undergone treatment for the condition. Statistical techniques such as univariate and multivariate logistic regression analyses have been successfully applied to risk prediction in clinical medicine. A commonly used instrument is the use of a prognostic score derived from logistic regression to classify a patient into a potential risk category [30]. This suggests that, using these techniques, an estimate of the associations between the various risk factors that cause rupture in an AAA can be encoded.

Many techniques have been used for data mining in medical and biomedical studies. A popular data mining technique is decision tree induction. The dataset is recursively partitioned into discrete subcategories. These subcategories are based on the value of an attribute in the dataset. The criteria for selecting these attributes in the dataset are based on its predictability within a certain subcategory. As a result of this, the final outcome is a set of series of categories based on values of the attributes. Each of these series generates a classification value. There are many algorithms developed for decision tree induction. Some clinical examples that apply this approach are diagnosis of central nervous system disorders [31], posttraumatic acute lung injury prediction [32], and acute cardiac ischemia [33].

Some of the parameters that may affect the ability to predict rupture risk in an untreated aneurysm are listed here. These parameters can be used in a model that learns from retrospective data and can be used in a prospective tool for patients.

(1) Diameter of lumen.

(2) Length of aneurysm.

(3) Aneurysm neck angle and length.

(4) Wall thickness.

(5) Presence and volume of intraluminal thrombus (ILT).

(6) Wall shear stress (WSS).

(7) Calcification of the aortic wall and calcium volume and percentage of aneurysm sac volume.

(8) Gender.

(9) Age of patient.

(10) Patient history (smoking, diabetes, specific occupations, and family history of aneurysms).

(11) CT scan slice thickness.

(12) Aortic and iliac vessel tortuosity.

(13) Imaging system used for diagnosis.

(14) AAA ILT index.

(15) Patient ethnicity.

(16) Body mass index (BMI).

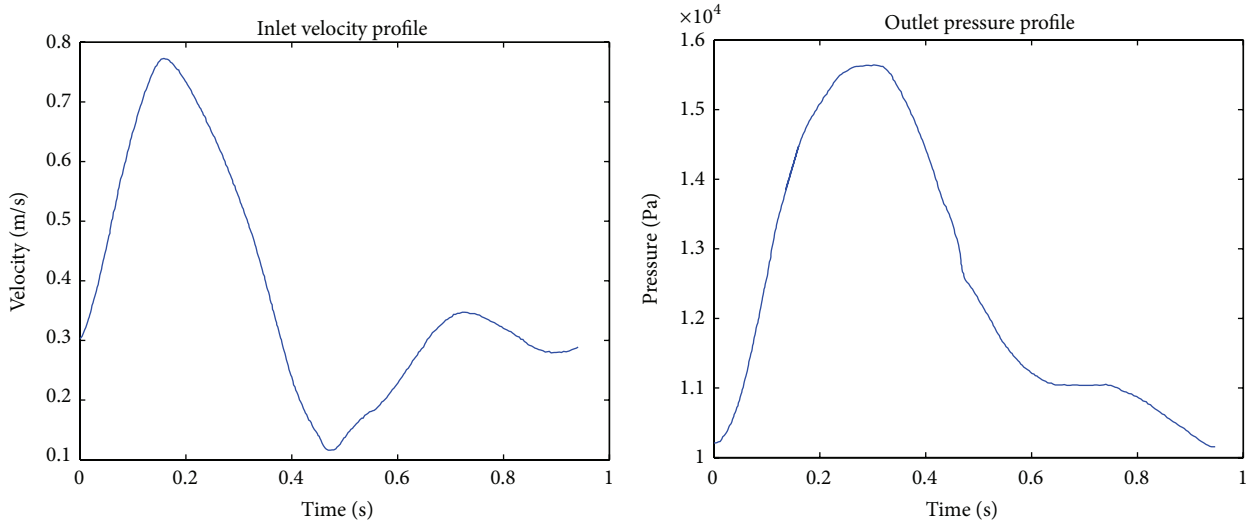

Figure 3: Inlet velocity and pressure outlet waveforms [43].

(17) AAA surface area.

(18) Aneurysm neck length/height and supra- and infrarenal neck angles.

Whilst these may be some of the input variables for a machine learning model, the output is the risk of rupture that can be obtained as a result of training the model on such retrospectively gathered data. The said model will learn about the parameters that play a part in the rupture or nonrupture of a patient-specific AAA. A great amount of classified data, that is, a suitable spread of ruptured and nonruptured retrospective cases, will allow the model to gauge the parameters that are the most important factors among many that lead to rupture in an AAA case. This model can then be applied prospectively where the model, having learnt from a large amount of data in the past, can to a degree of accuracy make a prediction of rupture risk in the patient.

When comparing different classifiers [34, 35] the key issues to address in such a model are

(1) predictive accuracy;

(2) interpretability of the classification models by the domain expert;

(3) handling of missing data and noise;

(4) ability to work with different types of attributes (categorical, ordinal, and continuous);

(5) reduction of attributes needed to derive the conclusion;

(6) computational cost for both induction and use of the classification models;

(7) ability to explain the clinical decisions made when models are used in decision making;

(8) ability to perform well with hitherto unseen (prospective) cases.

3. Computational Methods in AAA Analysis

The risk of rupture of an AAA has been studied in several different ways. Some researchers have performed experiments on phantoms made of silicone rubber [36] and distensible tubes [37–39], on cadavers, and during surgery to ascertain the properties of the AAA configuration [18]. Some of them have come up with analytical approaches of a simplified AAA problem [40–42]. But CFD approaches have dominated the landscape in the analysis of risk of rupture of AAA. Experiments are difficult to set up due to ethical considerations of sample retrieval from patients. Material properties need to be accurately obtained for the simulation to be of any use clinically. This has been a major challenge in AAA rupture studies.

3.1. Boundary Conditions. Unsteady boundary conditions of velocity and pressure are generally obtained from phase contrast MRI methods. There is a paucity of such studies and, in most instances, a few waveforms that are available in literature are reused by others, thereby taking away the patient-specific nature of the problem being solved. Figure 3 shows such a sample waveform that has been used by Soudah et al. [43], which in itself has been adapted from Ouriel et al. [9].

Flow in the aorta is pulsatile in nature. Hence, time varying waveforms of velocity and pressure are needed in order to accurately model the flow physics. The inlet flow velocity profile has two peaks as seen in Figure 3, each visualizing the systolic and diastolic phases in a pulse. The outlet pressure shows only one peak in the pulse.

Several different kinds of outlet boundary conditions have been used in literature such as constant pressure [44, 45] and flow specification at particular instances [46]. The boundary conditions for the velocity V are imposed as follows: (i) no slip at the walls, (ii) uniform (slug) profile at the inlet, and

(iii) zero-traction outflow at the exit. For pulsatile flow, the inflow mean velocity is time dependent and the volume flow rate is oscillatory as suggested by Finol and Amon, 2003 [47]. Although newly developed imaging methods, such as 4D MRI, have proved to be successful in measuring the flow, they still have limited spatial resolution (of the order of 2 mm), which restricts their applicability in calculating WSS parameters, and do not provide pressure information. Therefore, in the usage of Windkessel parameters based on patient-specific clinical data, the simulation takes into account the essential characteristics of the rest of the vasculature, which can be expected to improve the correlation between the simulation results and the true in vivo characteristics [48].

The Womersley number is a nondimensional number that describes the ratio of pulsatile flow to viscous effects and is used extensively in computations related to biofluids. Womersley profiles generally regard only the centerline velocity of the inlet. They incorporate the Bessel functions to derive the velocity profile in the inlet. But, it is not accurate as the geometry of the artery is patient-specific and affects the velocity values. The Womersley number is as shown below [49]:

$$\alpha = r\left(\frac{\omega}{\nu}\right)^{1/2}, \tag{3}$$

where α is the Womersley number, r is the radius of the artery, ω is the angular frequency of flow oscillation, and υ is the dynamic viscosity of the fluid.

Flow boundary conditions are critical to computing the flow solution in AAA. Hardman et al. [49] surmise that the Womersley number-based studies are inaccurate in computing solutions of flow fields and hence an axial velocity component is needed. They suggest that it is sufficient for the axial component to be used in the simulation and radial components are needed only when it is needed to understand the secondary flow components in the flow, such as particle tracking. Multiple outlet systems are, however, more complex and need better description of boundary conditions. Though this seldom occurs in the case of AAA, scalability, simplicity, and accuracy are paramount as there could be multiple outlets in an arterial network [50].

3.2. Material Properties. There are other boundary conditions to be considered in the simulation of AAA fluid mechanics. These are the material properties of the arterial wall. This requires the arterial wall material to be properly modeled, an issue which is complex given that the wall stiffness increases when lumen diameter increases and calcification and medial sclerosis occur with aging and in disease. Furthermore, the aneurysmal wall has been found to be mechanically anisotropic, a factor to be taken into account if a rational estimate of the wall stresses is to be made [51].

Generally, an isotropic properties assumption is made of the arterial wall when carrying out an analysis of the AAA. Whilst this may be a reasonable assumption in most cases, a more accurate constitutive model is needed to describe the properties of the wall. Constitutive models are obtained from experimental tests of actual tissue specimens. A uniaxial test specimen is generally made from two rectangular strips of tissue that has been cut out from the arterial layer. The flat arterial tissue layer is assumed to be a fiber-reinforced material with relatively stiff collagenous fibers embedded in a homogeneous isotropic (soft) ground matrix [56].

Most elastic tissues studies in literature have used the strain energy function approach. But arterial tissue exhibits highly nonlinear, nonisotropic, and possibly hyperelastic properties, and a computational model needs to incorporate all these properties as the elastic modulus is inadequate. Also, the assumption of strain energy functions holds good only for single continuous medium tissues. This is not the case with arterial tissue [57]. Since the mechanical properties of soft biological tissues depend greatly on their microstructure, proposing a reliable mechanical model for these tissues, including the arterial wall, depends on the level of microstructure integration attained in the constitutive model [58]. Taghizadeh et al. [58] proposed a new biaxial constitutive model based on microstructural properties as opposed to the simple uniaxial tests carried out by Sokolis et al. [59] and Karimi et al. [60]. The uniaxial tests only look at a single layer of the tissue that is in contact with the blood flow. The biaxial tests however consider a second layer as well, which is important in assessing the response of the arterial wall in a fluid-structure interaction scenario. In general, whilst using the simplified boundary conditions is computationally inexpensive and is accurate in most cases, incorporation of the nonlinear properties when custom codes are written makes the solution more accurate.

Wall thickness is another aspect that is central to the response of the blood vessel to flow. Since aneurysmal rupture occurs at a specific site of the aortic lumen, the properties of the wall affect the computed solution. This is because the wall thickness is seen to reduce as the disease progresses. In general, the aortic wall thickness in computational methods is assumed to be in the range of 1.5–2 mm. While this may be largely accurate for most simulations, it has been acknowledged as a major limitation in the completeness of the prediction solution [61]. Some previous measurements of thickness are listed in Table 1 [52].

The thickness from Thubrikar et al. [55] has been extensively used in literature as the value that accounts for the posterior and anterior sections of the AAA. Most ruptures are seen to occur in the proximal posterior part of the AAA. Hence, this value is assumed to accurately describe the wall thickness.

Raut et al. [52] suggest that the wall thickness as a constant value is not accurate and described a novel method that incorporated the regionally varying wall thickness, especially in the area of rupture. A comparison of uniform thickness, patient-specific uniform thickness, and the varying thickness was carried out in 28 samples. This showed a statistically significant difference on FE analysis with principal stresses and strains and strain energy density being the output parameters. As stated previously, rupture does not occur at the region of the aortic wall with maximum lumen diameter. This method can thus be used to obtain the carrying wall thickness that reflects the true thickness at the site of rupture.

Table 1: Wall thickness measurements as reported in literature [52].

Author	Reported thickness (mm)	Method of measurement	Remarks
Di Martino et al., 2006 [53]	Elective AAA 2.5 ± 0.1 Ruptured AAA 3.6 ± 0.3 Mean 2.9	Optical (laser)	Thickness is inversely correlated with local strength; only anterior wall tested; use of laser measurement eliminates compression due to caliper
Raghavan et al., 2006 [54]	Min 0.23 Max 4.26 Median 1.48	Caliper	No discernible difference in thickness for small and large aneurysm; thickness slightly lower in posterior and right walls; thickness low in ruptured aneurysm near site of rupture
Thubrikar et al., 2001 [55]	Posterior 2.73 ± 0.46 Lateral 2.52 ± 0.67 Anterior 2.09 ± 0.51	Customized micrometer with resistivity meter	Thickness decreases from posterior to lateral to anterior walls; accuracy 0.05 mm

3.3. Flow Physics. The physics of blood flow through the AAA is perhaps the most important aspect of the analysis of rupture risk. Data about material properties from experimental studies and boundary conditions from advanced MRI and CT technology have led to CFD methods being used to understand the flow fields in AAA. Accurate hemodynamic simulations can lead to better treatment planning and improved stent design for the diseased aorta. The interaction between blood flow and the arterial wall is a challenging problem. The studies reported below have used the rigid wall assumption to overcome the computational expense of the fluid-structure interaction solution.

Blood flow in the normal aorta can be ascribed to be laminar, similar to that in a pipe. Flow development is also well understood. In contrast to the normal aorta however, the flow in an aneurysmal segment is highly disturbed and maybe nonlaminar. Specifically in the aortic segment immediately beyond the aneurysm neck, flow separation involving regions of high streaming velocities and high shear stresses is observed [62]. At the expanded aneurysmal segment, average flow velocity and wall shear stress are much lower compared to those in the normal aorta [62]. Early studies used steady flow computations as the norm as pulsatile boundary conditions were hard to obtain. Taylor and Yamaguchi in 1994 [63] were amongst the earliest to compare the steady and unsteady flows in aneurysms. A set of symmetric vortices that were different in behavior downstream were observed in both the steady flow and the unsteady flow conditions. Symmetric vortices induce high pressure at certain locations in the lumen and were seen later on to be possible sites of rupture of the aneurysm.

Blood flow induces wall shear stress (WSS) in the aneurysm. This is the main parameter that determines the risk of rupture in an AAA lumen. WSS is related to the properties of the blood flowing through the lumen. Apart from blood pressure, WSS in AAA is also influenced by the aneurysm diameter, shape, wall thickness, wall mechanical properties, and the presence of intraluminal thrombus (ILT) [64].

ILT is an important component of AAA that is difficult to incorporate into any computational model. Most aneurysms have ILT within their lumen. Stenbaek et al. [66] argued that the development of ILT may be a better predictor than the maximum diameter of the AAA as a rupture risk parameter. But Di Martino and Vorp [67] postulated that ILT might protect the AAA wall from the pressure applied by blood flow. A finite element study by Li et al. [68] on the effect of ILT on wall shear stress showed that the non-ILT models had higher stress development than the ILT models. This is in keeping with Laplace's Law, which is often applied in fluid mechanics studies on AAA. It must however be emphasized here that Laplace's Law was originally used to describe bursting stresses in cylindrical shells and to apply it in this situation may not be wholly accurate. Thus, the role of ILT in the biomechanics of AAA is yet to be ascertained clearly. This might be because of the different types of ILTs that develop inside the AAA. O'Leary et al., 2014 [69], performed mechanical tests on 356 samples and classified them into 3 morphologies, type 1 which was a multilayered ILT whose strength and stiffness decreased gradually, type 2 whose strength decreased abruptly, and a single layered ILT with lower strength and stiffness compared to the other two types. This may partially explain why there is a difference of opinion amongst researchers about the effect of ILT in AAA. There are differences that crop up in ILT even due to gender. This aspect has been brought out by Tong et al. in 2013 [70] when they carried out biomechanical behavioral studies on 90 AAA samples (78 men and 12 women). They observed that the female ILT luminal layer showed a lower stiffness in the longitudinal direction than the males and, consequently, the thrombi may have different wall weakening effects in males and females.

While most methods have assumed laminar flow regimes in the aneurysm, there is a possibility that turbulent flow can also develop due to the presence of ILTs or wall calcification. Turbulent flow is common in the heart and the upper airway unlike the smaller diameter aorta where a discernible turbulent regime of flow is not seen. However, turbulence may well be a factor in very large aneurysms where there is the possibility for a turbulent mixing phenomenon to occur. In a study using a rigid wall Newtonian fluid approach considering turbulent conditions carried out on 3 symmetric aneurysm geometries with different diameters, the smallest

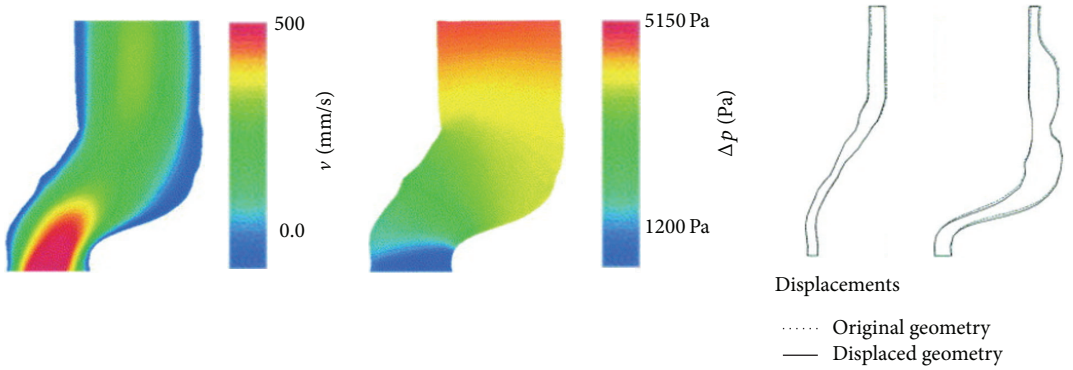

Figure 4: Velocity and pressure distribution along with wall displacement in a FSI calculation. (Adapted from Di Martino et al., 2001 [65].)

aneurysm did not show transition while the biggest diameter aneurysm shows flow separation and turbulent vortices [71]. It was seen that the vortex that impinges on the distal wall of the AAA generates secondary vortices, which then break up further. In the small aneurysm where flow remained laminar throughout the cycle, the high stress region was only seen in a relatively thin layer attached to the aortic wall. The large aneurysm, however, was seen to have islands of high stress in the middle of aneurysm due to the impact of turbulence transition [71]. This study on the impact of turbulence transition on WSS concluded that, with the onset of flow turbulence as seen in the large aneurysm models, the surface stress appeared to point to all directions and no dominant direction could be identified. The temporal and spatial gradient of WSS is significantly larger than the laminar flow (small aneurysm) situation. It is however important to consider the effect of turbulence as well which is seen to occur in larger aneurysms, especially above 5 cm. Given the inherent nature of flow, the transition to turbulence can occur even at resting conditions in large aneurysms.

3.4. Fluid-Structure Interaction (FSI). In computed solutions where the aneurysmal wall is assumed to be rigid, pressure values do not vary in time inside the lumen as the wall and blood flow are not coupled in the solution. But, in reality, the wall is not rigid and interacts with the flow during every pulse. The nature of this fluid-structure interaction is bidirectional; that is, the wall responds to blood flow pressure, shown by its displacement, and this in turn affects the flow through the aneurysm. FSI methods most accurately describe the flow physics occurring inside the aneurysm and in theory can precisely predict the site of rupture with the wall shear stress values reflecting the most realistic conditions.

Di Martino et al. [65] first carried out a FSI calculation on a realistic geometry obtained from segmentation of CT images (Figure 4). They were able to establish local stresses on the wall due to the structural and blood flow conditions. An ALE based FSI numerical method was used in commercial software Fidap to obtain the solution. The motion of the wall was not homogenous as would be seen in a rigid wall calculation. The asymmetry of the aneurysm also seemed to play a part in the development of wall stresses. They found that a larger area of maximum velocity corresponds to the systolic peak and that a flow deceleration is always discernible at the site where the aneurysm is larger, suggesting a higher pressure at these sites. This along with a weakening of the wall itself may be the genesis of continued aneurysm enlargement.

These studies have shown that a FSI method would be most accurate in predicting wall motion in the aneurysm as against only a FEM or a fluid dynamics model [75, 76]. This was further demonstrated by Scotti et al., who compared the FEM with a FSI method on 10 aneurysm models. It was observed that applying a realistic fluid pressure distribution to the arterial wall resulted in wall stresses that were 20% higher than if only peak systolic pressures were considered. This was in direct correlation with previous results that also showed a 21% increase in wall shear stresses when FSI methods were used [73, 77]. A limitation of this study was that this was an idealized model with asymmetry built into the geometry.

Using FSI methods, the various geometrical changes and material characteristics in patient-specific aneurysms can be accounted for. Scotti et al. compared aneurysm geometries having uniform wall thickness and variable wall thickness and also investigated the effect of 5 levels of asymmetry in the model. They observed that varying wall thickness increases the von Mises stress by up to 4 times as compared to when it is uniform. For asymmetrical variations too, the variation of thickness caused stresses to increase. This reinforces the fact that accurate thickness and FSI considerations will make the computed solution more accurate.

Li and Kleinstreuer [74] reinforced this by comparing different asymmetrical aneurysm geometries in a FSI solver. They concluded that assumption of symmetry leads to underestimation of wall shear stress. An iliac bifurcation angle also seems to have an effect. For iliac bifurcation angles less than 90 degrees, the wall shear stress is in the range of 0.57 to 0.63 mPa. But as it goes beyond 90 degrees, the stress increases by about 8%. At aneurysm neck angles of 12 degrees or so, the region with the higher wall stress is at the asymmetric bulge with the maximal stress being at the distal point of the bulge. As the neck angle increases, the proximal stresses move away from the bulge but the maximum point of stress remains the same. This, they argue, is due to the fact that

Table 2: Comparison of parameters investigated using FSI methods.

Reference	Method	Parameter	Remarks
Di Martino et al., 2001 [65]	FSI (ALE)	Maximum pressure	First accurate calculation of FSI
Scotti et al., 2008 [72]	FSI	Wall shear stress	20% more WSS if FSI method is used
Scotti et al., 2005 [73]	FSI	Asymmetry and wall thickness	Varying wall thickness and asymmetry increases von Mises stress
Li and Kleinstreuer, 2007 [74]	FSI	Neck angle, asymmetry, and bifurcation angle	Large neck angle leads to elevated von Mises stress; lateral asymmetry has higher stress

neck angles influence strong surface curvatures in the AAA neck leading to changes in the location of wall stress regions.

A summary of the different studies using FSI methods is listed in Table 2.

3.5. Machine Learning Methods. As described in the previous section, fluid-structure interaction methods greatly increase accuracy of simulations of AAA, leading potentially to more accurate surgical planning for clinicians. But FSI computations can take several days to complete depending on the computational power available. In addition, carrying out these computations needs an expert in fluid dynamics and programming to interpret the simulation results for the clinician. Clinicians are often unable to obtain this kind of expertise or do not have the lead time to go through the detailed process to assess the prognosis of AAA in a particular patient. CFD is crucial in understanding the mechanics of aneurysm formation and rupture but it needs to be used in conjunction with other methods that can be faster than CFD to obtain a conclusion quickly. This can be done by the use of machine learning methods.

A summary of the machine learning method has been described before. It uses a combination of statistical methods, probability and optimization methods, to predict the direction of movement of a system. A "learning" model is created with the available retrospective data. A large quantity of such retrospective data is available at hospitals and this is helpful in developing learning models that can make prospective predictions having "learnt" from retrospective data. There are numerous statistics based machine learning methods that can be used along with CFD simulations so that more accurate and faster conclusions can be drawn for clinicians [78].

Data mining is a popular method used to derive conclusions in hemodynamic simulations. Kolachalama et al. [79] proposed a data mining technique that accounted for the geometric variability in patients for predicting cardiovascular flows. A Bayesian network-based algorithm was used to understand the influence of key parameters through a sensitivity analysis. Although Monte Carlo methods are suitable for output statistical analysis, the Bayesian approach brings out the relationships between the parameters using a multivariate approach. They tested the method on the human carotid artery bifurcation. A range of automated geometries were created for steady state 3D flow analysis. After CFD analysis was carried out, the output maximum wall shear stress was approximated as a function of the geometric variables. The data from the runs was used as a training data set to build the Bayesian model. A probability plot of the maximum wall shear stress could then be generated.

Geometric parameters (especially diameter of the aneurysm) are critical in defining the risk of rupture and have undergone most investigation in the field of data mining. The variation of geometric parameters amongst patients gives an opportunity for models to be developed to predict the characteristics of disease progression. Martufi et al. [80] carried out a geometrical characterization of the wall thickness distribution in AAA. They were able to train a model to differentiate the wall thicknesses in ruptured and unruptured AAA. The thickness difference was seen to be 7.8% between ruptured and unruptured AAA. This could be an important parameter to determine the risk rupture and plan early intervention.

This work has been extended by Shum et al. [81] who developed a model from 66 ruptured data sets and 10 nonruptured data sets and their geometric indices and wall thickness variations. The results of this study showed that, in addition to maximum diameter, sac length, sac height, volume, surface area, bulge height, and ILT volume were all highly correlated with rupture status. It was also observed that parameters such as volume of ILT were also highly correlated with rupture in addition to the size of the AAA. In this study, the overall classification accuracy was 86.6%. It used a decision tree algorithm that is one of the possible machine learning methods that can be used for large data sets requiring a decision output.

The above mentioned studies have been limited by the use of geometric parameters and, in particular, the maximum diameter of lumen alone as factors contributing to the rupture of an AAA. But other parameters such as patient history and comorbidities and presence of stents or other geometric parameters such as the aneurysm neck angles, tortuosity, or even genetic factors could well play a significant part in contributing to the determination of rupture risk of an AAA. Here, machine learning methods in parallel with CFD can be used to develop a tool that can be of practical use to clinicians as a predictive tool in the management of an AAA.

4. Conclusions

This review of the computational methods used in the prediction of rupture risk in AAA reveals that several aspects of the problem need to be understood before computational methods can be used to reliably predict rupture. These include the boundary conditions and material properties

that weave in the patient specificity of the problem to be solved. These parameters are difficult to obtain in real clinical situations and have been approximated to a large extent in the literature. A suitable combination of these properties could lead to better prediction of the risk of rupture. But, given that measurement of boundary conditions and the material properties of the aortic wall in a particular patient is a very invasive process, computational methods have played a very big role in the biomechanical analysis of AAA. The combination of CFD and FEM has led to understanding of the flow pressure distribution, wall shear stress quantification, and effect of material properties and geometrical parameters. Computational methods have made patient-specific analyses possible, a feature essential for understanding the progression of AAA in a particular patient. Each patient has their own unique anatomy and pathophysiology that affects material properties and boundary conditions that can influence their management significantly.

Unfortunately, CFD/FEM methods, whilst providing crucial information on the pathophysiological mechanics of the aneurysm, can be very time consuming to undertake. This is made worse by the fact that FSI is a computationally expensive and complex method for routine use in real-life clinical situations. A much simpler interface therefore needs to be developed wherein the vascular clinician is in a position to assess the prognosis of the patient, rupture risk of the aneurysm and proceed to planning surgical, endovascular, or conservative treatments custom-made for that patient. This can be possible through the use of machine learning methods. The use of machine learning models in parallel with FSI computations can bridge the gap between translational and outcome research, thereby improving healthcare delivery and saving lives in aortic aneurysms.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

The Power of Phase I Studies to Detect Clinical Relevant QTc Prolongation: A Resampling Simulation Study

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Concentration-effect (CE) models applied to early clinical QT data from healthy subjects are described in the latest E14 Q&A document as promising analysis to characterise QTc prolongation. The challenges faced if one attempts to replace a TQT study by thorough ECG assessments in Phase I based on CE models are the assurance to obtain sufficient power and the establishment of a substitute for the positive control to show assay sensitivity providing protection against false negatives. To demonstrate that CE models in small studies can reliably predict the absence of an effect on QTc, we investigated the role of some key design features in the power of the analysis. Specifically, the form of the CE model, inclusion of subjects on placebo, and sparse sampling on the performance and power of this analysis were investigated. In this study, the simulations conducted by subsampling subjects from 3 different TQT studies showed that CE model with a treatment effect can be used to exclude small QTc effects. The number of placebo subjects was also shown to increase the power to detect an inactive drug preventing false positives while an effect can be underestimated if time points around t_{max} are missed.

1. Introduction

A specifically designed thorough QT/QTc (TQT) study has been identified by the E14 guideline of the International Conference on Harmonization as a crucial element for clinical assessment of potential cardiac risks of any drug [1]. A dedicated study to determine whether a drug has the potential to prolong the QT interval is conducted in later phases of drug development after proof-of-concept has been established and the pharmacokinetic profile, maximum tolerated dose, and proposed therapeutic dose are determined in Phase I/II studies. The study is solely designed to demonstrate if a drug-induced effect on the heart rate corrected QT interval beyond an upper bound of 10 ms—"the threshold of regulatory concern" [1] can be excluded. This needs to be demonstrated by showing that for each time point the 2-sided 90% confidence interval for the difference of the mean effect and that under time matched placebo are completely below this threshold. As this is the only aim of a TQT study and considering that relatively large sample sizes are required for the study, the cost-effectiveness of this type of approach has been discussed [2]. An important component of a TQT study is the use of an active control to demonstrate the sensitivity of the assay [3]. Moxifloxacin is commonly used in this role and the use of this antibacterial fluoroquinolone outside its indication has contributed to a search for alternatives to conventional TQT studies [4].

It has been conjectured that, without compromising the QT assessment, increased efficiency can be attained by collecting the same quality QT data in single ascending dose (SAD) and multiple ascending dose (MAD) first in human studies. Cardiac safety assessment is not the primary objective of these early studies but as these studies often use doses up to the maximum tolerated dose (MTD) achieving plasma concentrations above those that will be seen during later stages of development, SAD and MAD studies are the ideal candidates for incorporation of early QT assessment [5–8].

This search for alternatives to a TQT study and the use of QTc data obtained in Phase I studies have been extensively discussed [9]. One question of outstanding interest is whether

analyses based on data obtained from these studies will have a sufficient power to reliably show QTc prolongation and, more importantly, to reliably predict the absence of such an effect. Substantial differences between a TQT study and a SAD or MAD study must be considered. Although the total number of subjects involved in a SAD study may not be much less than in a crossover TQT study, only a fraction of them are exposed to drug doses that are at or above the level that will be used in future therapies. Moreover, while in a TQT study one proposed therapeutic dose and one supratherapeutic dose of the drug are used, in a SAD or MAD study several doses are employed and only a few subjects are given each of the doses. Furthermore, ECG monitoring in SAD/MAD studies may be limited as these studies are primarily designed to address subject safety and to exclude only large electrocardiographic abnormalities which implies a modification of these Phase I studies to integrate robust ECG monitoring and analyses.

Concentration-effect (CE) modelling is a well-established method already used as a secondary analysis in TQT studies [10]. The appropriateness of a CE analysis based on the change from baseline was shown to be a valid alternative recognizing that model selection can be improved with experience and more analysis of data from drugs with a known effect on QTc can help to substantiate the model [11].

Even though the potential of applying CE analysis to QTc data generated from SAD and MAD studies has been recognised, the level of confidence and the power of such an analysis in a situation such as a Phase I study are still one of the key points to be addressed. Ferber et al. [11] used subsampling from crossover TQT study data to simulate small studies and showed that sample sizes of 9 subjects on active drug and 6 on placebo provide sufficient power to detect or exclude an effect similar to the one of moxifloxacin. In this publication, we replicate these findings based on a different set of TQT studies and, in addition, we investigate the role of design features in the power of the analysis. In other words, we attempt to broaden the understanding of the power of CE analysis for detecting clinically significant QTc prolongation and exclude such an effect for an inactive drug.

2. Methods

The simulation work was based on data from three crossover TQT studies in healthy volunteers. In these studies, a single dose of 400 mg of moxifloxacin was given as a positive control. Data from subjects with available ECG and PK data from moxifloxacin and placebo treatment were used.

Study 1. This randomised, placebo-controlled, double blind crossover study consisted of 96 volunteers. Moxifloxacin was given in the fasting state on day 16 of the moxifloxacin study period (placebo given on 15 preceding days). ECG data were collected on day 16 of the moxifloxacin period at 12 time points: predose, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 12, and 24 h postdose [12].

Study 2. This randomised, placebo-controlled, double blind crossover study consisted of 64 volunteers. Moxifloxacin was administered in the fasting state on day 2 of the moxifloxacin study period (placebo given on the preceding day). ECG data were collected at 12 time points: predose, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 8, 12, and 24 h postdose [13].

Study 3. This randomised, placebo-controlled, double blind crossover study consisted of 49 volunteers. Moxifloxacin was given in the fasting state on day 1 of the moxifloxacin study period, preceded by placebo on a baseline day. ECG data were collected at 14 time points: predose, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, 12, and 24 h postdose [14].

For all studies, 12-lead ECGs were recorded and stored electronically on the MUSE CV information system (GE Healthcare). Before any ECG recording, the subjects maintained an undisturbed supine resting position for at least 10 minutes and avoided postural changes during the ECG recordings. At each time point, the ECGs were recorded in triplicate at 1-minute intervals during 3 minutes. Each ECG lasted 10 seconds.

Automatic ECG analysis was performed by the Marquette 12SL ECG Analysis Program (MEAP). All ECGs and their associated automated interval measurements were subsequently reviewed by qualified cardiologists. If manual adjustments of the automated measurement became necessary, a second cardiologist confirmed the assessment. Any disagreement between first and second readers was adjudicated by a third and most senior cardiologist. Details of this process have been described in [15]. For further analysis, the mean across the triplicates was used.

In our simulation studies, we used QT corrected according to Fridericia (QTcF) [16]. In particular, we did not consider subject-individual corrections, which may contribute to an undue complexity of a Phase I study and may be unnecessary in the presence of small heart rate effects.

2.1. Data Analysis. The analysis method used has been described elsewhere [11]. By taking a subsample of subjects, data under placebo and under active drug (moxifloxacin) can be obtained. To simulate a drug that does not prolong QTc, PK data obtained under moxifloxacin was combined with the time matched QTcF values from the same subjects under placebo. Data from all time points or only data from a subset of time points were used.

Each simulated study was assessed for a QT-prolongation of regulatory concern using a concentration-effect modelling approach according to the methods described in [9]. It was considered negative if the two-sided 90% confidence interval for the effect predicted at the geometric mean $C_{\max}$ was completely below 10 ms. More specifically, two concentration-effect models were fitted to each simulated study as follows:

Fixed effects:

$$\begin{aligned} &\Delta\text{QTcF} \sim C + \text{time} + \text{teatment} \\ &\Delta\text{QTcF} \sim C + \text{time} \end{aligned} \tag{1}$$

Random effects: intercept per subject.

The models use the change from predose baseline of QTcF as dependent variable and concentration as a covariate. In

Table 1: Scenarios used to investigate the influence of selection of time points.

Designation	Maximum total number of time points	Number of time points in time window			
		$0 < t < 2$ h	$2\text{ h} \le t \le 4$ h	$4\text{ h} < t \le 8$ h	$8\text{ h} < t \le 24$ h
All	All	All	All	All	All
Equi 8	8	At most 2	At most 2	At most 2	At most 2
Few t_{max}	7	At most 2	At most 1	At most 2	At most 2
Exclude t_{max}	6	At most 2	None	At most 2	At most 2
Sparse	4	At most 1	At most 1	At most 1	At most 1

order to correct for spontaneous circadian effects, a factor representing time was also added [17]. The two models differ in the inclusion of an additional treatment effect not forcing the slope through the origin (zero) [11]. Only an intercept per subject was included as a random effect. From each model, the effect at the observed geometric mean C_{max} was predicted together with a two-sided 90% confidence interval. The random variability of the C_{max} estimate was not taken into account in order to keep the computational burden within reasonable limits. A study was declared negative if the upper bound of the confidence interval was below the threshold of 10 ms, as per ICH E14 guideline [1].

One thousand simulations were performed for each configuration and each TQT study by sampling N_{act} + N_{pla} subjects without replacement, where N_{act} and N_{pla} are the number of subjects used under active drug and under placebo, respectively. The fraction of negative studies out of these simulations was determined. For the method to produce reliable results, this fraction of negative studies should be below a threshold of 5% for an active drug like moxifloxacin, while for an inactive drug, at least 85 or 90% of the simulated studies should be negative.

In a first step, data from simulations with the same number of subjects on active drug and on placebo was used and 6, 9, 12, 15, and 18 subjects per group were selected. The fraction of negative studies was displayed for the two types of models and for moxifloxacin and the simulated inactive drug.

To investigate the role of subjects under placebo in the CE analysis, simulations were conducted by fixing the number of subjects on active drug to 9 and varying the number of subjects on placebo from 3 to 6 and 9. Results for the model with a treatment effect were given for moxifloxacin and for the simulated inactive drug.

Finally, the number of time points included in the models was reduced to investigate the influence of the number of time points in general and the importance of sampling around C_{max}. Therefore, the time points were subsampled according to the scenarios depicted in Table 1. This investigation was performed for a scenario with 9 subjects on drug and 6 on placebo and was based on a model with a treatment effect. The fraction of false negatives was displayed for moxifloxacin and the fraction of false positives for the simulated inactive drug. All computations were performed using *R* [18] and in particular the package nlme [19].

3. Results

Using the model with treatment effect, the fraction of negative studies as function of the sample size (per treatment group) is displayed in Figures 1 and 2. Figure 1(b) shows that the CE method using a model with treatment effect reliably excludes an effect in an inactive drug, while Figure 1(a) shows that it detects an effect, such as the one caused by moxifloxacin. The rate of false negatives is below 5% for all studies and all sample sizes considered (Figure 1(a)), and studies based on the simulated inactive drug are correctly classified as negative in more than 95% of the case for sample sizes of $N \ge 9$.

When a concentration-effect model without a treatment effect is used, a clear effect on the fraction of negative studies becomes apparent. Figure 1(c) shows that with a model without a treatment effect the fraction of negative studies was never below 5% for all studies while with a model with treatment effect it was below 5% for all cases. In the "no-effect scenario," the fraction of negative studies was higher with a model without treatment effect, above 95% even with 6 subjects (Figure 1(d)).

Figure 2 gives the fraction of negative studies with 9 subjects on active drug as a function of the number of subjects on placebo. It shows that, using the model with a treatment effect and only 3 subjects on placebo, a "moxi-like" effect can reliably be detected, while a larger number of subjects on placebo improve the power to exclude a QT-prolonging effect in an inactive drug from above 80% to >95%. With 9 subjects on active drug and 6 subjects on placebo, the fraction of false positive studies can be reduced below 5%, providing a power of >95%.

The results of simulations based on a reduced number of time points are given in Figure 3. Overall, the reduction of the number of time points does not seem to have a strong influence on the performance of the method when the scenario "All" is compared with the remaining scenarios. However, reducing the number of time points around t_{max} results in a slight increase of false negative studies, as represented by the "Exclude t_{max}" scenario. This increase of false negatives is apparent in the results from Study 1 and, to some extent, by Study 3, but not suggested by Study 2. The influence of the selection of time points on the fraction of false positives is not very pronounced.

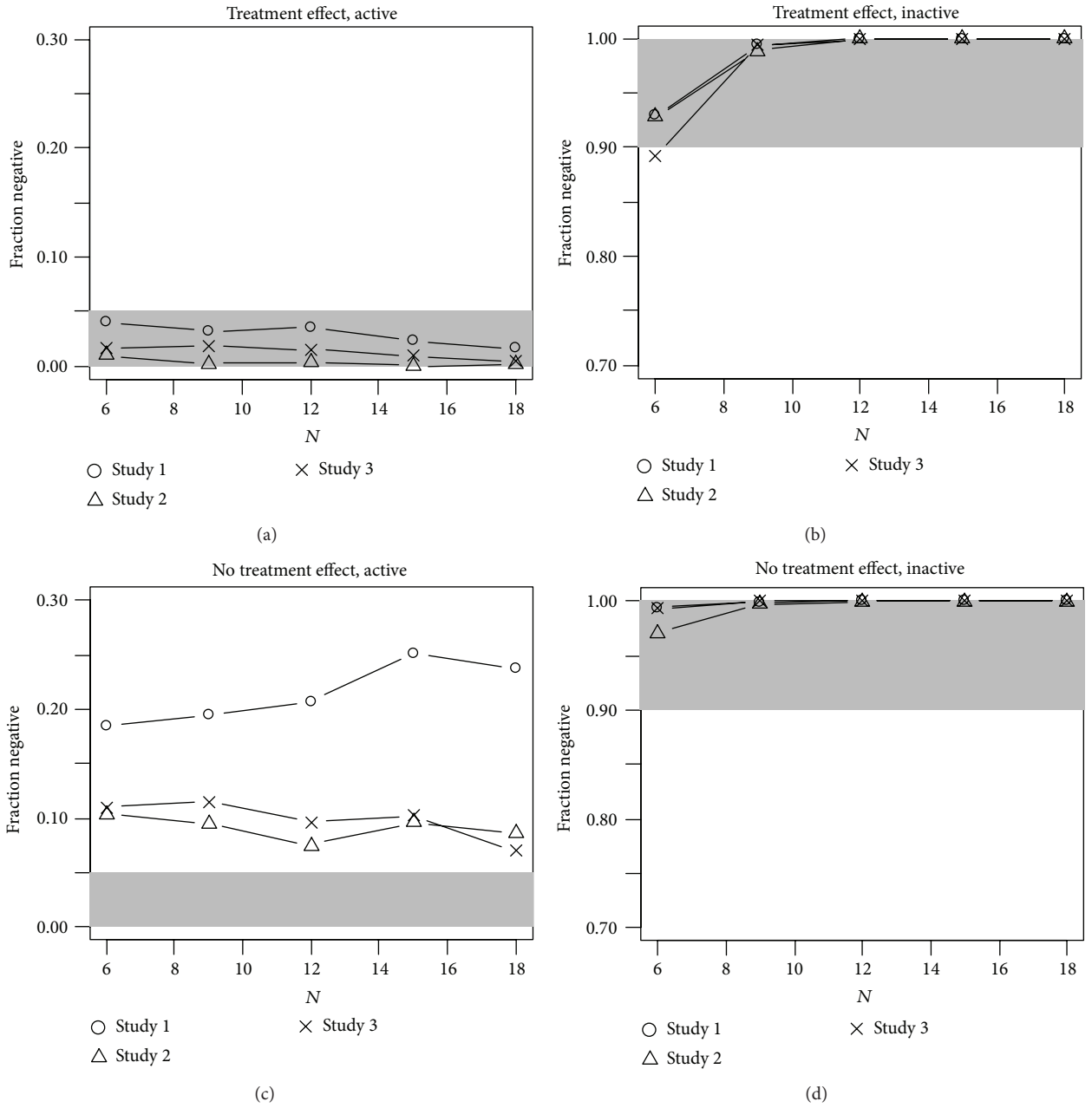

Figure 1: Fraction of negative studies by number of subjects per treatment arm. (a) and (b) Analysis with a model with a treatment effect; (c) and (d) analysis with a model without a treatment effect. Shaded range is considered acceptable.

4. Discussion

In this paper, we used real data from 3 published TQT studies to confirm the findings from Ferber et al. [11], based on another set of studies and, in addition, we investigate the dependence of the results on the selection of time points and on the role of placebo subjects for excluding an effect for an inactive drug. With this approach, we intend to broaden the basis of knowledge on the applicability and the behaviour of CE modelling in small studies.

In order to be acceptable to regulators as an alternative to a TQT study, the rate of false negatives in the analysis of QTc data from SAD or MAD studies must be low. On the other hand, if the method has a substantial risk for a false positive result with respect to QTc prolongation, it becomes unattractive for the sponsor, since, as a minimum, an additional TQT study has to be performed in such a case. Therefore, a good control of the false positive rate is in the interest of the sponsor.

The subsampling simulations presented here confirm that with 9 subjects on active drug and 6 on placebo, the fraction of false negatives under moxifloxacin and that of false positives under a simulated drug with no effect on QT and the pharmacokinetic properties of moxifloxacin are well controlled. All

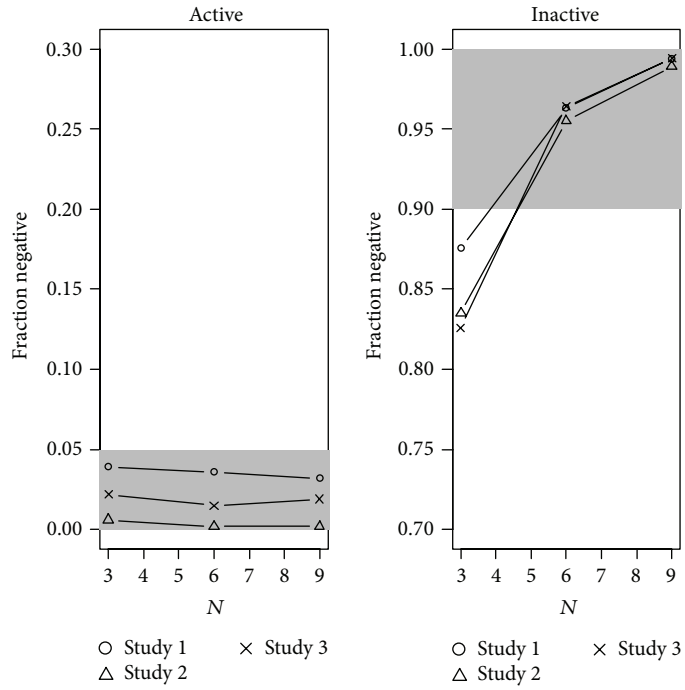

Figure 2: Power of CE modeling as a function of the number of subjects on placebo. In all simulations, 9 subjects were used in the active group, while the number on placebo was varied as given on the x-axis. Shaded range is considered acceptable.

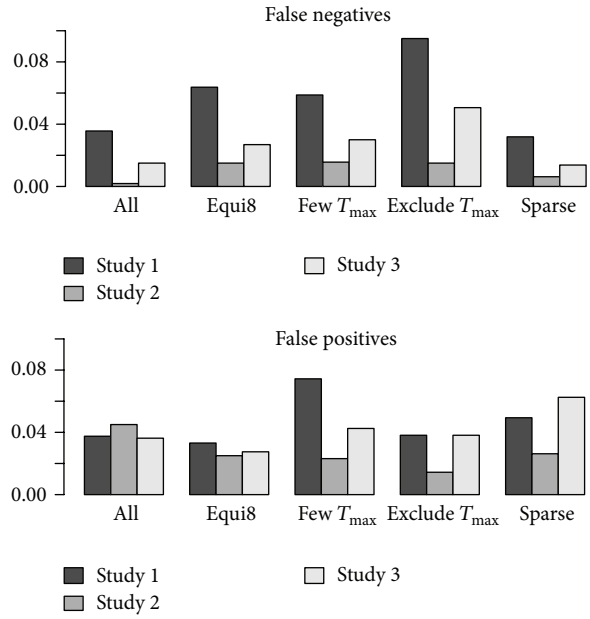

Figure 3: Performance of CE analysis as a function of the time points included. Simulations were based on model with treatment effect with 9 subjects on active drug and 6 on placebo.

studies presented a rate of false negatives and false positives, respectively, below 5% (Figure 2). These sample sizes are close to or below what is usually achieved in the highest dose groups of a SAD study and in the pooled placebo.

The importance of the treatment effect in the model to control the fraction of false negatives has been observed on different data [11]. In terms of the rate of false negatives, when treatment effect is omitted in the CE model and the linear regression is not forced to pass through zero, a rate increase is observed (Figure 1(c)) while the likelihood of false positive results is lower (Figure 1(d)). At this point in time, one can only speculate about the reasons for this phenomenon. A significant treatment effect is usually taken as a sign that model fit can be improved by taking into account nonlinearity and/or hysteresis. However, it should be kept in mind that the goal of CE analysis in Phase I studies is the reliable detection of a QT effect of regulatory concern and not an explanatory description of the PK-PD relationship. Therefore, using a model with treatment effect as default seems to be a reasonable choice. In a real Phase I study, a significant treatment effect would probably trigger further investigations into the appropriateness of the model used. The prospective definition of criteria to ascertain model fit with respect to a greater delay between pharmacokinetics and pharmacodynamics as well as to linearity is a key feature of CE analysis in Phase I studies and one of the topics of current research [9].

The results on the number of subjects on placebo included reinforcing the importance of placebo to obtain a reliable prediction. It may be speculated that the subjects on placebo provide the basis for a reliable estimate of the spontaneous variability over time and allow discriminating this from a drug-mediated effect if the time course of drug concentration is similar to the spontaneous changes.

Another feature varied in this study was the creation of different scenarios with selected time points to determine the importance of the time points around C_{max}. Bearing in mind that in a TQT study the estimated QTc effect at the highest clinically relevant plasma concentration will define the QTc

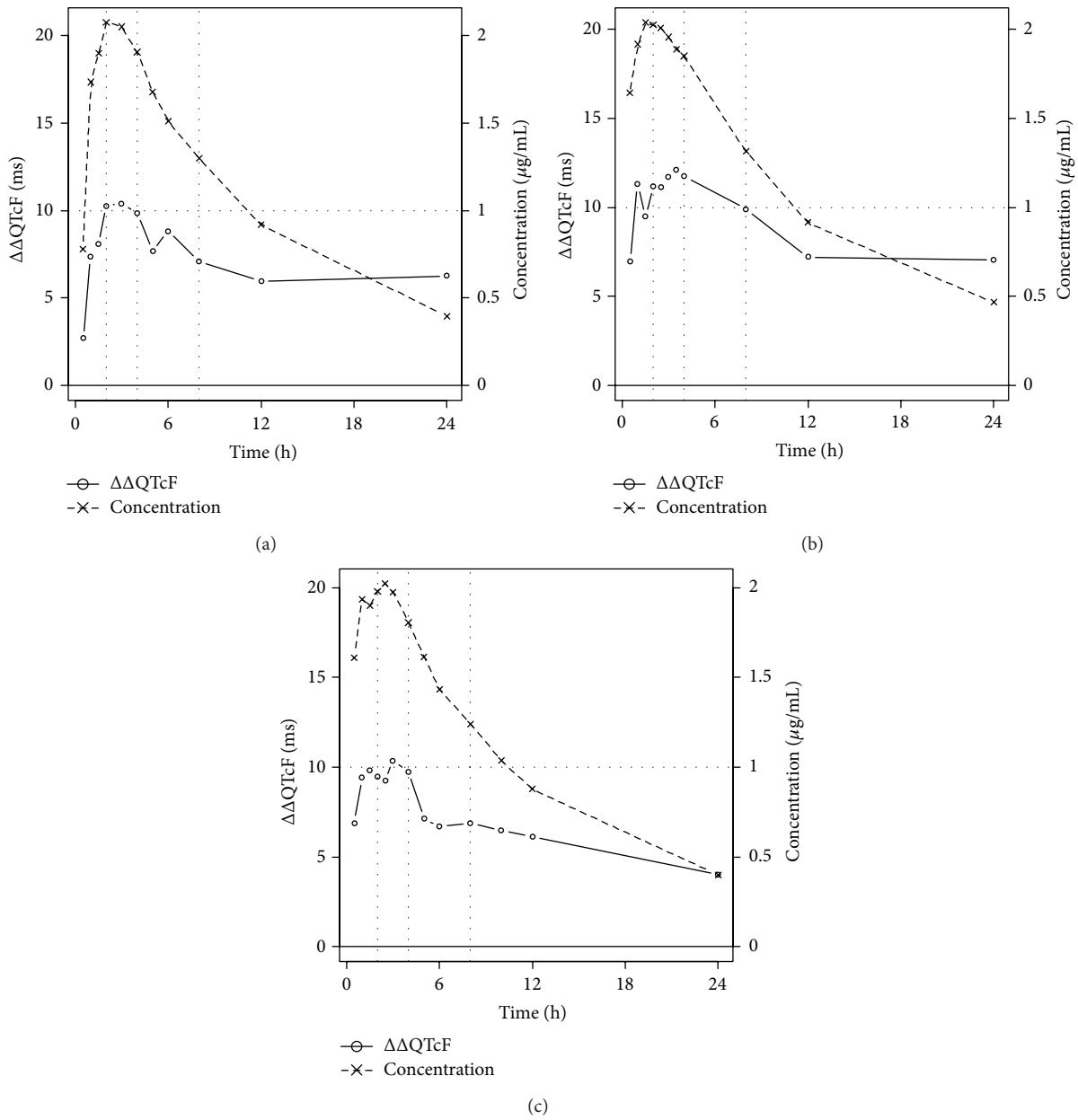

Figure 4: Time course of moxifloxacin plasma concentration and placebo corrected change from baseline of QTcF for each of the three studies, based on all subjects included. (a) Study 1, (b) Study 2, and (c) Study 3.

effect of a drug, the appropriate selection of time points around C_{max} is important to predict the maximum effect. Surprisingly, the method seems relatively stable against the selection of time points. However, a closer look at the characteristics of the three studies explains this finding.

Figure 4 presents the mean time course of the plasma concentration of moxifloxacin as well as the placebo corrected change from baseline of QTcF (ΔΔQTcF) based on all subjects in the respective TQT study. As demonstrated, in all three studies the rise of plasma concentrations and of placebo corrected QTcF starts early and values close to C_{max} are already reached in the first two hours. In particular, t_{max} for Study 2 is well before 2 h (Figure 4(b)). Excluding values in the time window 2–4 h therefore will not remove high concentrations from the model in Study 2 and an effect on the number of false positives is not observed. Only for Study 1 the concentrations outside the window 2–4 h are clearly lower than those in this window. Accordingly, this study shows the highest number of false negatives under the "Exclude t_{max}" scenario, indicating that if t_{max} is missed, the effect will be underestimated.

On the other hand, even reducing the number of time points to 4 per subject (Sparse scenario) seems to have little influence on the quality of the model fit as can be judged from the fraction of misclassifications presented here (Figure 3).

A limitation of this investigation is that all simulations are based on moxifloxacin and, furthermore, 400 mg is

not a supratherapeutic dose. Similar investigations using drugs with other kinetics or with a more complex PK/PD relationship would therefore be useful.

In early phase studies, the doses investigated are usually higher than all doses used in later phases and more than one cohort will contribute to the analysis. As a criterion for a negative QT assessment based on SAD data for CE analysis is to evaluate the QTc effect at plasma concentrations that cover levels seen in patients with impaired clearance and high plasma concentrations of the drug [6], it seems reasonable to expect that, using data from a SAD study, a QTc effect above 10 ms can be excluded with a likelihood of less than 5%.

Also important to note is that this study, as well as other similar simulations studies [9, 11], is based on data from traditionally designed TQT studies primarily focused on ECG analysis. Phase I studies are mainly focused in the pharmacokinetic and safety assessments and often include pharmacodynamics assessments which all can interfere with the accuracy of ECGs measurements. Additionally, eventual adverse events caused by high doses and general nervousness surrounding a first-in-human drug administration can affect the autonomic responses altering the QT/RR relationship which can also present a limitation for the use of Phase I data in simulation studies to detect clinical relevant QTc prolongation.

CE modelling with varied number of subjects on placebo was carried out and showed the importance of placebo for the power of the method, that is, the ability to reliably exclude an effect in an inactive drug. Placebo is routinely included in Phase I studies and, therefore, obtaining a sufficient number of subjects under placebo is not considered an issue.

The apparent robustness of the method against misplacement of time points—as long as values near the maximum concentration are not completely absent—is reassuring. However, at least until a more in depth understanding of the method is reached, the absence of a positive control remains an issue. SAD and MAD studies do not typically include a pharmacological control to confirm ECG assay sensitivity. This is considered a major limitation when using their data to exclude an effect as systematic errors may have occurred limiting the sensitivity of a study thereby giving a false negative result. Unlike random error, which will lead to wider confidence intervals and thereby will not allow excluding a 10 ms change in QTc, systematic errors cannot be reliably detected other than by including a positive control.

Several paths to overcome this lack are being investigated, but up to now, no solution has been generally accepted. We have demonstrated previously that one to four hours after the intake of a carbohydrate rich meal, a physiological QTc-shortening can be reliably observed [4]. The change in QTc is closely correlated with the release of c-peptide in response to raising blood glucose concentrations after a meal and thus is a physiological response rather than the effect of a blocking drug that may be differently metabolised in a significant proportion of the study population [20].

In the setting of a Phase I study, this effect can be estimated from the time course of the change from baseline of QTc that is a byproduct of the CE analysis. We therefore suggest that assessing this shortening of QT by about 5 ms 2–4 h after meal intake compared to the predose value can be developed into a proof of assay sensitivity [21]. It should be noted that the proposed test for assay sensitivity is based on the same data as the investigation of the drug effect on QTcF and is estimated by the same model as the primary analysis [17]. In this way, it becomes unlikely that a systematic error acts only on the drug effect, but not on that of food.

5. Conclusions

This study describes the power of simulated small studies and their use towards the acceptance of alternative approaches that can provide data at the same level of confidence of a TQT study. Here, we focused on a suitable analysis method for the setting of a Phase I study.

Our approach was based on the performance of CE analysis to investigate the confidence and the power of the analysis for detection of QTc changes of clinical relevance by subsampling subjects from three different TQT studies. This simulation confirmed that QTc prolongation can be reliably detected with a small sample size and a drug not causing any QTc prolongation can be identified with a power of more than 90%. Additionally, the power to detect an inactive drug is increased with the number of subjects on placebo, with 6 subjects on placebo showing good power. Although it is important to have a sufficient number of PK and QTc samples around t_{max} to avoid false negative findings, the simulations underline the robustness of the model even if the number of time points is reduced. The choice of an adequate statistical model that includes a treatment effect seems to be important to fully exploit the potential of this method. Our study supports that a model with a treatment effect can be used to exclude a QTc effect similar to moxifloxacin as the fraction of negatives studies is below 5%. However, the influence of pharmacokinetic parameters on the rate of false positives should be further explored to evaluate how lower doses can influence the precision of the concentration/QTc effect model to be used in QT assessment studies.

The proposal to use the effect of food as positive control fits well with the use of a CE model as it can be based on the same data. The results therefore support the assumption that, in many cases, this methodology, based on high quality ECG data, could be used instead of the TQT study that constitutes significant financial burden on clinical stages of drug development.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Two-Step Pseudomaximum Amplitude-Based Confidence Interval Estimation for Oscillometric Blood Pressure Measurements

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Blood pressure (BP) is an important vital sign to determine the health of an individual. Although the estimation of average arterial blood pressure using oscillometric methods is possible, there are no established methods for obtaining confidence intervals (CIs) for systolic blood pressure (SBP) and diastolic blood pressure (DBP). In this paper, we propose a two-step pseudomaximum amplitude (TSPMA) as a novel approach to obtain improved CIs of SBP and DBP using a double bootstrap approach. The weighted median (WM) filter is employed to reduce impulsive and Gaussian noises in the step of preprocessing. Application of the proposed method provides tighter CIs and smaller standard deviation of CIs than the pseudomaximum amplitude-envelope and maximum amplitude algorithms with Student's t-method.

1. Introduction

The maximum amplitude algorithm (MAA) based on oscillometric measurement is the most widely used technique to estimate the average arterial blood pressure [1–5]. The MAA approximates the mean blood pressure as the cuff pressure (CP) at which the maximum oscillation occurs and then linearly relates the systolic blood pressure (SBP) and diastolic blood pressure (DBP) [2, 6]. The blood pressure is constantly changing because of intrinsic physiological oscillations and in response to factors such as stress, exercise, disease, and food. Thus, the SBP and DBP can shift up to 20 mmHg within a few heartbeats and have larger variations over the course of the day [7]. This phenomenon and its serious consequences on blood pressure (BP) measurement are not recognized by most physicians, and it is what makes accurate measurements of BP a difficult task [8]. The American National Standard Institute (ANSI)/Association for the Advancement of Medical Instrumental (AAMI) [9] recommends a maximum allowable system error of ±5 mmHg with standard deviation of 8 mmHg compared to a reference reading done simultaneously by at least two trained nurses. However, the actual physiological variability, which could reach up 20 mmHg, is used to be ignored [8].

Although the oscillometric blood pressure devices are popularly used to estimate the SBP and DBP, these devices provide only one estimate with no confidence interval (CI) and users are not able to distinguish the statistical variance in the estimates from the intrinsic variability exhibited by physiological processes [10]. Since there is no golden standard technique except for auscultatory method, there is no method to determine the variance in BP estimates. If the CI in the BP estimates is too wide, an alert can recommend discarding the measurements and initiating another measurement. Without the CI, it is difficult to make any meaningful decision with the BP estimates. Based on some aggregate statistics, in a home-based monitoring setting, the repeated wide CI can trigger an alarm and alert either the nurse station or the family doctor. Even though this is an important factor of blood pressure estimation, until very recently, there was no

research investigating the estimation of the CI for these blood pressure measurements. Recently, Krakoff [11] proposed that the CIs were computed for SBP, DBP, pulse rate, and heart rate obtained from an Omron HEM725CIC monitor over a period of 7 days, with four measurements per patient (28 measurements per patient), which is not considered large BP measurements. Hence, the Student's t-distribution (ST), instead of asymptotic normal distribution, was utilized to obtain the CI of the SBP and DBP [11]. Although the asymptotic normal approximation is generally used to derive CIs, a large sample size is inevitably required to obtain such CIs. However, it is not feasible to acquire a large number of measurements for each subject using a noninvasive oscillometric blood pressure measurement device, as repeatable conditions for reproducible measurements cannot be guaranteed [12]. As a consequence, standard methods of obtaining the CI such as the one presented in [11] cannot be used for obtaining the CI in blood pressure measurements. This calls for an innovative method that can obtain the CI from a smaller sample size. In this regard, bootstrap approaches with oscillometric blood pressure measurements were presented in [12]. On the other hand, in our previous study [12], we confirmed that the CI using the bootstrap method sometimes becomes too wide or too narrow or too wide in one direction and too narrow in the other because only five measurements for each subject are used. That is, the standard variation of CIs is larger than the average of CIs for the SBP and DBP. Therefore, it is necessary to develop a method that can correct the problem of the CI obtained using a small number of measurements [13]. In this paper, we propose a two-step pseudomaximum amplitude (TSPMA) as a novel method to obtain improved CIs of SBP and DBP using a double bootstrap method [14]. The CIs based on the double bootstrap are significantly to reduce coverage rate errors obtained from single bootstrap method [15]. In particular, the TSPMAs are efficiently obtained from the pseudomaximum amplitudes (PMAs) which are large resample vectors due to the increase in the number of samples using the double bootstrap. Thus, we address the problem of CIs using the TSPMA based on the double bootstrap for the SBP and DBP. Moreover, we perform various experiments in the impulse and Gaussian noisy environments to evaluate the performance of the proposed algorithm. Summarizing our approach, this paper can be regarded as an expanded version of the previous paper [12] with the following enhancements:

(i) developing a method that reduces the standard deviation of the CI of PMA [12] using a small number of measurements;

(ii) using the weighted median (WM) filter to reduce impulsive and Gaussian noises.

Extensive simulation results show that the proposed algorithm offers tighter CI and smaller CIs' standard variation than the conventional algorithms.

2. Methods

Indeed, it is not feasible to obtain a large sample from each subject in BP measurement due to cost reasons. Even when cost is not the core issue, experimental conditions may not provide reproducible BP measurements. In such scenarios, one may have to resort to the method of employing pseudomeasurements as introduced in [12]. In this study, pseudomeasurements are also used to obtain the CI using the double bootstrap technique. The proposed method consists of two main steps to obtain the TSPMA and pseudoenvelope (PE) so that our approach is called two-step pseudomaximum amplitude-pseudoenvelope (TSPMAE).

The block diagram of the proposed approach is given in Figure 1. The upper path of the block diagram shows the first step including the PMA and TSPMA parts of the algorithm, whereas the lower path of the block diagram shows the second step regarding the PEs. These two steps are then utilized to get the CI estimate of BP. The envelopes of oscillometric BP are smoothed using the Gaussian curve fitting and separated into systolic BP and diastolic BP parts of the envelope. These envelopes are used in the lower path as shown in Figure 1. In the upper path, we obtain the maximum amplitude (MA) locations using the MAA technique. Then, the PMA locations are obtained using the nonparametric bootstrap (NPB) [12, 17]. We then also obtain the TSPMA locations reusing the PMA locations based on the NPB. In the next step, the upper, middle, and the lower PMAs and TSPMAs and the locations corresponding to those PMAs and TSPMAs are determined by using the CI technique [12].

In the lower path where the Gaussian curve fitted envelopes are adapted to get the same lengths, two sets of envelope matrices, which are systolic BP and diastolic BP parts, are constructed. The PEs are obtained for systolic BP and diastolic BP parts using the bootstrap technique. By employing the NPB for CI, the upper, middle, and lower PEs are then obtained. Following this, the results from TSPMA path are used to link the PEs and TSPMAs; then the CI estimates are obtained using the mean cuff pressure (MCP) which is computed based on the CP of the five measurements. For more details on the PMA and PEs, the interested readers are referred to [12].

In particular, the preprocessing component is to suppress a noisy signal using the WM filter [18], which is a set of K BP envelope valued weight $\langle W_1, W_2, \ldots, W_K \rangle$ and the observation peak vector $X = [X_1, X_2, \ldots, X_K]^T$. Thus, the WM filter's output is given by the use of the median operator such that

$$\widehat{\beta} = \text{median}\left(|W_1| \lozenge \text{sgn}\left(W_1\right) X_1, \ldots, |W_K| \lozenge \text{sgn}\left(W_K\right) X_K\right), \tag{1}$$

where $\lozenge$ denotes the replication operator and $W_i \in R$ denotes the weighted value for $i = 1, 2, \ldots, K$. Note that the weight signs are uncoupled from the magnitude values of BP envelope and are merged with the observation BP envelope sample as follows [19]:

(1) calculate the threshold $T_0 = (1/2) \sum_{i=1}^{K} |W_i|$;

(2) sort the signed observation sample $\text{sgn}(W_i)X_i$;

(3) sum the magnitude of the weights corresponding to the sorted "signed" samples beginning with the maximum and continuing down in order;

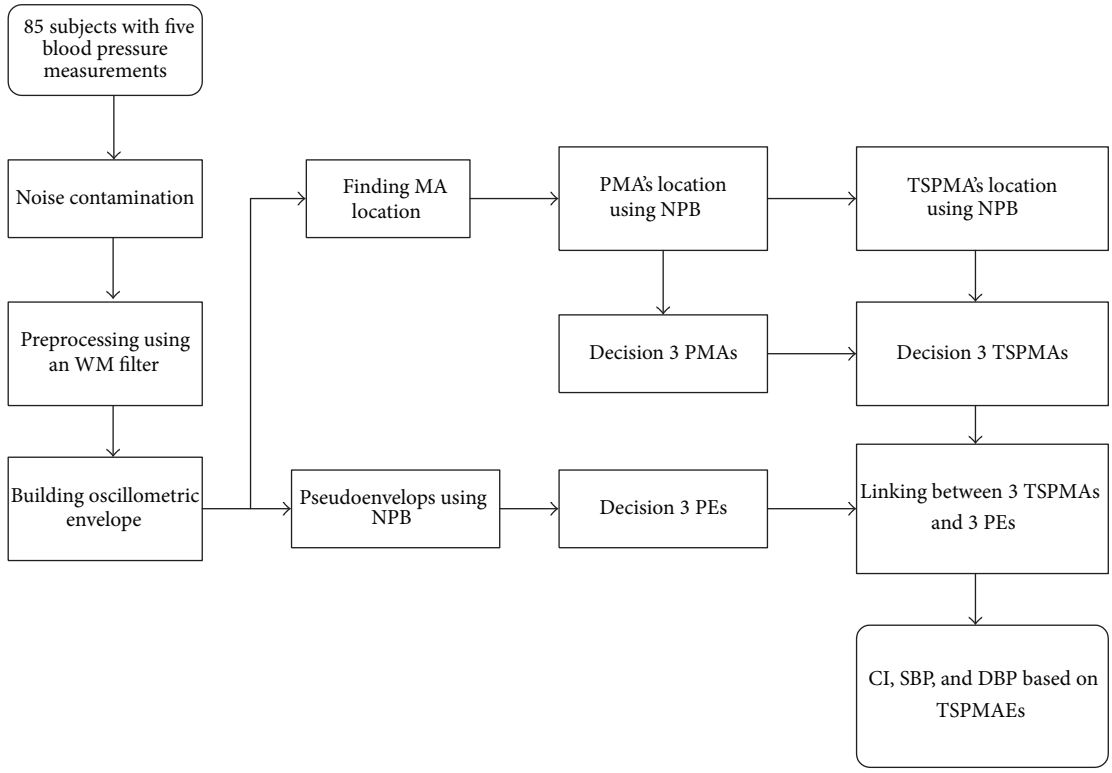

Figure 1: Procedure of TSPMAE based on NPB for improved confidence interval (CI) estimator.

(4) output is the signed sample whose weight magnitude causes the sum to become $\geq T_0$.

2.1. Short Review of Bootstrap. The fundamental concept of the bootstrap technique [17] is to provide a large number of independent bootstrap BP estimates by resampling the original BP estimate $X = (x_1, x_2, \ldots, x_n)$ of n measurements at random from an unknown probability distribution F. Bootstrap resamples $X_1^*(= x_1^*, x_2^*, \ldots, x_n^*), \ldots, X_B^*(= x_1^*, x_2^*, \ldots, x_n^*)$ are acquired by sampling n time randomly drawn with replacement from the original sample X with elements occurring zero, once, or multiple times, where n denotes an original sample size and B denotes a number of resamples. Based on the approach done by Efron and Tibshirani [17], we use $B = 1000$ for CIs. However, this number relies on the particular application. Specifically, as we have only five measurements for each subject, the number of all the possible bootstrap resamples is given as $(2n-1)!/[n!(n-1)!]$. This indicates that the number of bootstrap resamples achieves stability [20] as B approaches 126.

In this paper, we determine the CIs of oscillometric BP measurements using the nonparametric bootstrap [17, 21]. Specifically, the CI $\widehat{\theta}_\alpha^*$ with nonparametric bootstrap (NPB) represents the $100 \cdot \alpha$th percentile of B bootstrap replications $\widehat{\theta}_{(1)}^*, \widehat{\theta}_{(2)}^*, \ldots, \widehat{\theta}_{(B)}^*$. Percentile limit $\widehat{\theta}_l$, $\widehat{\theta}_u$ of intended range $1 - 2 \cdot \alpha$ is simply obtained such that

$$\left(\widehat{\theta}_l, \widehat{\theta}_u\right) = \left(\widehat{\theta}_{(\alpha(B+1))}^*, \widehat{\theta}_{(1-\alpha)(B+1)}^*\right), \quad (2)$$

where l and u are the lower and upper bounds of the CI and α is set to 0.05.

2.2. Review of PMA Using NPB [12]. In this section, we briefly represent the methodology to determine the upper and lower bounds on the CI for MA and the length position of the MA on the oscillometric BP envelope. The NPB method is used to determine the mean of the CI and the range for the SBP and DBP [12]. Firstly, we obtain the MAs and the length of occurrence of the MAs from all the five measurements per subject, respectively, as shown in Figure 2. These raw parameters of the MAs are utilized to find the final PMAs which are then used to obtain the TSPMA using the NPB method [12] as a special case of the double bootstrap method [14].

Suppose that $X = \{x_1, \ldots, x_5\}$ denote the set of five length positions of the MAs and $Y = \{y_1, \ldots, y_5\}$ denote the set of the corresponding five MAs. Based on the NPB on these two sets, we create B of resamples, X_j^*, Y_j^*, $j = 1, \ldots, B$, where $X_j^* = \{x_{1j}^*, \ldots, x_{5j}^*\}$ and $Y_j^* = \{y_{1j}^*, \ldots, y_{5j}^*\}$, respectively. We then compute the mean of all measurements in X_j^* and Y_j^* to obtain $\widehat{\mu}_{X(j)}^*$ and $\widehat{\mu}_{Y(j)}^*$ given by $\widehat{\mu}_{X(j)}^* = (1/N)\sum_{k=1}^N x_{k,j}^*$ and $\widehat{\mu}_{Y(j)}^* = (1/N)\sum_{k=1}^N y_{k,j}^*$, where $N = 5$ and $j = 1, \ldots, B$. The histograms of the bootstrap estimates $\widehat{\mu}_{X(j)}^*$ and $\widehat{\mu}_{Y(j)}^*$ are expressed in Figures 3(a) and 3(b) which represent the length of occurrence of the maxima and PMA from all the five measurements per subject, respectively. We then sort the bootstrap estimates, $\widehat{\mu}_{X(j)}^*$ and $\widehat{\mu}_{Y(j)}^*$, according

to ascending order. Therefore, the sorted PMAs are acquired as $\widehat{\mu}^*_{Y(1)} \leq \widehat{\mu}^*_{Y(2)} \leq \widehat{\mu}^*_{Y(3)} \cdots \leq \widehat{\mu}^*_{Y(B-1)} \leq \widehat{\mu}^*_{Y(B)}$ and the length locations of the PMAs are acquired as $\widehat{\mu}^*_{X(1)} \leq \widehat{\mu}^*_{X(2)} \leq \widehat{\mu}^*_{X(3)} \cdots \leq \widehat{\mu}^*_{X(B-1)} \leq \widehat{\mu}^*_{X(B)}$. Indeed, the desired $100 \cdot (1-\alpha)\%$ nonparametric CIs for position of PMA and the PMA are, respectively, acquired as $(\widehat{\mu}^*_{X(Q_1)}, \widehat{\mu}^*_{X(Q_2)})$ and $(\widehat{\mu}^*_{Y(Q_1)}, \widehat{\mu}^*_{Y(Q_2)})$, where Q_1 is the quotient of $B \cdot \alpha/2$, $Q_2 = B - Q_1 + 1$, and $Q_3 = B/2$. We therefore obtain $Q_1 = 25$, $Q_2 = 976$, and $Q_3 = 500$ with $\alpha = 0.05$ and $B = 1000$. Finally, we get the three positions of the PMA that will be used by the algorithm to estimate CIs of the SBP and DBP, respectively.

2.3. Proposed TSPMA Using Double Bootstrap. The main goal of the TSPMA technique based on the double bootstrap is to provide improved CIs of SBP and DBP with respect to the subject using only five measurements.

In practice, the first step is to abandon the mean as a measure of center in favor of a statistic that is more resistant to outliers [13]. The trimmed mean is the mean of only the center observations in a data set. In particular, the 25% trimmed mean ignores the smallest and largest 25% of the observations [13]. Thus, we acquire pseudomeasurements $\zeta^* = \{\widehat{\mu}^*_{X(Q_4)}, \ldots, \widehat{\mu}^*_{X(Q_5)}\}$ and $\eta^* = \{\widehat{\mu}^*_{Y(Q_4)}, \ldots, \widehat{\mu}^*_{Y(Q_5)}\}$ from (3) as vectors of bootstrap resample, respectively, where Q_4 is $251(= 0.25 \times B + 1)$ and Q_5 is $750(= B - Q_4 + 1)$. Herein, we also create a number of $B(= 1000)$ of resamples $\zeta^{**}_j = \{\chi^{**}_{1j}, \ldots, \chi^{**}_{500\,j}\}$ and $\eta^{**}_j = \{\psi^{**}_{1j}, \ldots, \psi^{**}_{500\,j}\}$ applied from the PMA's set obtained by trimmed means which are $\zeta^*(1 \times 500)$ and $\eta^*(1 \times 500)$, using the NPB for $j = 1$ to B, respectively. Thus, we obtain two matrices (500×1000) such as ζ^{**}_j and η^{**}_j which are calculating the mean of all measurements, where the mean of all resample measurements is given as follows:

$$\widehat{\mu}^{**}_{\chi(j)} = \frac{1}{B_2}\sum_{k=1}^{B_2} \chi^{**}_{kj}, \qquad \widehat{\mu}^{**}_{\psi(j)} = \frac{1}{B_2}\sum_{k=1}^{B_2} \psi^{**}_{kj}, \tag{3}$$

where $\widehat{\mu}^{**}_{\chi(j)}$ denotes the TSPMA and $\widehat{\mu}^{**}_{\psi(j)}$ denotes the length positions of TSPMA, which become vectors (1×1000). Also, $B_2(= 500)$ is the number of the resamples obtained from Q_4 to Q_5.

In the next step, we also sort the TSPMAs and the length positions of the TSPMA in increasing order. The desired $100 \cdot (1-\alpha)\%$ double bootstrap's CIs for position of TSPMA and the TSPMA are, respectively, given by $(\widehat{\mu}^{**}_{\chi(Q_1)}, \widehat{\mu}^{**}_{\chi(Q_2)})$ and $(\widehat{\mu}^{**}_{\psi(Q_1)}, \widehat{\mu}^{**}_{\psi(Q_2)})$, where Q_1 is the quotient of $(B \cdot \alpha)/2$, $Q_2 = B - Q_1 + 1$, and $Q_3 = B/2$, where $\alpha = 0.05$ and $B = 1000$.

2.4. Review of PE Using NPB [12]. In order to obtain the PEs for estimating the CI of the SBP and DBP using NPB, we construct a BP measurement matrix **E** as shown in [12] composing BP envelopes for five measurements for the systolic and diastolic parts of each subject [12]. Each column of BP measurement matrix **E** denotes an BP envelope obtained from the oscillometric measurement. Particularly,

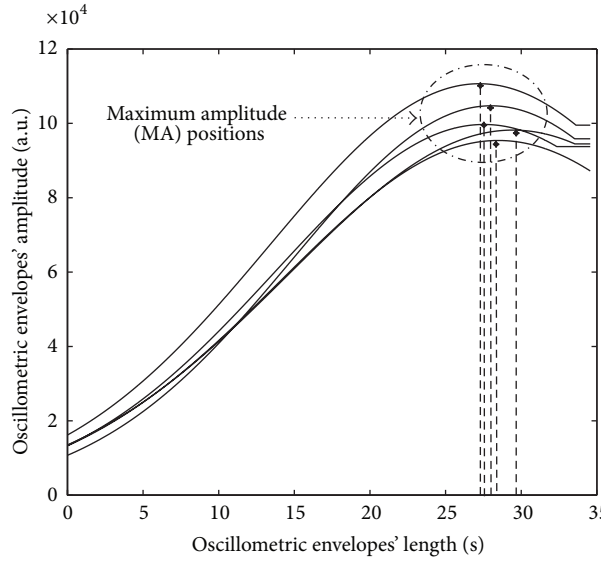

Figure 2: The MA's positions from a subject with five measurements [12].

all measurements are forced to be of length, either by extrapolating length if the measurement is shorter or by truncating the length if the measurement is longer. From the BP envelope matrix **E**, employing NPB method, we acquire B resample envelope matrices $\mathbf{E}^*_1, \ldots, \mathbf{E}^*_B$, where $j = 1, \ldots, B(= 1000)$. The SBP and DBP parts of the envelope are identified utilizing the peak of the envelope. From the beginning to the peak of the BP envelope (corresponding to the decreasing cuff pressure) represents the SBP part and from the peak to the end of the BP envelope represents the DBP part. We then reorder the resampled BP envelope matrices (for systolic and diastolic parts of the envelopes) using the ascending and descending sort techniques (for SBP and DBP parts of the envelopes, resp.). It is noted that each of the sorted matrices has five columns, each corresponding to a BP measurement of length L. We then obtain a single BP envelope per subject as shown in [12]. For more details on the PE, the reader is referred to [12].

In the previous subsection, we obtain the value of the TSPMA utilizing the NPB approach. As the TSPMA estimates may not connect with the end (start) point of the systolic (diastolic) PEs and also in the amplitudes, it may be needful to use signal processing (padding and clipping) to ensure that the location values (both in amplitude and in length) of the TSPMA are based on the PEs. In the final step, we need to obtain the MCP to find the CI estimates of SBP and DBP. In order to estimate the SBP and DBP, systolic and diastolic ratios must be determined. The systolic and diastolic ratios used in our algorithm are 0.70 and 0.45, respectively, which were experimentally decided [1, 2]. Using these ratios, the SBP and DBP points are identified on the TSPMAE, and they are mapped back to the MCP in the SBP and DBP values in mmHg.

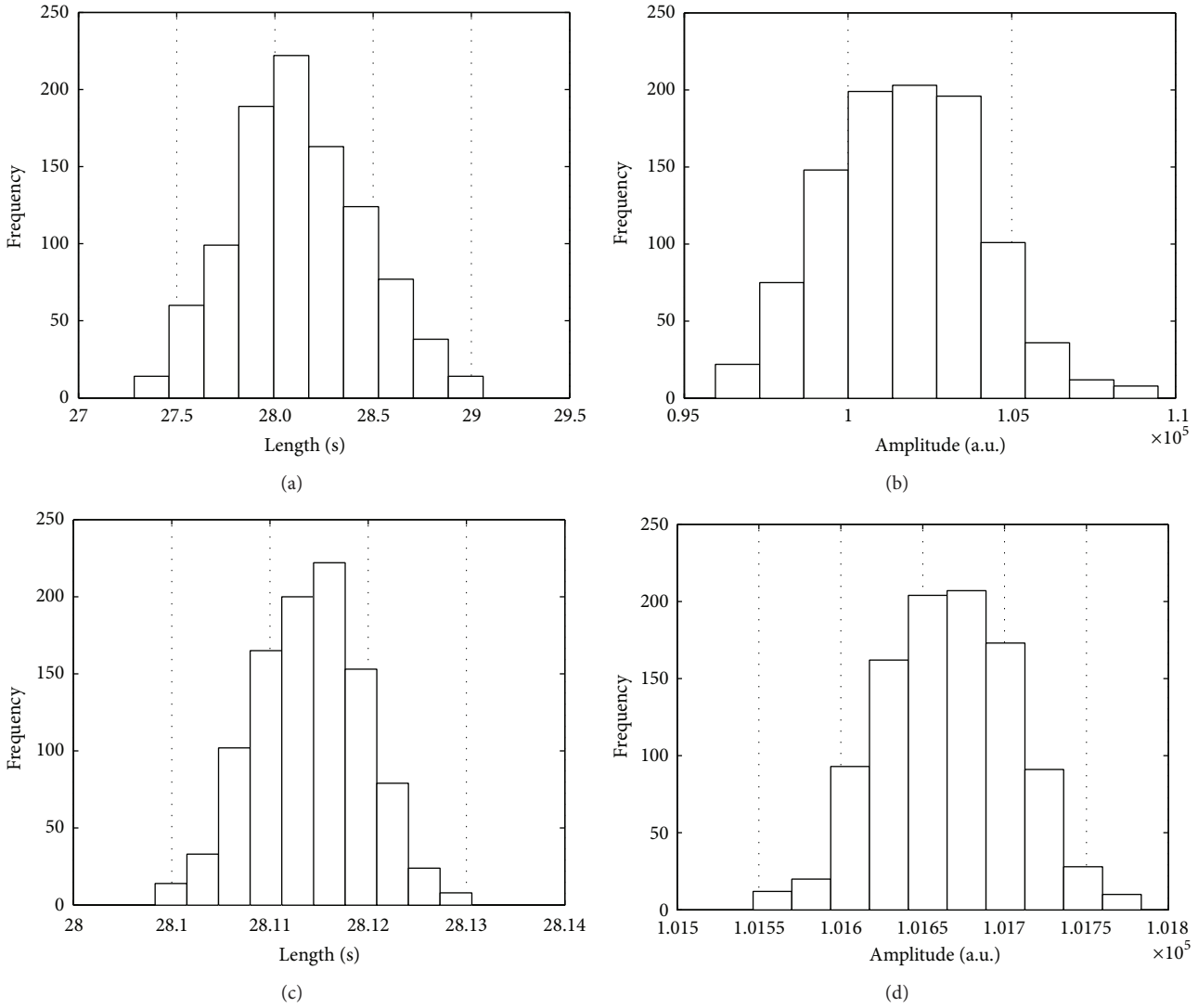

Figure 3: Histograms of the PMA and TSPMA using NPB for one subject. (a) The length positions of the amplitude of the PMA, (b) the amplitude of the PMA, (c) the length positions of the amplitude of the TSPMA, and (d) the amplitude of the TSPMA.

3. Results

This work was approved by the local research ethics committee, and all subjects offered informed consent prior to the BP measurement on the basis of the protocol of the institutional research ethics board. The oscillometric measurements were provided by Biosign Technologies Inc., Toronto, Ontario, Canada, for this work. The experimental BP data set was acquired from 85 healthy subjects aged from 12 to 80, out of which thirty-seven were females and forty-eight were males. Oscillometric BP measurements were obtained from each volunteer (5 set ×85 subjects = 425 total measurements based on a wrist worn UFIT TEN-10 blood pressure device) (Biosign Technologies Inc., Toronto, Ontario, Canada) in accordance with the recommendations of the ANSI/AAMI SP 10 standard [9]. In particular, the two nurse reference readings at the same time are averaged to supply one SBP and one DBP reading. Nurse reference reading of SBP ranged from 78 to 147 mmHg and DBP ranged from 42 to 99 mmHg across total subjects [22]. Note that our procedure of BP measurements consists of an oscillometric blood pressure recoding, followed by readings of SBP and DBP with the help of two trained nurses after a one-minute pause. This was then followed by another one-minute break. The procedure was repeated again four more times to build the recoding of five measurements [22]. Thus, we only acquired the five measurements for each subject because it is not practically possible to obtain a large number of measurements [12].

In order to verify the performance of BP estimation, the mean absolute error (MAE) and the standard deviation (SD) between the estimated BP and the auscultatory nurse measurements were calculated [9, 23, 24] as shown in Table 1. The MAE of the proposed TSPMAE algorithm was compared to that of the PMAE and MAA methods as in Table 1. In addition, the standard deviation (SD) was used to describe a measure of error variability between

Table 1: Summary (averaging 85 subjects with five measurements) of the MAE and SD between the auscultatory nurse measurements of TSPMAE, MAA, and PMAE.

BP (mmHg)	MAE (nurse versus MAA)	MAE (nurse versus PMAE)	MAE (nurse versus TSPMAE)	SD (nurse versus MAA)	SD (nurse versus PMAE)	SD (nurse versus TSPMAE)
SBP	6.52	6.49	6.48	5.94	5.91	5.72
DBP	5.63	5.63	5.60	5.32	5.33	5.10

Table 2: Comparison of average results (85 subjects with five measurements) in CIs (95%) of SBP and DBP using the MAAST, MAAGUM, PMAE, and TSPMAE, where σ is a standard deviation and L and U are lower and upper limits, respectively. MAA with ST is MAAST. MAA with the Guide to the Expression of Uncertainty in Measurement (GUM) is MAAGUM [16].

BP (mmHg)	SBP (σ) CI	DBP (σ) CI	SBP (σ) L	SBP (σ) U	DBP (σ) L	DBP (σ) U
MAAST	13.5 8.1	9.3 5.7	106.7 14.3	120.2 16.5	62.4 10.4	71.7 11.0
MAAGUM	14.1 7.8	10.1 5.3	106.4 14.3	120.5 16.4	62.0 10.4	72.1 10.9
PMAE	2.6 3.1	1.5 2.3	112.4 13.9	115.0 14.9	66.7 10.5	68.2 9.9
TSPMAE	2.4 0.9	1.3 0.5	111.4 14.5	113.7 14.8	68.8 10.2	70.1 10.5

the auscultatory nurse measurements and the estimates obtained using the proposed method. The range of the CI (mean) of the proposed TSPMAE with the bootstrap is smaller than that of the pseudomaximum amplitude-envelope (PMAE) [12] with bootstrap for both SBP and DBP, most likely because of the decrease in the standard deviation through the increase in the pseudomeasurements using the bootstrap method for each subject as shown in Table 2. Figures 3(c) and 3(d) show that the plot of histograms has a small bias though they are roughly normal by the TSPMA using double bootstrap.

Occasionally, the oscillometric wave signal is contaminated by additive noise such as impulsive and Gaussian noises generated from subject's moving artifact, electronic device, and environmental conditions in the processes of BP measurements. However, it is not well defined with the Gaussian model [19]. Thus, to evaluate the robustness of the TSPMAE algorithm in impulsive and Gaussian noisy environments, we generated the impulsive and Gaussian noise. First, the impulsive noise is represented by four parameters: a scale parameter $\gamma > 0$, an index of stability $\alpha \in (0, 2]$, a skewness parameter $\delta \in [-1, 1]$, and a location parameter $\beta \in R$. The scale parameter γ is a key factor to generate impulsive noise, which is similar to the variance of the Gaussian distribution. The stability parameter $\alpha = 0.5$ in our paper measures the thickness of the tails of the distribution. When the skewness parameter is set to $\delta = 0$ in our paper, the stable distribution is symmetric about the location parameter $\beta = 0$ [19]. Based on the generated impulsive noise, we presented simulation results under impulsive and Gaussian noise environments. Indeed, Figure 4 shows an example of the preprocessing of the proposed methodology using the WM filter to reduce the impulsive noise of the oscillometric wave signal with respect to one subject. Figure 5 also shows an example of the processing of the WM filter for a noise artifact caused by subject movement.

In Table 3, we have presented the CIs of the proposed method for SBP and DBP, respectively, in impulsive noisy environments, and also compared the proposed TSPMAE with weighted median (TSPMAEWM) method with the conventional methods (MAAST and MAAGUM) in order to verify the robustness of the proposed method TSPMAEWM. Here, we can not find that the CI of the TSPMAEWM is varied due to the decrease of the γ from 2.0 to 0.5. In this section, we omitted the explanation of white Gaussian noise's generation because it is a basic method. The conventional MAAST and MAAGUM do not work well in white Gaussian noise contaminated environments for all SNRs. However, the proposed TSPMAEWM works well except for SNRs of 5 and 10 dB in white Gaussian noise contaminated scenarios as given in Table 4.

4. Discussion

The goal of this paper is to derive the improved CIs for SBP and DBP estimates when only a small number of blood pressure measurements are available. The degree of error variability between the readings obtained with the proposed method and those obtained with the auscultatory nurse method as the reference (Table 1) was investigated. The MAE of the SBP and DBP obtained through the TSPMAE is similar to that obtained with the MAA. The proposed TSPMAE method has a MAE about 5-6 mmHg with respect to the auscultatory nurse measurements. Although the proposed approach in this paper does not focus on providing robust blood pressure estimates, the result of the MAE does not fall within the 5 mmHg recommendations of the AAMI SP 10, but the result of the SD is satisfied by the AAMI [9]. In addition, we note that the TSPMAE method has also much smaller spread (i.e., small standard deviation) in the CI when compared with the MAAST, MAAGUM, and PMAE based on the average results for 85 subjects in Table 2. We also note that the SDs of the SBP and DBP of the TSPMAE method are similar to those of SBP and DBP of the MAAST as shown in columns 4 to 7 of Table 2. According to the bootstrap principle, the distributions of the SBP and DBP

Table 3: Comparison of average results (85 subjects with five measurements) in CIs of SBP and DBP using the PMAEWM and TSPMAEWM in impulsive noisy environments within $\gamma = 2.0$ and $\gamma = 0.5$ where σ is a standard deviation and N/A denotes not available.

BP (mmHg)	γ	SBP (σ)	DBP (σ)	CI SBP (σ)	CI DBP (σ)
MAAST and MAAGUM	2.0	N/A	N/A	N/A	N/A
MAAST and MAAGUM	1.5	N/A	N/A	N/A	N/A
MAAST and MAAGUM	1.0	N/A	N/A	N/A	N/A
MAAST and MAAGUM	0.5	N/A	N/A	N/A	N/A
PMAEWM	2.0	114.1 14.2	67.6 10.3	2.6 3.0	1.6 2.4
PMAEWM	1.5	114.2 14.2	67.5 10.2	2.6 3.0	1.5 2.4
PMAEWM	1.0	114.2 14.3	67.5 10.2	2.6 3.0	1.5 2.3
PMAEWM	0.5	114.1 14.2	67.5 10.2	2.6 3.0	1.6 2.3
TSPMAEWM	2.0	113.5 14.1	68.6 10.3	2.5 1.0	1.4 0.6
TSPMAEWM	1.5	113.2 14.2	68.5 10.2	2.4 0.9	1.3 0.6
TSPMAEWM	1.0	113.2 14.1	68.6 10.2	2.4 0.9	1.3 0.5
TSPMAEWM	0.5	113.1 14.2	68.6 10.2	2.4 0.9	1.2 0.5

Table 4: Comparison of average results in CIs of SBP and DBP using the PMAEWM and TSPMAEWM under Gaussian noisy environments within SNR 5 dB to SNR 20 dB where n (= 85) is the number of subjects with five measurements and σ is a standard deviation and N/A denotes not available.

BP (mmHg)	SNR	SBP (σ)	DBP (σ)	CI SBP (σ)	CI DBP (σ)
MAAST and MAAGUM	5 dB	N/A	N/A	N/A	N/A
MAAST and MAAGUM	10 dB	N/A	N/A	N/A	N/A
MAAST and MAAGUM	15 dB	N/A	N/A	N/A	N/A
MAAST and MAAGUM	20 dB	N/A	N/A	N/A	N/A
PMAEWM	5 dB	N/A	N/A	N/A	N/A
PMAEWM	10 dB	N/A	N/A	N/A	N/A
PMAEWM	15 dB	114.3 14.8	67.4 10.1	3.5 4.4	1.7 2.5
PMAEWM	20 dB	114.1 14.1	67.4 10.2	3.1 5.3	1.5 2.1
TSPMAEWM	5 dB	N/A	N/A	N/A	N/A
TSPMAEWM	10 dB	N/A	N/A	N/A	N/A
TSPMAEWM	15 dB	113.3 14.8	68.4 10.1	3.5 1.5	1.7 0.8
TSPMAEWM	20 dB	113.1 14.1	68.4 10.2	3.1 1.4	1.5 0.7

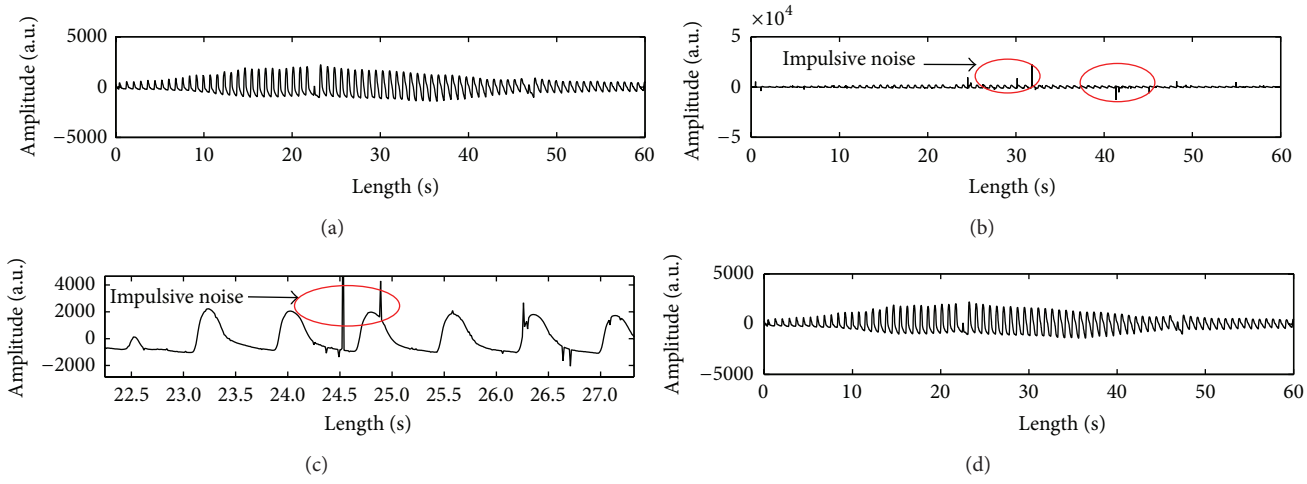

Figure 4: This figure shows examples as (a) oscillometric waveform (OMW); (b) OMW with impulsive noise ($\gamma = 2.0$); (c) enlarged figure of an OMW with impulsive noise ($\gamma = 2.0$); (d) cleaned OMW used the WM filter, where γ is a scale parameter.

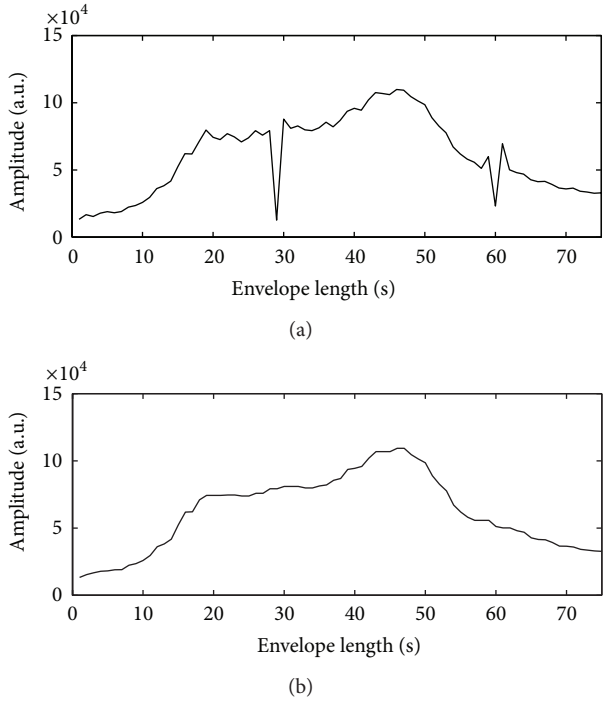

Figure 5: Comparison of envelops: (a) top panel, envelope, and (b) bottom panel, envelope with the WM filter.

of the TSPMAE represent the sampling distribution of the original measurement successfully. An interesting point is that the SDs of the CI obtained from the TSPMAE method have much smaller SDs than the CI obtained from the PMAE, but the average range of the CIs for both methods is very similar. This indicates that the range of the CI fluctuates higher for the method which uses the PMAE. The range of the CI developed using the TSPMAE method is more stable across the 85 subjects. The decrease of the standard deviation in the CI results obtained by our method is clear and it demonstrates the advantage of the proposed TSPMAE method over the existing PMAE method for obtaining the CI from a small set of measurements as shown in Table 2.

The bottom histograms of Figure 3 confirm that TSPMA produces the distributions tighter than the PMA. Note that Figures 3(c) and 3(d) represent the histograms' plot along with frequency closer to normal than PMA as shown in Figures 3(a) and 3(b). Thus, we also confirmed TSPMA to overcome the weakness of small measurement of the PMA [12]. And Table 3 shows that the comparisons of CIs of the PMAE with WM (PMAEWM) and TSPMAEWM are almost unaffected in impulsive noise contaminated scenario. On the contrary, it is found that the conventional methods (MAAST and MAAGUM) represent a very weak characteristic on impulsive noise contaminated scenario. In Table 3, the PMAEWM and TSPMAEWM methods are well represented in that the robust characteristic is made regardless of the variation of the impulsive noise at contaminated scenarios from $\gamma = 2.0$ to $\gamma = 0.5$. However, the proposed TSPMAE also does not work well in low SNRs of 5 and 10 dB white Gaussian noise contaminated scenarios as given in Table 4. Unfortunately, the conventional methods (MAAST and MAAGUM) also do not work in white Gaussian noise contaminated environments from SNRs of 5 to 20 dB. In Figures 4 and 5, we present simulation results under impulsive and Gaussian noise environments. Indeed, we used the calculation of the correlation coefficient to verify the robustness of the proposed TSPMAEWM in impulsive noise contaminated oscillometric waveform. As a result, the correlation coefficient between the top panel of Figure 4(a) and the bottom panel of Figure 4(d) was 0.99, which can be considered relatively very high. In Figure 5, the measured envelope abruptly fluctuates (compared to envelopes in top and bottom panels). As a result, the measured envelope becomes smooth (compared to envelopes in top and bottom panels at envelope's length from 18 to 30 sec and from 57 to 60 sec), and all small notches in the contaminated oscillometric envelope are eliminated. Therefore, the proposed TSPMAEWM is quite effective in impulsive and Gaussian noise environments.

5. Conclusion

In conclusion, we demonstrated that the CI obtained using the proposed method is narrower and has a narrower standard deviation than CIs obtained using other methods. Note that this paper does not focus on accuracy directly while the accuracy in the estimates can be obtained through the standard error from the golden reference. If the standard deviation of the estimate is low and if there is no bias, then the estimates may be deemed to be accurate. The decrease of the standard deviation in the CI results is attributed to the increase in the effective number of samples due to resampling using bootstrap principles. The results indicate that the proposed methodology reduces the standard deviation and consequently improved the accuracy. Our results imply that the proposed methodology is the best available to deal with small samples of blood pressure measurements. Our proposed method outperformed conventional methods for obtaining the CI under regular recording, impulsive, and white Gaussian noisy conditions. Indeed, the proposed technique can be used extensively as a potential application for self- and home-based monitoring scenario. We expect further studies to extend this methodology for older people with stiff arteries and wide pulse pressures and we will present these results in a near future.

Abbreviations

MAA: Maximum amplitude algorithm
CP: Cuff pressure
SBP: Systolic blood pressure
DBP: Diastolic blood pressure
BP: Blood pressure
ANSI: American National Standard Institute
AAMI: Advancement of Medical Instrumental
CI: Confidence interval
ST: Student's t-distribution
TSPMA: Two-step pseudomaximum amplitude
PMA: Pseudomaximum amplitude

WM:	Weighted median
PE:	Pseudoenvelope
TSPMAE:	Two-step pseudomaximum amplitude-pseudoenvelope
MA:	Maximum amplitude
NPB:	Nonparametric bootstrap
MCP:	Mean cuff pressure
MAE:	Mean absolute error
SD:	Standard deviation
ME:	Mean error
MAAST:	Maximum amplitude algorithm with Student's t-distribution
MAAGUM:	Maximum amplitude algorithm with Guide to the Expression of Uncertainty in Measurement
PMAE:	Pseudomaximum amplitude-envelope
PMAEWM:	Pseudomaximum amplitude-envelope with weighted median
TSPMAEWM:	Two-step pseudomaximum amplitude-envelope with weighted median.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

MicroRNAs Based Therapy of Hypertrophic Cardiomyopathy: The Road Traveled So Far

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Hypertrophic cardiomyopathy (HCM) is an autosomal dominant disease characterized by variable expressivity, age penetrance, and a high heterogeneity. The transcriptional profile (miRNAs, mRNAs), epigenetic modifications, and posttranslational modifications seem to be highly relevant for the onset of the disease. miRNAs, small noncoding RNAs with 22 nucleotides, have been implicated in the regulation of cardiomyocyte function, being differentially expressed in several heart diseases, including HCM. Moreover, a different miRNA expression profile in the various stages of HCM development is also observed. This review summarizes the current knowledge of the profile of miRNAs characteristic of asymptomatic to overt HCM patients, discussing alongside their potential use for diagnosis and therapy. Indeed, the stability and specificity of miRNAs make them suitable targets for use as biomarkers for diagnosis and prognosis and as therapeutical targets.

1. Introduction

Hypertrophic cardiomyopathy (HCM) is the most common familial form of cardiomyopathies, occurring mainly due to mutations in genes encoding for the cardiac contractile apparatus [1, 2]. Among the 1,400 mutations identified has responsible for HCM, 70% of them are in sarcomere genes cardiac β-myosin heavy chain (*MYH7*) and cardiac myosin binding protein C (*MYBPC3*) [3]. Mutations are inherited in an autosomal dominant pattern affecting 1 : 500 individuals in the general population, with 60% of the sarcomere gene mutations being described as familial HCM [1, 4]. The progression of symptoms in HCM is not straightforward, due to morphological and pathological heterogeneity, age dependency and incomplete penetrance, resulting in prognosis uncertainty. Accordingly, the clinical outcome of HCM is diverse, ranging from asymptomatic patients to cardiac arrhythmias, congestive heart failure, and sudden cardiac death [5]. A high percentage of patients are asymptomatic or mildly symptomatic and diagnosis is made during family screening or incidentally by the observation *via* conventional echocardiography of an unexplained left ventricular wall thickening [6]. Due to the heterogeneity of the pathology, the diagnosis is made in the middle or late adulthood when the morphology and functional debility of the heart have already progressed [6]. This is particularly devastating for asymptomatic or mildly symptomatic young patients that may experience sudden cardiac death [7].

One of the most striking features of HCM is the inexistence of a correlation between genotype and phenotype, has family members carrying the same mutation developed distinct symptoms [4, 8, 9]. The clinical outcome of HCM is likely to be the sum of genetic mutations with age-related decline in protein-protective mechanisms and environmental factors such as lifestyle, degree of physical exercise, and blood pressure [10, 11]. In this regard, the transcriptional profile, epigenetic modifications, and protein posttranslational modifications seem to be crucial to the events in the cardiomyocyte that will trigger the onset of HCM [11, 12]. In the last decade microRNAs (miRNAs), small noncoding endogenous RNAs

that regulate gene expression by directing their target mRNAs for degradation or translational repression, were revealed as important regulators of the heart physiology [12–15], with a characteristic expression profile in different cardiovascular diseases [16–18]. The small size (22 nucleotides in length) and stability make miRNAs suitable targets for silencing by antisense oligonucleotides or by restoring their function by using synthetic double stranded miRNAs or viral vector based overexpression [16, 19]. Moreover, the discovery of the presence of miRNAs in the bloodstream [20] highlighted the possibility of their use as circulating biomarkers for HCM. However, the role of miRNAs in the progress of HCM is still not completely understood. In this review the possibility of the use of miRNAs based therapy throughout the course of events occurring in the path from asymptomatic to overt HCM will be discussed.

2. MicroRNAs

Since their discovery in 1993, miRNAs have been increasingly recognized as an important class of regulatory small noncoding RNAs that function as negative regulators of gene expression [17, 21]. Approximately 60% of protein coding genes are regulated by miRNAs [22]. Concerning the function of miRNAs, some act as key regulators of a particular cellular process, affecting the expression of hundreds of genes simultaneously, while others may regulate specific individual mRNA targets or regulate target mRNAs cooperatively [22]. These miRNA regulatory networks are important in the "fine tuning" of the overall protein expression in cells and are important in the cellular responses to stress [23].

miRNAs are transcribed by RNA polymerases II and III from different genomic locations [24–27] and can be located in introns of protein coding genes, such as the miR-25-miR-93-miR-106b cluster in an intron of the *MCM7* gene, or in exons of protein coding genes, as miR-985 located in the last exon of *CACNG8* gene [27]. miRNAs are also transcribed from introns of protein noncoding genes such as the miR-15a-miR-16-1 cluster in an intron of the *DLEU2* gene and from exons of noncoding genes such as miR-155 coded by an exon of *BIC* gene [27]. Pri-miRNA are long molecules (more than 1 Kbp) that fold into a characteristic secondary structure, comprising a terminal loop, a stem of approximately 33 base pairs (bp), and flanking segments of single stranded RNA [27]. In the nucleus, the microprocessor complex which contains Drosha RNase cleaves the pri-miRNAs 11 bp away from the junction of single stranded RNA with double stranded RNA [28, 29] (Figure 1). This cleavage generates precursor miRNAs (pre-miRNAs) that maintain the stem-loop conformation [30]. Pre-miRNAs are then exported from the nucleus to the cytoplasm by the RanGTP-binding export receptor, exportin 5 [31] (Figure 1). In the cytoplasm, the loops from pre-miRNAs are then cleaved by Dicer RNase, thus forming the mature double stranded miRNA with 22 nt [16, 32, 33] (Figure 1). These miRNAs are incorporated as single stranded RNAs into the RNA-induced silencing complex, RISC [30] (Figure 1). Genetic silencing is accomplished by base pairing between the seed region (nucleotides 2–8 in the 5′ region) of the miRNA in the RISC complex and the complementary region in the 3′-untranslated region of the target mRNAs [34].

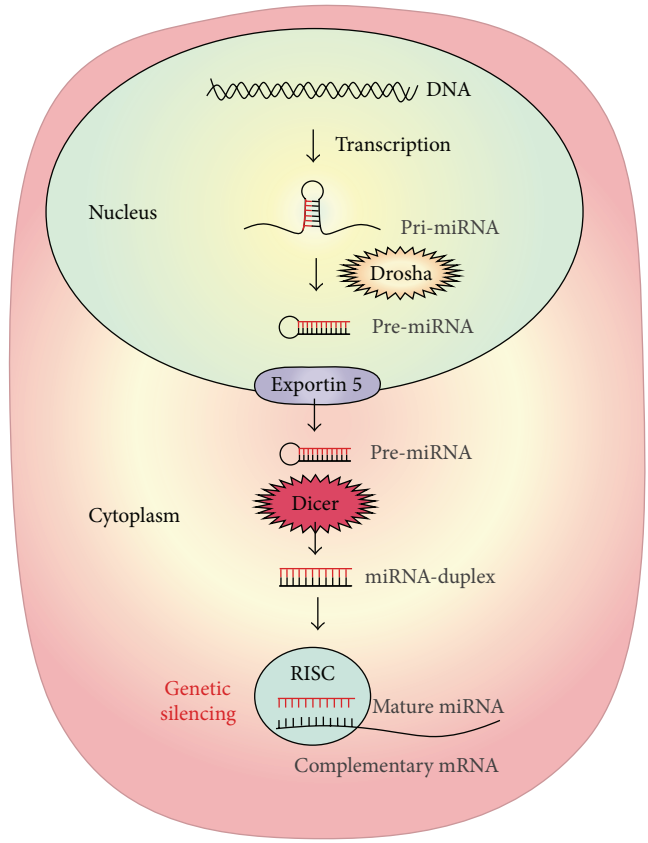
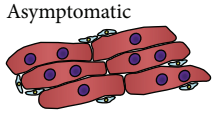
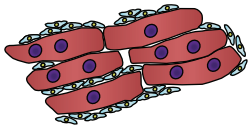
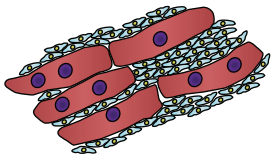


Figure 1: Schematic representation of miRNA biogenesis and function. The biogenesis of miRNAs is initiated in the nucleus by the transcription of pri-miRNAs that, after hydrolyzation mediated by the RNAse Drosha, form pre-miRNA. After transport to the cytoplasm mediated by exportin 5, the loop of pre-miRNAs is cleaved by Dicer RNAse, forming the miRNA-duplex, which is incorporated in the RISC complex. The miRNA-duplex is then separated forming the mature miRNA that will inhibit translation by base pairing with the 3′UTR of the target mRNA.

3. miRNAs in Hypertrophic Cardiomyopathy

Perturbations of the sarcomere function due to HCM related mutations result in an energy deficiency that ultimately compromise the relaxation capacity of the cardiomyocyte and consequently the force of contractibility [35, 36]. These events will develop distinct cardiac histopathological features, such as cardiomyocytes hypertrophy, which are indicators of an early stage of HCM pathology [7, 37]. Further events that precede cardiomyocyte hypertrophy include myocyte disarray, consisting in an asymmetric distribution of the hypertrophic cardiomyocytes within the ventricle, and interstitial fibrosis, mainly due to an increased synthesis of collagen as a consequence of sarcomere mutations and accumulation of fibroblasts after cardiomyocyte death [3, 37, 38]. This maladaptive cardiac remodeling contributes to the development of HCM pathology, such as left ventricular (LV) hypertrophy and

Table 1: miRNA expression profile in the development of hypertrophic cardiomyopathy (HCM). The development of the pathology is associated with the stress imposed to cardiomyocytes due to mutations in genes of the cardiac contractile apparatus. The passage from asymptomatic to mildly asymptomatic stages is related to the heart morphology (schematically represented). In mildly asymptomatic patients, a cardiac remodeling, consisting in cardiomyocyte (red cells) hypertrophy, fibrosis mediated by an increased synthesis of interstitial collagen, cardiomyocytes spatial misalignment, and substitution of dead cardiomyocytes by fibroblasts (blue cells), is performed [3, 41]. Overt HCM is characterized by a cardiac left ventricular hypertrophy higher than 15 mm. miRNAs whose expression is consistently altered in tissues and in circulation are in bold.

	Murine heart	Human heart		Circulating miRNAs
Asymptomatic 	*Downregulated* **miR-1** [40] miR-133 [40]			
Mildly asymptomatic (cardiac remodeling) 	Upregulated **miR-21** [40] miR-132 [40] miR-214 [40] miR-331 [40] *Downregulated* **miR-1** [40]			*Upregulated* *Fibrosis* miR-29a [15] *Cardiomyocyte hypertrophy* miR-27a [15] miR-29a [15] miR-199a-5p [15] *Other functions* **miR-21** [15] miR-26a [15] miR-30a [15] miR-126-3p [15] miR-133a [15] miR-143 [15] miR-145 [15] miR-155 [15] miR-199a-3p [15] miR-483-5p [42]
Overt HCM 	*Upregulated* **miR-21** [40] miR-31 [40] miR-34b-3p [40] miR-132 [40] miR-142 [40] miR-214 [40] miR-222 [40] *Downregulated* **miR-1** [40] miR-30b-5p [40] miR-30c [40] miR-30e [40] miR-133a [40] miR-133b [40] miR-150 [40] miR-486-5p [40]	*Upregulated* **miR-21** [43] miR-34b* [44] miR-92a [45] miR-96 [44] miR-130b [43] miR-132 [44] miR-181a-2* [44] miR-184 [44] *Downregulated* **miR-1** [45] miR-10b [44] miR-10a [44] miR-10b* [44] miR-30b [45] miR-133b [45] miR-139-5p [43] miR-139-3p [43] miR-144* [43] miR-150 [43] miR-191 [45]	miR-204 [44] miR-222* [44] miR-383 [44] miR-371-3p [44] miR-497 [44] miR-590-5p [45] miR-708 [44] miR-208b [45] miR-218 [45] miR-363 [43] miR-374 [45] miR-451 [43] miR-454 [45] miR-486-3p [43] miR-495 [45] miR-1246 [43] miR-3141 [43]	

miR-X*: antisense miRNA star.

arrhythmias, and subsequent appearance of several clinical manifestations, such as obstruction of the outflow tract in the LV or LV systolic dysfunction, which ultimately result in poorer prognosis due to an increased risk of heart failure or sudden death [3, 7, 37, 38]. The morphological heterogeneity and incomplete penetrance of HCM render the study of the pathology progression and the establishment of an analogy between mutation and miRNA profile difficult. Nevertheless, several miRNAs were described as being important regulators of the cardiomyocytes hypertrophy and fibrosis [39], and a different miRNA expression profile in ventricle cardiomyocytes between the early and end stages of HCM pathology was described [40]. Hence, three major phases can be considered in HCM progression as follows: (i) Asymptomatic phase, considered as the early stage of the pathology where no major pathophysiological changes have been developed; (ii) mildly asymptomatic phase, considered as the onset of the disease where the cardiomyocyte relaxation ability is compromised resulting in an expansion of cardiomyocyte hypertrophy and myocardium fibrosis; and (iii) overt HCM phase, which is the symptomatic stage of HCM, characterized by overt LV hypertrophy and arrhythmias. Table 1 resumes the differential expression profile of miRNAs throughout the course of HCM pathology and the following chapters will discuss their possible use in the diagnostics and prognostics of HCM phenotype.

3.1. Asymptomatic HCM. Due to obvious limitations in the study of asymptomatic patients, no studies have been published regarding the miRNA profile during the asymptomatic stage of HCM related mutations carriers. However, a recent study of Bagnall and coworkers [40] revealed a differential miRNA expression profile since the predisease state of the animals (after 5 days). In this study, the miRNA profile of the ventricles of a transgenic HCM double mutant mouse model suggested downregulation of miR-1 and miR-133 in a primary stage of the disease prior to a pathophysiological change [40]. Interestingly, the expression profile of both miRNAs seems to be maintained throughout the course of HCM progression (Table 1) [40, 42, 46]. Downregulation of miR-1 is a well-described event that occurs during cardiomyocyte hypertrophy [46–50], which is a response of the terminally differentiated cardiomyocytes to mechanical or pathological stress, consisting in an increase in cell size and concomitant reinduction of the fetal gene program [51]. While miR-1 is highly abundant in terminally differentiated cardiomyocytes, its level is lower in the developing embryonic hearts of mice and hypertrophic cardiomyocytes [46, 49].

Despite the tempting suggestion for the use of miR-1 and miR-133 as biomarkers to identify subjects who are at risk of the development of HCM symptoms and/or as therapeutic targets, this will only be conceivable if the alteration of abundance of these miRNAs in asymptomatic patients can be identified through minimal-invasive procedures. Interestingly, an increased circulating concentration of miR-1 and miR-133 in patients with overt HCM was registered [15, 52]. The contradictory higher levels of circulating miR-1 and miR-133 in HCM patients and lower concentration in hypertrophic cardiomyocytes reflect ischemic episodes and consequent cardiomyocyte death [42]. Because of the irreversible mutation-induced perturbations of the cardiomyocyte structure and function established in the postnatal period [53] it is plausible that ischemic death might occur in a higher frequency throughout life of an HCM patient. Hence, an increased concentration of circulating miR-1 and miR-133 even in asymptomatic patients can be hypothesized.

3.2. Mildly Asymptomatic HCM. The knowledge of regulation of cardiac hypertrophy and fibrosis mediated by miRNAs, particularly in HCM related pathologies, represents novel signatures of disease that can be targeted for restraining clinical phenotypes [15, 39, 54]. Gain-of-function/loss-of-function approaches *in vitro* using cardiomyocytes cultures and *in vivo* studies using mutant mouse heart as a model allowed taking insights into the role of miRNAs in the mechanisms involved in HCM development and revealing miRNAs that can be used as biomarkers of different traits of the pathology. miRNAs involved in cardiomyocyte hypertrophy can be classified as being prohypertrophic and antihypertrophic, if the mechanisms they regulate increase or decrease the degree of hypertrophy [55]. Examples of prohypertrophic miRNAs are miR-23a [56] and miR-499 [18], while miR-22 [57], miR-26b [58], miR-451 [43], and miR-98/let-7 [59] are antihypertrophic miRNAs.

3.3. Overt HCM. Efforts have been made in order to find the relation between HCM related mutations and cardiomyocyte miRNA profile. However, they are limited by the use of animal models or by studies performed only in an advanced state of HCM, which is mainly due to the invasive procedures for sampling.

Nevertheless, a differential miRNA expression profile induced by HCM pathologies was observed by Kuster and coworkers [44] that analyzed the miRNA expression profile in affected heart tissues of patients carrying mutations in *MYBPC3* and by Palacín and coworkers [45], which, inclusively, were able to find a different miRNA profile between the hearts of HCM patients with distinct mutations (Table 1). Furthermore, Vignier and coworkers [60] suggested a distinct circulating miRNA profile between cardiovascular ischemic pathologies and cMyBP-C related cardiomyopathy.

There are some limitations imposed by the current murine models used for HCM studies. Although animal models can provide information about the release kinetics of miRNAs, these findings cannot be directly transferred to humans, because of the different physiological parameters and species specific differences in miRNA expression. However, despite the species specific differences, several miRNAs were found to be equally regulated in murine and humans with overt HCM (Table 1), with upregulation of miR-21, miR-132, and miR-222 and downregulation of miR-1, miR-30b, miR-133b and miR-150.

3.4. Circulating miRNAs in HCM. One of the most exciting possibilities of the characterization of circulating miRNA expression profile is its use as biomarkers for diagnosis and prognosis. Indeed, circulating miRNAs fulfill several criteria that make them suitable for use as clinical biomarkers, such as accessibility through minimal-invasive procedures, a long half-life within the sample, possibility of a rapid and accurate detection, high degree of specificity and sensitivity, and ability to differentiate pathologies [61]. Interestingly, studies of the miRNA profile of the plasma of HCM patients performed by Roncarati and coworkers [15] suggested a disease specific profile that distinguished between HCM and aortic sclerosis hypertrophies. These authors found that 3 miRNAs (miR-199a-5p, miR-199a-27a, and miR-199a-29a) correlated with hypertrophy but only miR-29a is significantly associated with both hypertrophy and fibrosis, identifying it as a potential biomarker for myocardial remodeling assessment in HCM [15]. However, further studies are needed that may reveal an HCM specific circulating miRNA profile that could distinguish primary HCM even before development of symptoms. The quality and integrity of RNA extracted from hearts or biological fluids are fundamental for miRNA profiling based on microarrays and real-time PCR. This is a destructive testing that limits the number of samples available for the HCM studies. Differences in the sample quality and integrity may justify some discrepancies between studies.

Recently, gold nanobeacons have been proven useful tools for diagnostics [62]. Due to the design of an antimiR oligonucleotide bound to a gold nanoparticle it was possible to detect the hybridization of the miR-21 to its respective antimiR due to an increase of the gold nanobeacons' fluorescence [63].

Table 2: Resume of miRNAs based therapies targeting cardiomyocytes hypertrophy and fibrosis. The oligonucleotide modification, miRNA, the model used in the study, and the obtained result are depicted. LNA: locked nucleic acid.

	Oligonucleotide modification	miRNA	Model	Result	Ref.
AntimiR	AntagomiR	miR-133	C57BL/6 mice	Repression resulted in cardiac hypertrophic phenotype	[46]
	AntagomiR	miR-21	Heart failure induced by pressure overload mice	Repression reduces cardiac ERK-MAP kinase activity, inhibits interstitial fibrosis, and attenuates cardiac dysfunction	[66]
	AntagomiR	miR-132	Heart failure induced by pressure overload mice	Repression rescues heart hypertrophic phenotype	[67]
	LNA-modified oligonucleotide	miR-208a	Diastolic heart failure rats	Repression resulted in reduction of cardiac remodeling	[68]
RNA-mimic	Adenoviral vector containing miR-133a-2 precursor sequence	miR-133	AKT induced heart hypertrophy mice	Overexpression resulted in attenuation of cardiac hypertrophic phenotype	[46]

This methodology allows simultaneously the detection of a specific miRNA directly on a blood sample without the need of disrupting cells for RNA extraction and at the same time permits a specific silencing [63]. These gold nanobeacons have also the potential to be further functionalized with specific targeting moieties (e.g., antibodies) in order to achieve specific targeting [64]. The application of these nanoformulations for simultaneous detection and silencing of miRNAs directly from blood samples can overcome the bias introduced by sample quality and integrity that may justify some discrepancies between studies, opening a new avenue for future early diagnosis, prognosis, and therapy in hypertrophic cardiomyopathy.

4. Therapies Based on miRNAs

The ability of miRNAs to be naturally transported in biological fluids and delivered to cells makes them good targets for therapy in the context of cardiomyopathy [23, 65].

Therapies based on miRNAs involve the restoration of miRNA function that has been done through the use of synthesized miRNA-duplexes (miRNA-mimics), which are double stranded oligonucleotides including the mature miRNA sequence and the complementary passenger strand [19, 65]. The passenger strand can be chemically modified, with cholesterol, for instance, to improve cellular uptake [19]. Targeting of these miRNA-mimics can be augmented through the use of lenti-, adeno-, or adeno-associated viruses (AAV) [19, 20, 65]. On the other side, miRNAs silencing has been accomplished by the use of synthetic oligonucleotides with 8–25 nt of length, complementary to the seed sequence of the miRNA of interest, called antimiRs [19, 65]. Chemically modified antimiRs have been successfully used in order to increase their binding affinity to the target miRNA, biological stability, and pharmacokinetic properties [19]. The antagomiRs are a class of antimiRs in which the oligonucleotides are conjugated with cholesterol [65]. Other frequent chemical modifications of the antimiRs include 2-O-methyl-modified oligonucleotides and locked nucleic acid-(LNA-) modified oligonucleotides [19, 65].

HCM therapeutics based on miRNAs involves the modulation of expression of these noncoding RNAs in cell, by inactivating the function of prohypertrophic miRNAs using synthetic miRNAs or by using miRNAs mimics oligonucleotides of antihypertrophic miRNAs [19].

The possibility of the use of miRNAs based therapy in treatment of HCM is highlighted by several *in vivo* studies [46, 66, 67] that were able to inhibit cardiomyocyte hypertrophy and fibrosis (Table 2). With the purpose to study the effect of miR-133 in the cardiomyocyte hypertrophy, Carè and coworkers [46] successfully used a miR-133 RNA-mimic combined with an adenoviral vector and an antagomiR in the hearts of a mouse model of AKT induced heart hypertrophy and in a normal C57BL/6 mouse line, respectively. Similarly, Thum and coworkers [66] and Ucar and coworkers [67] were able to inhibit cardiomyocyte hypertrophy using an antagomiR for miR-21 and for miR-132, respectively, in mice models with heart failure induced by pressure overload of the left ventricle. An antimiR for miR-208a was successfully used to reduce miR-208a expression in a rat model of diastolic heart failure [68]. However, possibly due to the limited knowledge on HCM progression, miRNAs based therapies are yet to be made.

Despite the promising use of therapy based miRNAs, modulation of the function of miRNAs raises several concerns. The uptake of miRNA-mimics can result not only in the restoration of miRNA function in affected cells but also in the overexpression of the miRNA in other cells. In the same way, the inhibition of miRNA activity by antimiRs can be done on off-target locations. Therefore, targeting would be important in order to meliorate miRNA therapy, which can be accomplished with nanotherapies [62]. Furthermore, the alteration of systemic levels of miRNAs can perturb the homeostasis of circulating miRNA and disturb normal functions in cells and tissues and thus create unwanted side effects.

5. Conclusions

The study of miRNAs expression profiles that will allow its use for diagnosis, prognosis, and therapeutics of HCM is still in its infancy. The high heterogeneity of HCM pathology renders the detection of a disease specific miRNA profile difficult. Furthermore, due to the invasive procedures required for human cardiomyocytes analysis, the analysis of the cardiac miRNA profile is only possible when the disease reached a terminal state. The use of murine models has proven to be fruitful to study the several stages of the disease. Nanobiotechnology allows the simultaneous detection and silencing of miRNAs directly from blood samples will speed up early diagnosis, prognosis, and therapy in hypertrophic cardiomyopathy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Hypoglycaemia, Abnormal Lipids, and Cardiovascular Disease among Chinese with Type 2 Diabetes

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We recruited a group of 6713 consecutive Chinese patients with T2D but normal renal and liver function who were admitted to one of 81 top tertiary care hospitals in China. Mild hypoglycaemia was defined as having symptomatic hypoglycaemia in one month before hospitalization. Severe hypoglycaemia was defined as having hypoglycaemia that needed assistance from other people in three months before hospitalization. Prior cardiovascular disease (CVD) was defined as having coronary heart disease, stroke, or peripheral arterial disease. Of 6713 patients, 80 and 304 had severe and mild hypoglycaemia episodes, respectively, and 561 had CVD. Patients with severe and mild hypoglycaemia episodes were more likely to have prior CVD (32.5% versus 16.5% versus 7.7%, $P < 0.0001$). Both mild and severe hypoglycaemia were associated with increased risk of CVD (adjusted odds ratios (ORs): 2.64, 95% CI: 1.85–3.76 for mild hypoglycaemia; 6.59, 95% CI: 3.79–11.45 for sever hypoglycaemia) than those patients free of hypoglycaemia. Further adjustment for lipid profile did not change these two ORs. In the same way, the ORs of lipid profile for CVD were similar before and after adjustment for hypoglycaemia. We concluded that hypoglycaemia and lipid profile were independently associated with increased risk of CVD.

1. Introduction

Hypoglycaemia is the most common acute episode in the management of type 2 diabetes (T2D) [1]. Recent several large randomised clinical trials (RCT) rekindled a strong interest in hypoglycaemia. In the ACCORD (Action to Control Cardiovascular Risk in Diabetes) [2], the intensive management aiming at achieving glycated haemoglobin (HbA1c) target (<6.0%) resulted in more frequent hypoglycaemia that needed assistance and unexpected more deaths in the intensive management arm. The ADVANCE (Action in Diabetes and Vascular Disease: PreterAx and DiamicroN Modified Release Controlled Evaluation) also found that intensive glucose control aiming at achieving HbA1c <6.5% led to more frequent severe hypoglycaemia (2.7% versus 1.5% in the standard-control group; hazard ratio, 1.86; 95% CI, 1.42 to 2.40) but all-cause mortality was similar in both groups [3]. The Veterans Affairs Diabetes Trial (VADT) [4] also reported significantly more episodes in the intensive-therapy group than in the standard-therapy group ($P < 0.001$). The epidemiological analysis of the ACCORD trial data [5] found that symptomatic severe hypoglycaemia was associated with an increased risk of death in both the intensive glucose control arm and the standard glucose control arm. Similarly, epidemiological analysis of the ADVANCE trial data [6] also reported that severe hypoglycaemia was associated with a significant increase in the risks of macrovascular and microvascular events and death from cardiovascular and any causes. These findings from epidemiological analysis of trial data are consistent from observational studies [7, 8]. Additionally, hypoglycaemic episodes may increase the risk of cardiac arrhythmias [9], dementia [10], and accidents [11].

However, detailed analysis of the ACCORD trial data suggested that the increased death risk in the intensive glucose arm could not be attributable to more frequent hypoglycaemia [5], and the authors of the ADVANCE group [6] also suggested that hypoglycaemia might be a marker of vulnerability to those clinical events. Indeed, the data of Hong Kong Diabetes Registry [12] suggested that severe hypoglycaemia that needed hospitalization identified vulnerable patients with T2D at high risk of cancer and mortality due to sharing some cancer phenotypes including low low-density lipoprotein cholesterol (LDL-C) plus low triglyceride [13] and low high-density lipoprotein cholesterol (HDL-C) [14]. Also noted is that a recent meta-analysis of cohort studies suggests that the association between hypoglycaemia and cardiovascular disease could not be entirely attributable to comorbid severe illness [8]. It remains to be known whether hypoglycaemia and abnormal lipid profile are independent in their associations with cardiovascular disease (CVD). Thus, our study aimed to investigate whether symptomatic or severe hypoglycaemia and abnormal lipid profile identify different groups at increased risk of CVD, that is, both having independent associations with CVD.

2. Methods

2.1. Patients. In 2013, Chinese Hospital Association (CHA) set up a systematic management program of hyperglycemia in inpatients with type 2 diabetes (T2D) admitted to top tertiary hospital to improve the care of inpatients with T2D in China and, in particular, to learn the profile of hypoglycaemia and associated factors. A total of 81 top tertiary care hospitals in 27 cities from 21 provinces were invited and agreed to participate in the study. The inclusion criteria were as follows: (1) patients with T2D admitted to the department of endocrinology; (2) using a basal bolus plus meal time insulin insensitive management scheme; and (3) being between 18 and 80 years of age. The exclusion criteria were as follows: (1) liver dysfunction defined as alanine aminotransferase (ALT) or aspartate aminotransferase (AST) ≥2.5-fold of the upper limits of the normal range, 0–40 U/L; (2) renal dysfunction defined as serum creatinine ≥125 μmol/L in male and ≥110 μmol/L in female or chronic kidney disease (CKD); (3) pregnancy or lactation; and (4) inability to communicate in a normal way.

From May 2013 to August 2013, we successfully recruited 6713 patients with T2D from the 81 hospitals and used them in the final analysis. Ethical approval was obtained from the People's General Army (PLA) Hospital Clinical Research Ethics Committee and written informed consent was obtained from all patients for data analysis and research purpose.

2.2. Clinical Measurements. Measured parameters included body weight, body height, and sitting BP (after 5 minutes of rest). Fasting blood was taken for measurement of glycated hemoglobin (HbA_{1c}), lipids including total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglyceride, renal and liver function, complete blood count, and HbA_{1c}. The first-morning urine was used to calculate urinary albumin to creatinine ratio (ACR). Albuminuria was defined as ACR ≥2.5 mg/mmol in men and ≥3.5 mg/mmol in women. The abbreviated Modification of Diet in Renal Disease (MDRD) Study formula recalibrated for Chinese [15] was used to estimate glomerular filtration rate by the following equation: estimated GFR $= 186 \times [\text{SCR} \times 0.011]^{-1.154} \times [\text{age}]^{-0.203} \times [0.742 \text{ if female}] \times 1.233$, where SCR is serum creatinine expressed as μmol/L (original mg/dL converted to μmol/L) and 1.233 is the adjusting coefficient for Chinese [16]. Chronic kidney disease (CKD) was defined as eGFR <60 mL/min/1.73 m^2. The cut-off points recommended by the American Diabetes Association (ADA) [17] were used to define abnormal lipids, that is, LDL cholesterol <2.6 mmol/L, triglyceride <1.7, and HDL cholesterol ≥1.0 mmol/L in male and ≥1.3 mmol/L in female.

2.3. Definition of Hypoglycaemia and Cardiovascular Disease. Patients were asked whether they had asymptomatic hypoglycaemia defined as plasma glucose ≤3.9 mmol/L but without any symptoms in one month before hospitalization, whether they had symptomatic hypoglycaemia with or without plasma glucose ≤3.9 mmol/L in one month before hospitalization, and whether they had severe hypoglycaemia defined as having hypoglycaemia that needed assistance from other people in three months before hospitalization. In this study, mild hypoglycaemia was defined as symptomatic hypoglycaemia in one month before hospitalization and severe hypoglycaemia defined as hypoglycaemia that needed assistance from other people in three months before hospitalization. A prior coronary heart disease (CHD) was defined as having CHD. CHD included myocardial infarction, ischemic heart disease, coronary revascularization, percutaneous transluminal coronary angioplasty, or coronary atherectomy. Similarly, a prior stroke (but not transient ischemic attack) before or at this visit was defined as having stroke no matter whether the stroke was completely or incompletely recovered. Peripheral arterial disease (PAD) was defined by lower limb amputation, revascularization for PAD, or absence of foot pulses as confirmed by an ankle : brachial ratio <0.90 measured by Doppler ultrasound examination. In this study, CVD was defined as having either CHD or stroke or PAD.

2.4. Statistical Analysis. The Statistical Analysis System (Release 9.30; SAS Institute, Cary, NC) was used to analyse the data. All continuous variables were expressed as mean (standard error, SE) or median (interquartile range, IQR) as appropriate while categorical variables were expressed as percentages (number, *n*). Body mass index (BMI) was calculated as body weight in kilograms divided by squared body height in meters. Normality of distribution of continuous variables was checked using Q-Q plots. Log transformation of continuous variables was performed before comparisons if they were not normally distributed.

General linear model was used to perform analysis of variance to compare the differences among the three groups of patients with mild hypoglycaemia and severe hypoglycaemia and without mild or severe hypoglycaemia. Bonferroni

test was used to adjust for multiple comparisons. The binary logistic regression was used to obtain odds ratios (ORs) of severe hypoglycaemia and mild hypoglycaemia versus nonhyperglycemia for CVD before and after adjusting for levels of LDL-C, HDL-C, and triglyceride as well as ORs of LDL-C, HDL-C, and triglyceride for CVD before and after adjusting for hypoglycaemia. This procedure was also used to obtain ORs of abnormal lipids for either severe hypoglycaemia or mild hypoglycaemia. The generalized logit model was used to obtain ORs of abnormal lipids for severe hypoglycaemia and mild hypoglycaemia, respectively. A structured adjustment scheme was used to adjust for confounding effects of other variables, as shown in Tables 2 and 3.

As adjustment for use of lipid lowering drugs might fail to completely remove the confounding effects of use of these drugs, we further perform a sensitivity analysis for the main analysis after exclusion of 408 users of lipid lowering drugs.

3. Results

3.1. Characteristics of the Study Patients. Patients with severe hypoglycaemia had an older age but those with mild hypoglycaemia had a younger age than those without hypoglycaemia. Patients with severe hypoglycaemia or mild hypoglycaemia had a longer duration of disease and lower BMI than patients without. The percentages of obesity and overweight were decreased steadily from nonhypoglycaemia to mild hypoglycaemia and then to hypoglycaemia. HbA1c was observed to be lower in mild hypoglycaemia than in severe hypoglycaemia. There were marked increases in the levels of LDL-C and triglyceride and the percentages of high LDL-C and high triglyceride from nonhypoglycaemia to mild hypoglycaemia and then to hypoglycaemia. However, the level of HDL-C was also increased and the percentage of low HDL-C decreased from nonhypoglycaemia to mild hypoglycaemia and then to hypoglycaemia. A total of 561 patients had prior CVD, that is, CHD, stroke, and PAD. There were marked increases in the percentages of CVD from nonhypoglycaemia to mild hypoglycaemia and then to hypoglycaemia (Table 1).

3.2. Abnormal Lipids for Hypoglycaemia. Increased LDL-C was associated with mild hypoglycaemia, severe hypoglycaemia, or both in multivariable analysis. The OR of LDL-C per mmol/L was 1.17 (95% CI: 1.04–1.31) in multivariable analysis. However, increased but not decreased HDL-C was associated with increased risk of mild hypoglycaemia (OR per mmol/L: 1.10, 95% CI: 1.03–1.17) and either mild or severe hypoglycaemia (OR: 1.09, 95% CI: 1.02–1.16) but not associated with severe hypoglycaemia (Table 2).

3.3. Hypoglycaemia for Cardiovascular Disease. Severe hypoglycaemia and, to a lesser extent, mild hypoglycaemia were associated with increased risks of CVD in univariable and multivariable analyses. After adjusting for traditional risk factors and drug use, patients with severe hypoglycaemia were more likely to have developed CVD (OR: 6.59, 95% CI: 3.79–11.45) than those patients free of hypoglycaemia. Patients with mild hypoglycaemia were also at markedly increased risk of CVD than those patients without hypoglycaemia (OR: 2.64, 95% CI: 1.85–3.76). Further adjustment for lipids did not change the ORs of hypoglycaemia for CVD (Table 3).

3.4. Lipids for Cardiovascular Disease. Increased LDL-C and triglyceride and decreased HDL-C were associated with increased risk of CVD in univariable analysis. After adjusting for traditional risk factors and drug use, decreased HDL-C and increased triglyceride were still significantly associated with CVD (OR of HDL-C per mmol/L: 0.89, 95% CI: 0.80–0.99; OR of triglyceride per mmol/L: 1.08, 95% CI: 1.02–1.14). Further adjusting for hypoglycaemia only slightly changed the sizes of ORs of HDL-C and triglyceride (Table 3).

3.5. Sensitivity Analysis. In the sensitivity analysis, exclusion of lipid lowering drug users slightly enhanced the associations between hypoglycaemia and CVD while it did not change the effect sizes of lipids for CVD although the latter did not reach statistical significance (Table 4).

4. Discussions

Our study found that although levels of lipids were associated with hypoglycaemia among Chinese patients with T2DM, hypoglycaemia and levels of lipids were independently associated with increased risk of CVD, that is, the association between hypoglycaemia and CVD not being attenuated by levels of lipids and the association between levels of lipids and CVD not being attenuated by hypoglycaemia.

Many studies [7, 8] including the epidemiological analysis of the large trial data [5, 6] reported that severe hypoglycaemia was associated with increased risk of cardiovascular disease and mortality. Consistently, our findings confirmed that Chinese T2D patients with normal kidney dysfunction and liver function who had either mild or severe hypoglycaemia were associated with markedly increased risk of cardiovascular disease. On the other hand, there were a few studies that investigated risk factors for hypoglycaemia or demographic and clinical features of patients with hypoglycaemia. For example, advanced age, low BMI, poor glycaemic control [12], cognitive impairment, current use of sulphonylureas and current insulin use [18], and renal failure [12, 18, 19] were reported to be associated with hypoglycaemia. Even few studies investigated whether hypoglycaemia is characterized by abnormal lipids. In this regard, a Hong Kong group reported that cancer subphenotypes including low LDL-C, low triglyceride, or low HDL-C as well as copresence of low LDL-C and low triglyceride predicted hospital admission due to hypoglycaemia among Hong Kong Chinese with T2D [12]. In our study, increased LDL-C was associated with increased risk of CVD. However, unexpectedly, increased HDL-C but not decreased HDL-C was associated with increased risk of CVD. Of note, the data from Hong Kong showed that severe hypoglycaemia was associated with increased risk of cancer specific death but not associated with CVD specific death [20]. Thus, the data of Hong Kong study were likely to suggest that shared lipid profile of hypoglycaemia and cancer may be responsible for the increased risk of hypoglycaemia and

Table 1: Clinical and biochemical characteristics of study patients by hypoglycaemia.

Variables	Nonhypoglycaemia (n = 6329)	Mild hypoglycaemia (n = 304)	Severe hypoglycaemia (n = 80)	P value
	Mean or % (S.E. or n)	Mean or % (S.E. or n)	Mean or % (S.E. or n)	
Age, year	56.4 (0.13)†,‡	55.5 (0.60)†	61.5 (1.18)‡	<0.0001
Male gender	56.7% (3589)	52.0% (158)	62.5% (50)	0.1490
BMI, kg/m^2	24.0 (0.03)†,‡	22.7 (0.18)†,§	21.7 (0.35)‡,§	<0.0001
BMI ≥ 24.0 but <28 kg/m^2	39.5% (2497)	22.7% (69)	10.0% (8)	<0.0001
BMI ≥ 28 kg/m^2	9.2% (580)	7.2% (22)	0% (0)	
Duration of diabetes, year*	3.6 (0.05)†	5.0 (0.25)†	4.4 (0.48)	<0.0001
Systolic BP, mmHg	131 (0.2)	131 (0.7)	131 (1.3)	0.8935
Diastolic BP, mmHg	81 (0.1)	81 (0.4)	81 (0.9)	0.6782
HbA1c, %	10.4 (0.02)†	10.0 (0.09)†	10.3 (0.17)	0.0006
LDL-C, mmol/L	3.15 (0.01)†,‡	3.36 (0.06)†	3.64 (0.11)‡	0.0001
LDL-C ≥ 2.6 mmol/L	80.0% (5064)	89.1% (271)	96.3 (77)	<0.0001
HDL-C, mmol/L*	1.90 (1.39–2.90)†,‡	2.60 (1.90–3.20)†	2.50 (2.00–2.90)‡	<0.0001
HDL-C <1.0 mmol/L in male and <1.3 mmol/L in female	14.6% (924)	6.9% (21)	0% (0)	<0.0001
Triglyceride, mmol/L*	2.30 (1.54–3.00)†,‡	2.60 (2.15–3.20)†	2.80 (2.40–3.20)‡	<0.0001
Triglyceride ≥ 1.7 mmol/L	70.8% (4483)	85.2% (259)	97.5% (78)	<0.0001
Urinary ACR, mg/mmol*	0.17 (0.15–0.20)†	0.16 (0.15–0.19)	0.16 (0.15–0.17)†	0.0025
Complications				
Coronary artery disease	4.9% (308)	11.8% (36)	16.3% (13)	<0.0001
Stroke	1.3% (85)	2.6% (8)	16.3% (13)	<0.0001
Peripheral artery disease	3.1% (199)	3.6% (11)	7.5% (6)	0.0830
Cardiovascular diseases	7.7% (485)	16.5% (50)	32.5% (26)	<0.0001
Drug use before admission				
Statins	5.2% (327)	5.3% (16)	1.3% (1)	0.2857
Other lipid lowering drugs	0.9% (59)	0.7% (2)	3.8% (3)	0.0312
Renin-angiotensin system inhibitors	5.9% (370)	8.2% (25)	3.8% (3)	0.1629
Other antihypertensive drugs	3.0% (190)	4.9% (15)	0% (0)	0.0449
Oral antidiabetes drugs only	38.9% (2461)	49.0% (149)	38.8% (31)	0.0019
GLP-1 based treatment	0.2% (13)	0.7% (2)	15% (12)	<0.0001
Basal insulin based treatment				<0.0001
No	87.3% (5524)	77.3% (235)	80.0% (64)	
Basal insulin	7.4% (471)	17.4% (53)	14% (17.5%)	
Basal + meal time insulin	5.3% (334)	5.3% (16)	2.5% (2)	
Premixed insulin based treatment				<0.0001
No	79.3% (5016)	81.6% (248)	73.8% (59)	
Once per day	0.8% (48)	1.0% (3)	16.3% (13)	
Twice per day	20.0% (1265)	17.4% (53)	10.0% (8)	

ACR: albumin to creatinine ratio; GLP: glucagon-like peptide; BMI: body mass index; BP: blood pressure; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol.

*Data were presented as median and their interquartile ranges.

P values were derived from Chi-square test or analysis of variance. For analysis of continuous variables, Bonferroni test was used to perform multiple comparisons with identical †, ‡, or § indicating statistically significant differences between two means.

Table 2: Odds ratio of abnormal lipids for mild and severe hypoglycaemia.

	Mild hypoglycaemia		Severe hypoglycaemia		Either mild or severe hypoglycaemia	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Model one						
LDL-C, mmol/L	1.12 (1.00–1.26)	0.0526	1.22 (1.05–1.43)	0.0139	1.14 (1.03–1.26)	0.0109
HDL-C, mmol/L	1.12 (1.00–1.26)	0.0526	1.22 (1.05–1.43)	0.0139	1.14 (1.03–1.26)	0.0109
Triglyceride, mmol/L	1.12 (1.05–1.19)	0.0002	1.02 (0.87–1.19)	0.7988	1.10 (1.05–1.17)	0.0005
Model two						
LDL-C, mmol/L	1.14 (1.01–1.29)	0.0331	1.21 (1.04–1.17)	0.0163	1.15 (1.03–1.28)	0.0110
HDL-C, mmol/L	1.10 (1.04–1.17)	0.0015	1.01 (0.87–1.17)	0.8922	1.09 (1.03–1.16)	0.0049
Triglyceride, mmol/L	0.96 (0.86–1.07)	0.4664	1.10 (0.94–1.28)	0.2313	0.98 (0.89–1.09)	0.7388
Model three						
LDL-C, mmol/L	1.14 (1.01–1.29)	0.0340	1.24 (1.05–1.48)	0.0129	1.17 (1.04–1.31)	0.0079
HDL-C, mmol/L	1.10 (1.03–1.17)	0.0030	1.02 (0.83–1.24)	0.8860	1.09 (1.02–1.16)	0.0084
Triglyceride, mmol/L	0.92 (0.82–1.04)	0.1858	1.02 (0.82–1.26)	0.8916	0.93 (0.83–1.04)	0.2238

Model one: not adjusted for other variables.

Model two: adjusted for age, sex, BMI, HbA1c, systolic BP, and log-transformed urinary albumin to creatinine ratio.

Model three: further adjusted for diabetes complications (coronary artery disease, stroke, and peripheral arterial disease) and drug use (statins, other lipid lowering drugs, rennin-angiotensin system inhibitors, oral antidiabetes drugs only, glucagon-like peptide-1 based treatment, basal insulin based treatment, and premixed insulin based treatment).

cancer [12]. In this regard, our data support the findings of the meta-analysis [8] that hypoglycaemia was a risk factor for CVD, independently of traditional CVD risk factors and comorbid severe illness. The major differences between the Hong Kong Diabetes Registry and the current study were as follows: (1) the Hong Kong Diabetes Registry is long term cohort study while our study is a cross-sectional study, and reverse associations between hypoglycaemia and abnormal lipids could be excluded; (2) CKD, a strong risk factor for hypoglycaemia, accounted for 10% of the patients but in our study these high risk patients were excluded but patients with cancer were excluded in our current survey; (3) the severe hypoglycaemia was defined by hospitalization due to hypoglycaemia but only 76 out of 384 patients reporting to have had mild or severe hypoglycaemia were hospitalised due to hypoglycaemia. These differences may partially account for the different findings in Hong Kong Diabetes Registry and our survey.

Typical abnormal lipids in T2D are increased levels of triglyceride, decreased levels of HDL-C, and increased levels of small dense LDL particles, due to increased free fatty acid flux subsequent to insulin resistance [21, 22]. Hyperglycemia led to albuminuria that was strongly predictive of end-stage renal disease [23, 24]. On the other hand, albuminuria increased the risk of high LDL-C but chronic kidney disease (CKD) increased the risk of low HDL-C [25] while albuminuria and CKD also modified the associations of total cholesterol (or LDL-C) and HDL-C with CHD [26]. Our study observed associations between LDL-C/HDL-C and hypoglycaemia but adjustment for hypoglycaemia did not attenuate the associations between lipids and CVD, suggesting that hypoglycaemia could not explain the increased risk of CVD with abnormal lipid profile, that is, not suggesting that abnormal lipids and hypoglycaemia had causal relationships between each other. In addition, we also noticed that patients with hypoglycaemia were an undertreated group, for example, few with severe hypoglycaemia taking statins, which might contribute to the observed associations between lipids and hypoglycaemia.

Hypoglycaemia is one of the most common acute complications that plays an important role in achieving optimal glycaemic control [27]. Although some drug treatments such as use of insulin and secretagogues [28] are associated with higher rates of hypoglycaemia, occurrence of hypoglycaemia itself may contribute to increased risk of CVD as well as multidimensional impairment [29] in T2D. Given the increasing prevalence of diabetes in China [30], it is critical to reduce the rates of diabetes complications in these high-risk patients to reduce the burden of disease. Clinicians need to balance the benefits of tight glycaemic control [31, 32] and possible harms of hypoglycaemia associated with tight glycemia control [2–4] in the management of T2D.

Our study has several limitations. First, this study was a cross-sectional survey of inpatients being hospitalised due to T2D. The study cannot establish time relationship regarding the associations between hypoglycaemia and abnormal lipids. Second, patients with abnormal liver function or CKD were excluded. Albuminuria was the strongest predictor of renal endpoint [33] and most of the patients with albuminuria and high BP might have been excluded due to exclusion of patients with CKD. Thus, our study could not examine the associations of urinary ACR, eGFR, or BP with hypoglycaemia, which had been shown to be associated with hypoglycaemia in Hong Kong Diabetes Registry [12]. Third,

Table 3: Odds ratio of hypoglycaemia and lipid profiles for cardiovascular disease.

	OR (95% CI)	*P* value
Hypoglycaemia for CVD		
Model one		<0.0001
Nonhyperglycemia	Reference	
Mild hyperglycemia	2.37 (1.72–3.26)	
Severe hyperglycemia	5.80 (3.60–9.35)	
Model two		<0.0001
Nonhyperglycemia	Reference	
Mild hyperglycemia	2.64 (1.85–3.76)	
Severe hyperglycemia	6.59 (3.79–11.45)	
Model three		
Nonhyperglycemia	Reference	<0.0001
Mild hyperglycemia	2.64 (1.85–3.76)	
Severe hyperglycemia	6.59 (3.79–11.45)	
Lipid profile for CVD		
Model four		
LDL cholesterol, mmol/L	1.11 (1.01–1.24)	0.0351
HDL cholesterol, mmol/L	0.69 (0.63–0.77)	<0.0001
Triglyceride, mmol/L	1.12 (1.06–1.78)	<0.0001
Model five		
LDL cholesterol, mmol/L	0.95 (0.84–1.07)	0.3703
HDL cholesterol, mmol/L	0.89 (0.80–0.99)	0.0299
Triglyceride, mmol/L	1.08 (1.02–1.14)	0.0160
Model six		
LDL cholesterol, mmol/L	0.93 (0.82–1.05)	0.2462
HDL cholesterol, mmol/L	0.87 (0.78–0.97)	0.0097
Triglyceride, mmol/L	1.08 (1.01–1.14)	0.0166

Model one: not adjusted for other variables.
Model two: adjusted for age, sex, BMI, systolic blood pressure and log-transformed urinary albumin to creatinine ratio, and drug use (statins, other lipid lowering drugs, renin-angiotensin system inhibitors, oral antidiabetes drugs [OADs] only, glucagon-like peptide-1 based treatment, basal insulin based treatment, and premixed insulin based treatment).
Model three: further adjusted for LDL-C, HDL-C, and triglyceride.
Model four: not adjusted for other variables.
Model five: adjusted for age, sex, BMI, systolic blood pressure, LDL-C, HDL-C, triglyceride and log-transformed urinary albumin to creatinine ratio, and drug use (statins, other lipid lowering drugs, renin-angiotensin system inhibitors, and oral antidiabetes drugs [OADs] only).
Model six: adjusted for the variables listed in model five and hypoglycaemia.

Table 4: Sensitivity analysis of odds ratio of hypoglycaemia and lipid profiles for cardiovascular disease after exclusion of 408 patients who used lipid lowering drugs.

	OR (95% CI)	*P* value
Hypoglycaemia for CVD		
Model one		<0.0001
Nonhyperglycemia	Reference	
Mild hyperglycemia	2.86 (2.04–4.02)	
Severe hyperglycemia	7.72 (4.73–12.60)	
Model two		<0.0001
Nonhyperglycemia	Reference	
Mild hyperglycemia	2.95 (2.02–4.29)	
Severe hyperglycemia	6.75 (3.79–12.04)	
Model three		<0.0001
Nonhyperglycemia	Reference	
Mild hyperglycemia	2.95 (2.02–4.29)	
Severe hyperglycemia	6.75 (3.79–12.04)	
Lipid profile for CVD		
Model four		
LDL cholesterol, mmol/L	1.06 (0.94–1.20)	0.3604
HDL cholesterol, mmol/L	0.79 (0.70–0.89)	<0.0001
Triglyceride, mmol/L	1.09 (1.02–1.16)	0.0165
Model five		
LDL cholesterol, mmol/L	0.93 (0.80–1.09)	0.3755
HDL cholesterol, mmol/L	0.91 (0.82–1.03)	0.1201
Triglyceride, mmol/L	1.07 (0.99–1.16)	0.0739
Model six		
LDL cholesterol, mmol/L	0.91 (0.77–1.07)	0.2469
HDL cholesterol, mmol/L	0.88 (0.77–1.00)	0.0580
Triglyceride, mmol/L	1.08 (1.00–1.17)	0.0593

Model one: not adjusted for other variables.
Model two: adjusted for age, sex, BMI, systolic blood pressure and log-transformed urinary albumin to creatinine ratio, and drug use (statins, other lipid lowering drugs, renin-angiotensin system inhibitors, oral antidiabetes drugs [OADs] only, glucagon-like peptide-1 based treatment, basal insulin based treatment, and premixed insulin based treatment).
Model three: further adjusted for LDL-C, HDL-C, and triglyceride.
Model four: not adjusted for other variables.
Model five: adjusted for age, sex, BMI, systolic blood pressure, LDL-C, HDL-C, triglyceride and log-transformed urinary albumin to creatinine ratio, and drug use (statins, other lipid lowering drugs, renin-angiotensin system inhibitors, oral antidiabetes drugs [OADs] only, glucagon-like peptide-1 based treatment, basal insulin based treatment, and premixed insulin based treatment).
Model six: adjusted for the variables listed in model five and hypoglycaemia.

smoking and alcohol drinking habits were not collected in this survey. Their confounding effects cannot be adjusted. Fourth, the patients were patients being hospitalised due to T2D and the findings of this survey need to be confirmed in low risk patients with T2D. Fifth, it is noticed that there was much greater intraindividual variability in hypoglycaemia symptom reporting by patients with diabetes over a long period, that is, 12-month period [34]. Although we chose reporting hypoglycaemia symptoms over more recent short periods of time, intraindividual variability in reporting these symptoms by our subjects was unavoidable.

In conclusion, in a large survey of Chinese inpatients with T2D, we found that hypoglycaemia and lipid profile were independent risk factors for CVD and both factors may be useful in assessing CVD risk among Chinese patients with T2D. Further studies are warranted to validate these findings in cohorts of patients with T2D, including low risk Chinese with T2D.

Conflict of Interests

Yiming Mu, Yijun Li, Qiuhe Ji, Qin Huang, Hongyu Kuang, and Linong Ji received research grant(s) from Novo Nordisk China.

Authors' Contribution

Yijun Li and Yiming Mu equally contributed to the paper.

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Research Article

Resting Heart Rate and Auditory Evoked Potential

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The objective of this study was to evaluate the association between rest heart rate (HR) and the components of the auditory evoked-related potentials (ERPs) at rest in women. We investigated 21 healthy female university students between 18 and 24 years old. We performed complete audiological evaluation and measurement of heart rate for 10 minutes at rest (heart rate monitor Polar RS800CX) and performed ERPs analysis (discrepancy in frequency and duration). There was a moderate negative correlation of the N1 and P3a with rest HR and a strong positive correlation of the P2 and N2 components with rest HR. Larger components of the ERP are associated with higher rest HR.

1. Introduction

Cardiovascular diseases are the most frequent causes of morbidity and mortality around the world during the last decades [1–4]. The cardiovascular system is influenced by extrinsic stimuli through the autonomic nervous system (ANS) [5]. The central nervous system (CNS) [6] sends commands to the ANS, controlling the regulation of heart rate (HR) and blood pressure (BP); the sympathetic system is responsible for the increase in HR whereas the parasympathetic system is responsible for the decrease in HR.

Since the CNS actively regulates the cardiac system, auditory evoked-related potentials (ERPs) are an important method used to evaluate it because it captures electrical responses in the cortex due to an acoustic stimulus. Brainstem auditory ERPs are characterized by bioelectric responses of thalamic and cortical activity, measured in milliseconds; it assesses cortical activities involved in the skills of discrimination, integration, memory, and attention in the brain, besides the integrity of central auditory nervous system [7].

Few studies have focused on the cardiac and auditory systems through musical stimuli [8–10]; cardiac and auditory potentials [11, 12]; and white noise [13, 14], indicating that there is an association between hearing bioelectrical response and HR regulation. According to the literature, more studies are necessary to confirm these findings, particularly the interaction between auditory evoked potential and cardiac autonomic regulation [15].

Knowledge of physiological responses involved in the relationship between the central auditory pathways and cardiac autonomic regulation is important for the development of future therapies to propose intervention and prevent the development of disorders of the cardiovascular system. In this sense, it is important to have new studies to help to understand the association between cardiac autonomic control and the electrical activity in the auditory system. Therefore, we aimed to evaluate the association between HR and ERPs at rest in women.

2. Methods

2.1. Subjects. This research protocol was approved by the Ethics Committee in Research of the State University of São Paulo (case number 419/2012) and was in accordance with Resolution 196/96 of the National Health Council of 10/10/1996. The sample consisted of 21 apparent healthy female subjects between 18 and 24 years old. All volunteers were

informed about the procedures and objectives of the study and, after agreeing, signed a consent form.

2.2. Procedure. We excluded smokers, people with any degree of hearing loss or middle ear disorders, subjects with related cardiorespiratory, metabolic, neurological, and/or any condition that prevented the individual to perform the procedures, and those users of drugs that alter cardiac autonomic regulation. For this characterization, the following procedures were performed: medical history and research of hearing threshold (air and bone conduction) and acoustic impedance measurements; resting blood pressure and heart rate measurement.

The normal parameters of hearing assessments considered in this study were pure tone audiometry thresholds below or equal to 25 dB and airway below or equal to 15 dB bone conduction [16], with acoustic impedance tympanogram type A, indicating eardrum-ossicular normal [17] system and presence of ipsilateral and contralateral reflexes.

Data collection was conducted in a quiet room electric and sound-proof in temperatures between 21°C and 25°C and humidity between 50 and 60%. Volunteers were instructed to not ingest alcohol and caffeine in the 24 hours prior to evaluation. After the initial assessment, the heart rate receiver Polar RS800CX (Polar Electro, Finland) was placed on the chest of the volunteers in the sternum region for analysis. After placing the strap and the monitor, subjects were positioned in a chair and told to remain at rest for 10 minutes. Blood pressure was measured using a sphygmomanometer and stethoscope. Blood pressure and heart rate were measured at rest prior to the auditory ERPs. Only volunteers who showed no significant differences in the values of these two measures participated in the survey.

The examination of the auditory ERPs was performed with the individual in a state of alert watching a video (without sound) for distraction and recommended to not direct their attention to sound stimuli in oddball paradigm (two auditory stimuli, deviant-standard, presented in random order), unattended test. We used Biologic's Evoked Potential System (PE) for data acquisition and five disposable electrodes placed on Fz and Cz in reference to the right lobe (A2) and left (A1) and ground in Fpz, using the two channels of the equipment record.

The parameters used for the ERPs were filter between 0.5 and 30 Hz binaural stimuli in the frequency discrimination, the standard stimulus which was elicited at a frequency of 750 Hz and 1000 Hz for the deviant stimulus (tone burst with plateau 60 ms and rise/fall 20 ms), the duration discrimination on, the standard stimulus (tone burst with plateau 60 ms and rise/fall 20 ms), deviant stimulus (burst tone with plateau 30 ms and rise/fall 10 ms) both in the frequency of 1000 Hz (with a probability of 20%), intervals between stimuli of 1.1 ms, intensity of 70 dB HL, analysis time of 500 ms, prestimulus analyses time of 0 ms, a sensitivity of 100 microvolts alternating polarity, and number samples of 200 stimuli [18].

The wave identification ERPs followed criteria in the literature, including visualization of sequential peaks of negative-positive-negative waves, that is, N1, P2, N2 complex, respectively, between 60 and 300 ms, observed in do twice traces [19]. As the component P3a latency was marked before 350 ms/P3b, in unattended test [20], the P3b component was not analyzed in this study. The P3a is the AEP components related to early warning processes and auditory sensory processing, which occurs automatically in response to the large differences of the stimuli, independent of the active attention of the individual to the stimulus sequence [21].

Testing took approximately 50 minutes. In order to maintain a standard quality test, in the volunteers who showed myogenic interference, we suggested change positions and when necessary the examination was repeated.

The Shapiro-Wilk Test was applied to evaluate distributions; Person and Spearman correlation Tests were applied for parametric and nonparametric distribution, respectively, in order to investigate correlation between variables. The P values were considered statistically significant when $P < 0.05$ and all confidence intervals were constructed with 95% statistical confidence. For correlation values we considered strong correlation for $r > 0.5$, moderate correlation for r between 0.3 and 0.49 ($0.3 > r > 0.49$), and weak correlation for $r < 0.3$. The statistical software used was GraphPad Software StatMate 2:00 version for Windows, GraphPad Software, San Diego, California USA.

3. Results

Correlations between latencies and amplitudes of N1, P2, N2, and P3a and N2-P3a interamplitude, measured at Cz and Fz (Figure 1), related to ERP and HR in healthy women were studied.

The results obtained show correlation between some components of the ERP and HR as shown in Tables 1, 2, 3, 4 and in Figure 2.

4. Discussion

The ERP is one of the promising measures used in the research of central auditory processing that reflects cortical activity involving listening skills from the simple to the most complex. HR is mediated by the direct activity of the ANS through sympathetic and parasympathetic branches and is an indicator usually expressed as the number of heartbeats per minute (bpm).

The findings of the literature state that the decrease or increase in HR is caused by the selective activation of cardiac neurons in the amygdala and in the reticular formation, structures located in the cerebral cortex. The reticular formation is responsible for the regulation of alertness and subsidizes the attentional process and the amygdala is specifically related to emotions [22].

Anatomically and physiologically the link between cardiovascular and hearing impairment due to structures responsible for cardiovascular regulation and components of ERPs that presents similar functions in the regulation of alertness and sensory attentional process is evident. The N1 component of the ERP is associated with attention and initial decoding of the auditory stimulus. The P2 is related to temporal acoustic stimulus, which brings information to

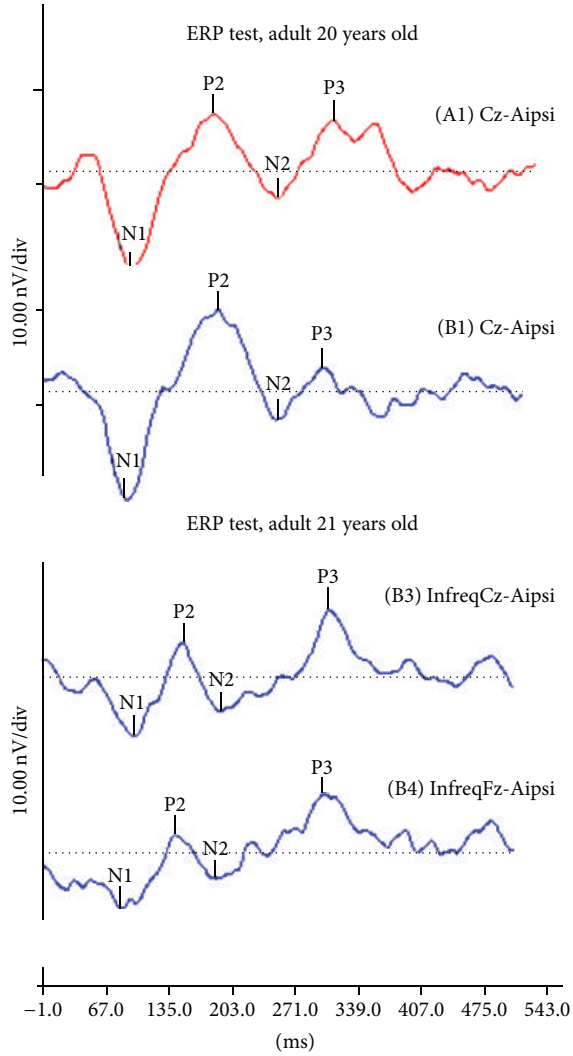

Figure 1: ERP test of two individual adults.

the level of the auditory cortex, the early cortical processing of sound and features. The N2 contributes to physical breakdown of the acoustic characteristics of the stimuli and is also responsible for passive, preattentional, automatic perception, discrimination, and sound recognition [7, 18] response activities. The P3a is related to the activity of alert during the initial allocation of attention or redirection of sensory attention and is triggered by distractor stimuli [23, 24].

It was possible to observe in the Cz scan that when the right and the left ear were aurally stimulated, there was a moderate negative correlation in the amplitude of N1 and P3a, respectively. It was reported that when the electrical activity reaches the cortex in areas associated with attention and initial decoding of the stimulus and during stimulation the stimulus starts to be processed in the auditory pathway through activity updates for automatic allocation of sensory attention, while HR increases the amplitude of N1 and P3a decreases.

Some authors [11, 12] related cardiac evoked potentials with long-latency auditory evoked potential and showed that there is a link between CNS and ANS in the reflection of some aspects of processing auditory stimulus, confirming our findings in relation to the auditory N1 component.

Moreover, we observed strong positive correlation of the amplitude of P2 and N2 amplitude and latency with HR in the scan Cz and Fz in the right and left ear. Therefore, the results showed that the lower the electrical activity of the auditory processing is, the lower the HR. P2 and N2 components reflect the acoustic characteristics of the stimuli [18], which can affect the ANS and interfere with the heartbeat.

A study of simulated driving mental fatigue testing associated with another type of auditory cortical potential observed significant reduction in P300 amplitude, indicating that when the individual was exposed to the mental fatigue there was a decrease of attention to the auditory task. The authors suggested that auditory electrophysiological response is related to mental fatigue, which has impact on the function

Table 1: Correlation between latency (ms) and amplitude (μV), ERPs and HR in frequency protocol on the right ear.

RE	Variables	Mean	SD	r	P
Cz	LAT N1	98,29	9,72	0,100	0,40
	AMP N1	−4,80	1,80	−0,492	0,02*
	LAT P2	169,93	29,84	0,300	0,60
	AMP P2	2,24	1,99	0,200	0,50
	LAT N2	221,63	37,84	0,200	0,50
	AMP N2	−1,77	1,98	0,100	0,70
	LAT P3a	289,05	32,49	0,100	0,40
	AMP P3a	2,03	1,82	0,300	0,10
	AMP N2-P3a	−3,68	3,47	0,090	0,90
Fz	LAT N1	97,85	9,60	0,040	0,30
	AMP N1	−4,45	2,19	0,100	0,30
	LAT P2	157,48	25,18	0,100	0,30
	AMP P2	0,76	2,17	0,557	0,00*
	LAT N2	201,04	32,15	0,200	0,50
	AMP N2	−1,49	2,28	0,563	0,00*
	LAT P3a	288,16	30,84	0,200	0,50
	AMP P3a	2,06	1,41	0,100	0,40
	AMP N2-P3a	−3,74	3,11	0,090	0,40

RE: right ear; LAT: latency; AMP: amplitude; SD: standard deviation; *$P < 0.05$; Shapiro-Wilk Test, Person Test, and Spearman Test.

Table 2: Correlation between latency (ms) and amplitude (μV), ERPs and HR in duration protocol on the right ear.

RE	Variables	Mean	SD	r	P
Cz	LAT N1	102,04	12,87	0,010	0,70
	AMP N1	−4,08	1,87	0,100	0,90
	LAT P2	148,41	15,52	0,200	0,20
	AMP P2	1,70	1,75	0,300	0,10
	LAT N2	209,63	20,11	0,070	0,50
	AMP N2	−3,06	1,15	0,200	0,40
	LAT P3a	301,99	23,87	0,100	0,40
	AMP P3a	2,25	1,48	0,100	0,30
	AMP N2-P3a	−5,31	1,84	0,100	0,60
Fz	LAT N1	103,30	14,74	0,200	0,50
	AMP N1	−3,92	2,07	0,100	0,50
	LAT P2	155,50	15,01	0,200	0,60
	AMP P2	0,09	1,19	0,200	0,60
	LAT N2	199,52	16,98	0,100	0,30
	AMP N2	−2,93	1,08	0,090	0,40
	LAT P3a	294,64	31,48	0,100	0,40
	AMP P3a	2,04	1,25	0,300	0,30
	AMP N2-P3a	−4,96	1,73	0,030	0,30

RE: right ear; LAT: latency; AMP: amplitude; SD: standard deviation; *$P < 0.05$; Shapiro-Wilk Test, Person Test, and Spearman Test.

Table 3: Correlation between latency (ms) and amplitude (μV), ERPs and HR in frequency protocol on the left ear.

LE	Variables	Mean	SD	r	P
Cz	LAT N1	97,55	13,65	0,080	0,40
	AMP N1	−4,70	1,84	0,090	0,20
	LAT P2	175,43	26,69	0,486	0,02*
	AMP P2	2,90	2,09	0,618	0,00*
	LAT N2	227,38	35,55	0,437	0,04*
	AMP N2	−1,32	1,18	0,100	0,60
	LAT P3a	282,06	32,19	0,090	0,90
	AMP P3a	1,92	2,17	−0,443	0,04*
	AMP N2-P3a	−3,29	2,72	0,200	0,60
Fz	LAT N1	99,04	15,66	0,100	0,50
	AMP N1	−4,58	2,09	0,090	0,80
	LAT P2	171,91	29,44	0,100	0,90
	AMP P2	1,17	1,92	0,516	0,01*
	LAT N2	214,24	35,06	0,523	0,01*
	AMP N2	−1,40	1,53	0,511	0,01*
	LAT P3a	277,70	25,54	0,100	0,70
	AMP P3a	2,11	1,59	0,200	0,90
	AMP N2-P3a	−3,51	2.10	0,090	0,50

LE: left ear; LAT: latency; AMP: amplitude; SD: standard deviation; *$P < 0.05$; Shapiro-Wilk Test; Person Test, and Spearman Test.

Table 4: Correlation between latency (ms) and amplitude (μV), ERPs and HR in duration protocol on the left ear.

LE	Variables	Mean	SD	r	P
Cz	LAT N1	95,17	13,88	0,080	0,50
	AMP N1	−3,37	1,11	0,100	0,70
	LAT P2	151,19	12,42	0,100	0,90
	AMP P2	1,91	1,47	0,200	0,80
	LAT N2	207,30	18,52	0,090	0,50
	AMP N2	−2,54	1,54	0,050	0,70
	LAT P3a	283,74	23,15	0,200	0,70
	AMP P3a	2,34	1,97	0,100	0,90
	AMP N2-P3a	−4,88	2,25	0,090	0,50
Fz	LAT N1	93,34	22,10	0,060	0,40
	AMP N1	−2,79	1,69	0,200	0,80
	LAT P2	145,88	17,28	0,090	0,60
	AMP P2	0,81	1,39	0,090	0,80
	LAT N2	199,02	17,15	0,200	0,60
	AMP N2	−2,73	1,63	0,100	0,90
	LAT P3a	291,73	23,95	0,100	0,80
	AMP P3a	2,50	1,33	0,090	0,70
	AMP N2-P3a	−5,26	2,01	0,040	0,90

LE: left ear; LAT: latency; AMP: amplitude; SD: standard deviation; *$P < 0.05$; Shapiro-Wilk Test; Person Test, and Spearman Test.

of the CNS, which consequently controls and regulates the cardiovascular system [14].

Finally, regarding the parameters of frequency and duration, we observed statistical correlation only in the frequency protocol in both ears. This fact can be explained because their stimuli were presented in an oddball paradigm and

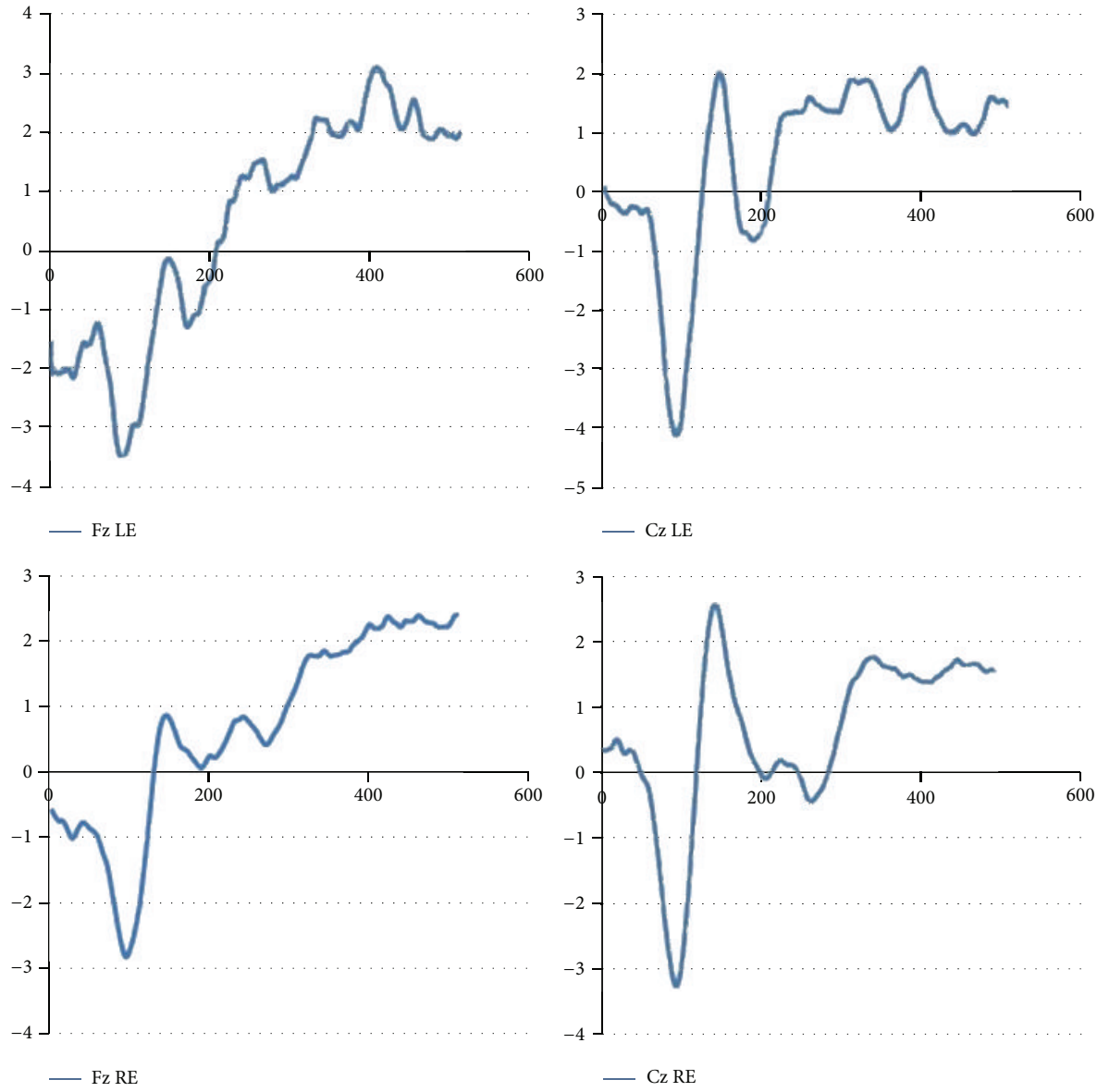

Figure 2: Grand average of ERP tests—frequency protocol. RE: right ear; LE: left ear.

differentiate in intensity, which can make them more discriminating and hence may affect the ANS and interfere with the heartbeat.

5. Conclusion

There was association between cardiovascular parameters and the central auditory pathways, indicating physiological responses of the studied variables and the relationship between them. We suggest further research in this field, in order to clarify and confirm this association.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

All authors participated in research design, writing of the paper, and data analysis, and all authors read and approved the final paper.

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Research Article

Renin-Angiotensin Activation and Oxidative Stress in Early Heart Failure with Preserved Ejection Fraction

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Animal models have suggested a role of renin-angiotensin system (RAS) activation and subsequent cardiac oxidation in heart failure with preserved ejection fraction (HFpEF). Nevertheless, RAS blockade has failed to show efficacy in treatment of HFpEF. We evaluated the role of RAS activation and subsequent systemic oxidation in HFpEF. Oxidative stress markers were compared in 50 subjects with and without early HFpEF. Derivatives of reactive oxidative metabolites (DROMs), F2-isoprostanes (IsoPs), and ratios of oxidized to reduced glutathione (E_h GSH) and cysteine (E_h CyS) were measured. Angiotensin converting enzyme (ACE) levels and activity were measured. On univariate analysis, HFpEF was associated with male sex ($p = 0.04$), higher body mass index (BMI) ($p = 0.003$), less oxidized E_h CyS ($p = 0.001$), lower DROMs ($p = 0.02$), and lower IsoP ($p = 0.03$). Higher BMI (OR: 1.3; 95% CI: 1.1–1.6) and less oxidized E_h CyS (OR: 1.2; 95% CI: 1.1–1.4) maintained associations with HFpEF on multivariate analysis. Though ACE levels were higher in early HFpEF (OR: 1.09; 95% CI: 1.01–1.05), ACE activity was similar to that in controls. HFpEF is not associated with significant systemic RAS activation or oxidative stress. This may explain the failure of RAS inhibitors to alter outcomes in HFpEF.

1. Introduction

Heart failure with preserved ejection fraction (HFpEF) accounts for up to 50% of heart failure (HF) cases [1, 2]. The prevalence of HFpEF is increasing [2], and nearly all patients with HF symptoms, including those with HF with reduced EF, have some component of HFpEF [3]. The pathogenesis of HFpEF is still incompletely understood. It is believed that before the advent of HF symptoms in HFpEF there is a latent phase of diastolic dysfunction (DD), associated with impaired left ventricular (LV) relaxation, elevated LV end diastolic pressure, and increased LV stiffness [4].

It has been shown that nitric oxide (NO) and nitric oxide synthase (NOS) have a role in cardiac relaxation, with a reduction in cardiac NO contributing to DD [5, 6]. The bioavailability of NO is dependent on the presence of reactive oxygen species (ROS) that oxidize NO and uncouple NOS, preventing NOS from producing NO [5]. Angiotensin II (Ang II) is known to cause uncoupling of NOS by activating NADPH (nicotinamide adenine dinucleotide phosphate) oxidase to produce ROS. This provides a possible link between RAS activation and DD [7]. Nevertheless, recent clinical trials have failed to show a benefit of RAS blockade in the treatment of DD [8–12].

Several convenient methods are available to measure oxidative stress in blood. Glutathione (GSH) is a major soluble intracellular peptide that eliminates peroxides and other oxidants [13]. GSH and its oxidized form (GSSG) can be reliably measured in plasma, and their ratio represents a redox couple, E_h GSH. Cysteine (CyS) comprises the major extracellular thiol, and, along with oxidized cysteine (CySS), it represents another measurable redox couple, E_h CyS [14]. Derivatives of reactive oxygen metabolites (DROMs) are a colorimetric assay for lipid peroxidation [15]. F2-isoprostanes (IsoPs) are a series of prostaglandin- (PG-) like compounds produced by the free radical-catalyzed peroxidation of arachidonic acid [16]. Recently, it has been shown that elevated levels of IsoPs are associated with incident cardiovascular events in patients with atrial fibrillation [17]. Additionally, we have used these assays in previous study to show increased systemic oxidative stress in patients with atrial fibrillation (AF) [18].

Preclinical studies have proposed that RAS and subsequent oxidation play a role in pathogenesis of DD in HFpEF. The cardiovascular effects of Ang II are believed to be because of its activation of NADPH oxidase [7]. Ang II also induces mitochondrial dysfunction, generating ROS such as superoxide ($O_2^{\bullet -}$). Overall, these are thought to lead to a reduction in NO bioavailability and a defect in myocardial relaxation [19]. Nevertheless, angiotensin convertase enzyme- (ACE-) inhibitors or angiotensin receptor blockers (ARBs) have not shown efficacy in treatment of DD [8–11]. This is in contrast to definite response seen with the use of RAS inhibitors in LV systolic HF [20, 21].

To help explain this paradox, we tested whether systemic RAS activation and associated oxidative stress were present in patients with DD in early HFpEF.

2. Methods

2.1. Study Design and Patient Recruitment. In a cross-sectional, case-control study, 50 subjects with NYHA functional Class I-II HF symptoms and echocardiographic evidence of HFpEF, as defined by preserved LV ejection fraction (EF) of >50% and abnormal echocardiographic LV relaxation pattern on pulsed-wave and tissue Doppler, and matched controls were recruited from the outpatient clinics and hospital at the Atlanta Veterans Affairs Medical Center and Emory University Hospital from July 2006 to February 2008 (https://www.clinicaltrials.gov/; NCT00142194). Cases and controls were matched for age in decades, smoking history, and diabetes mellitus, all known confounders in oxidative stress measurements. The protocol was approved by the Emory University Institutional Review Board. A written informed consent for participation in the study was obtained from all subjects.

Eligibility criteria for both cases and controls included age ≥18 years, an echocardiogram with mitral valve inflow velocities and tissue Doppler measurements within six months of enrollment, normal sinus rhythm, LV EF between 50 and 70%, and normal systolic and diastolic cardiac dimensions on qualifying echocardiogram. Exclusion criteria included systemic inflammatory disease, malignant neoplasm, severe valvular heart disease, HF NYHA Class III or IV, untreated hyper- or hypothyroidism, greater than mild cardiac hypertrophy, cardiomyopathy of any etiology, blood pressure (BP) > 180/100 mmHg on medications, concurrent illness resulting in life expectancy <1 year, and illicit drug or alcohol abuse.

2.2. Clinical Data. Demographic and clinical data were collected by review of medical records and physical examination upon enrollment. A qualifying standard 2D echocardiogram with Doppler examination was obtained at entry into the study. A single blood draw in a nonfasting state was obtained between 8:30 AM and 5:00 PM. Blood samples were collected from the antecubital vein and, for thiol measures, were immediately transferred to a microcentrifuge tube with 0.5 mL preservative solution of 100 mmol/L serine borate (pH 8.5), containing (per mL) 0.5 mg sodium heparin, 1 mg bathophenanthroline disulfonate sodium salt, and 2 mg iodoacetic acid. This was done to minimize autoxidation and hemolysis [22]. Samples were analyzed at the Emory Biomarkers Core Laboratory.

2.2.1. Echocardiographic Data. All echocardiographic studies were performed with a GE System Vivid 7 Echocardiogram with the patient in left lateral position. Standard echocardiographic views were obtained per protocol. Left ventricular ejection fraction was calculated using the biplane modified Simpson rule. Cardiac inflow velocities were obtained by pulsed-wave (PW) Doppler analysis performed in the apical-4 chamber (A4C) plane. Peak early filling (E) and late atrial contraction (A) wave velocities were measured; the proportion of E/A waves was calculated. Mitral annular velocities were measured in the A4C plane at the septal and lateral mitral leaflet insertion sites using PW tissue Doppler imaging (TDI) [23]. The proportion of inflow/annular early velocities (E/e') and annular early/annular late velocities (e'/a') were calculated per standard guidelines. Isovolumic relaxation time and deceleration time were recorded. An independent cardiologist interpreted the studies using standard protocols [24].

2.3. Measurement of Oxidative Stress Markers. Markers used to measure systemic oxidative stress were the same as those we have characterized previously [18]: redox potential of the ratios of oxidized to reduced glutathione (E_h GSH) and cysteine (E_h CyS) in plasma (thiol ratios) [18], DROMs [15], and IsoPs [16]. The samples were stored at −80°C. Samples from cases and controls were treated identically. Laboratory technicians were blinded to the clinical data. The redox states (E_h) of thiol/disulfide pools were calculated using Nernst equation:

$$E_h = E_o + \frac{RT}{nF} \ln \frac{[\text{disulfide}]}{[\text{thiol}]^2}, \quad (1)$$

where E_o is the standard potential for redox couple, R is the gas constant, T is the absolute temperature, n is the number of electrons transferred, and F is Faraday constant. E_o used for glutathione and cysteine redox couples was −264 mV and −250 mV, respectively. Less negative E_h numbers implied a more oxidized state.

For measurement of IsoPs, plasma samples were acidified and a deuterated standard was added. This was followed by C-18 and Silica Sep-Pak extraction [16]. IsoPs were then converted to pentafluorobenzyl esters which were subjected to thin layer chromatography. F2-IsoPs were quantified by gas chromatography/mass spectrometry by using an Agilent 5973 MS with computer interference. After dissolution of serum in acidic buffer, an additive (N-N-diethyl-para-phenylenediamine) was added for DROM measurements [15]. Concentration of DROMs was determined through spectrometry (505 nm).

2.4. Measurement of RAS Activation. ACE activity and protein levels were analyzed in 31 (15 cases and 16 controls) subjects not taking any form of RAS inhibitor, since these are known to alter the measures [25–27]. Heparinized human plasma (20–40 μL) was diluted 1 : 5 parts with phosphate buffered saline (PBS) and incubated at 37°C with 200 μL of substrate for 2 hours. ACE activity was determined fluorometrically with two different substrates, Hip-His-Leu (HHL, 5 mM) and Z-Phe-His-Leu (ZPHL, 2 mM), and expressed as mU/mL [28, 29]. Levels of ACE protein were determined using plate precipitation assay based on a monoclonal antibody to the epitope localized on the N domain of ACE (9B9) and expressed as a percentage (%) of gold standard from pooled human plasma [25–27].

Western blot analysis was used to measure extracellular copper-zinc superoxide dismutase (ec-SOD) and the copper-delivering protein, ceruloplasmin (Cp), expression in representative samples from both groups. Briefly, plasma ec-SOD or Cp was concentrated by concanavalin-A sepharose chromatography, and protein expression was examined by immunoblotting with antibody against ec-SOD or ceruloplasmin (Dako Cytomation, Carpinteria, CA) [28, 29].

2.5. Statistical Analysis. Statistical analyses were performed using SAS software 9.1 (SAS Institute, Inc.). Sample size was based on a 0.90 power to detect the same difference that we observed in the least sensitive measure of oxidative stress in our previous study using a two-tailed α-level of 0.05 [18]. Baseline characteristics with normal distribution were compared between cases and controls using a paired t-test for continuous variables and Chi-square/Fisher exact test for categorical variables. Nonparametric tests were used for variables with skewed distribution. Mean (and median, where appropriate) levels of oxidative stress and ACE markers in cases and controls were compared using t-test for normally distributed variables and NPAR1WAY procedure (SAS software 9.1) for variables with skewed distribution. All variables significant on univariate analysis were entered into multiple logistic regression models to calculate adjusted odds ratios.

3. Results

We enrolled 50 patients with and without echocardiographic evidence of DD in HFpEF. The groups were well matched for known confounders in measurement of oxidative stress markers, including age ($p = 0.96$), smoking ($p = 1.00$), and diabetes mellitus ($p = 0.77$). The mean age of cases and controls was 64.8 ± 10.8 years (range: 45–83 years) and 65.0 ± 11.3 years (range: 43–88 years), respectively.

Table 1: Clinical characteristics of the study population.

	Cases ($N = 25$)	Control ($N = 25$)	P value
Demographic variables			
Age	64.8 ± 10.8	65.0 ± 11.3	1
Gender			**0.04**
Females	7 (28%)	14 (56%)	
Males	18 (72%)	11 (44%)	
Race*			0.8
White	13 (54%)	12 (48%)	
Black	11 (46%)	12 (48%)	
Clinical variables			
Smoking	10 (40%)	10 (40%)	1
Diabetes	8 (32%)	9 (36%)	1
BMI	29.6 ± 4.8	25.3 ± 4.7	**0.00**
Hypertension	16 (64%)	14 (56%)	0.6
Mean SBP (mm Hg)	135.4 ± 19.2	127.4 ± 16.7	0.1
Mean DBP (mm Hg)	75.8 ± 13.0	74.0 ± 10.3	0.6
Hypercholesteremia	14 (56%)	10 (40%)	0.4
Medications			
Betablocker	15 (60%)	10 (40%)	0.3
ACEI	12 (48%)	9 (36%)	0.6
ARB	4 (16%)	1 (4%)	0.4
Diuretic	4 (16%)	8 (32%)	0.3
Statin	14 (56%)	16 (64%)	0.8

*1 (4%) Asian in each group; HFpEF: heart failure with preserved ejection fraction; DD: diastolic dysfunction; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; ACEI: angiotensin converting enzyme inhibitor; ARB: angiotensin II receptor blocker.

Cases and controls only differed in male sex and BMI (Table 1). The association of higher BMI with DD was maintained on a multivariate analysis (*model 1*) using all demographic and clinical parameters as predictive variables (adjusted OR: 1.3; 95% CI: 1.1–1.6; Figure 1).

Table 2 compares markers for oxidative stress, ACE activity, and ACE protein levels in patients with and without DD. Three of four oxidative stress measures suggested that early DD was associated with a more reduced systemic oxidative state as compared to controls. E_h CyS was significantly more reduced (more negative) in patients with early DD (mean, −70.1 ± 7.8 mV; median, −72.9 mV in cases versus mean, −50.3 ± 11.6 mV; median, −50.6 mV in controls, $p < 0.001$). There was no significant difference in E_h GSH (mean, −118.8 ± 14.0 mV; median, −114.6 mV in cases versus mean, −118.4 ± 16.5 mV; median, −117.0 mV in controls, $p = 0.93$), a less sensitive measure of plasma redox state [23]. IsoPs levels (mean, 1495 ± 663 pg/mL; median, 1345 pg/mL in cases versus mean, 7385 ± 3241 pg/mL; median, 2341 pg/mL in controls, $p = 0.03$) and DROMs (mean, 375.2 ± 132.4 Carr units; median, 341.3 Carr units in cases versus mean, 474.5 ± 167.5 Carr units; median, 462.1 Carr units in controls,

Table 2: Oxidative stress markers and ACE activity in study population.

	Cases Mean ± SD	Controls Mean ± SD	P value
Oxidative stress measures			
(E_h) CyS*	70.1 ± 7.8	50.3 ± 11.6	**0.001**
(E_h) GSH*	118.8 ± 14.0	118.4 ± 16.5	0.9
DROMs±	375.2 ± 132.4	474.5 ± 167.5	**0.02**
IsoP€	$15 \times 10^2 \pm 6 \times 10^2$	$73 \times 10^2 \pm 32 \times 10^2$	**0.03**
ACE measures			
ACE-HHL‡	42.6 ± 9.6	36.8 ± 9	0.1
ACE-ZPHL‡	39.0 ± 8.7	36.2 ± 8.9	0.4
ACE PROT§	134.5 ± 38.2	101.9 ± 22.2	**0.03**

*mV; ±Carr units; €pg/mL; ‡mU/mL; §percentage (%); DD: diastolic dysfunction; E_h CyS: redox potential of reduced to oxidized cysteine (negative); E_h GSH: redox potential of reduced to oxidized glutathione (negative); DROMs: derivatives of reactive oxygen metabolites; IsoPs: isoprostanes; ACE-HHL: angiotensin converting enzyme activity measured using Hip-His-Leu (HHL) substrate; ACE-ZPHL: angiotensin converting enzyme activity measured using Z-Phe-His-Leu (ZPHL) substrate; ACE PROT: angiotensin converting enzyme protein levels.

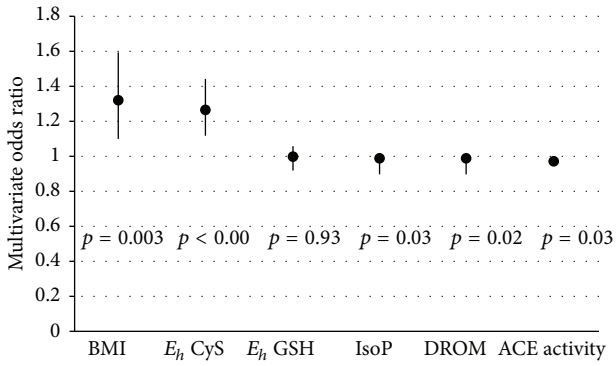

Figure 1: Multivariate odds ratios for association with diastolic dysfunction in early HFpEF. (BMI: body mass index; E_h CyS: redox potential of reduced to oxidized cysteine; E_h GSH: redox potential of reduced to oxidized glutathione; DROMs: derivatives of reactive oxygen metabolites; IsoPs: isoprostanes; ACE: angiotensin converting enzyme levels.)

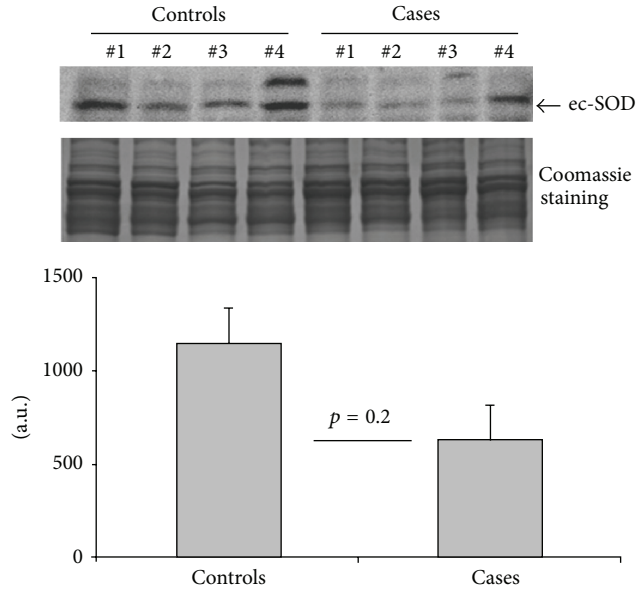

Figure 2: Protein expression of ec-SOD in blood samples from cases and control groups. The results depicted in the graph are presented as mean ± SE (cases ($n = 6$) and controls ($n = 12$)).

$p = 0.02$) were significantly lower in cases. The association between a more reduced E_h CyS and DD was maintained on a multivariate analysis (*model 2*) using gender, BMI, and oxidative stress measures as predictive variables (adjusted OR: 1.22; 95% CI: 1.08–1.37).

ACE activity, determined with HHL as substrate, demonstrated a mild but statistically insignificant increase in cases compared to controls (mean, 42.6 ± 9.6 mU/mL; median, 40.2 mU/mL in cases versus mean, 36.8 ± 9.0 mU/mL; median, 37.5 mU/mL in controls, $p = 0.1$). ACE protein levels were only marginally higher in patients with early DD (mean, 134.5 ± 38.2%; median, 112.8% in cases versus mean, 101.9 ± 22.2%; median, 104.3%; $p = 0.03$; adjusted OR: 1.05; 95% CI: 1.01–1.09).

We measured ec-SOD in representative samples from both groups. Although there was a mild decrease in ec-SOD activity in cases, this did not reach statistical significance ($p = 0.2$) (Figure 2). Since ec-SOD is a copper enzyme, serum Cp, a marker protein for systemic copper, was also measured in the same samples but was not found to be altered in cases.

4. Discussion

In this study, we found no evidence of significant RAS activation or systemic oxidative stress in cases compared to matched controls. This is consistent with a lack of effect of RAS inhibitors and antioxidants in DD [8–12, 30]. Plasma ec-SOD activity and its copper-delivering protein, Cp, were not raised in cases. This also suggests a lack of increase in ROS, since systemic oxidative stress is known to upregulate peripheral ec-SOD activity [31]. Inhibition of ACE activity has been associated with elevated ec-SOD levels in studies

[32], and unchanged ACE activity in the cases was consistent with unchanged ec-SOD.

There is a growing evidence that ROS signaling is compartmentalized [33]. It is possible that only local cardiac RAS activation or oxidation is required to generate DD. In a study by Inoue and group, peripheral oxidative stress was not detectable in HF after myocardial infarction (MI), even with progressive remodeling [34]. It was postulated that in the later phase of remodeling post-MI, ROS was generated mainly in the myocardium and multiple innate antioxidant defense mechanisms in the periphery stabilized levels of oxidative markers in blood and urine [34]. This might explain the contradiction between association of cardiac oxidative stress with DD and our findings. On the other hand, it is possible that systemic RAS activation and peripheral oxidative stress may occur late in the pathogenic cascade.

Alternatively, the lack of RAS activation and systemic oxidation may imply that other factors might be responsible for the onset of DD in HFpEF. Obesity has been associated with DD and may be one such factor [35, 36]. Our results are consistent with earlier studies showing correlation of obesity with DD [35, 36]. It has been shown that high BMI leads to a downregulation of adiponectin production in adipocytes [37]. Adiponectin, a member of complement factor C1q family, is believed to prevent endothelial injury in the heart and vasculature by multiple mechanisms, including promoting eNOS activity and preserving bioactive NO [38]. The lack of adiponectin in obesity may lead to progression of HFpEF [39]. Cardiac oxidation may be concentrated in obese individuals because of increased epicardial adipose tissue (EAT) [40, 41]. EAT is a source of adipocytokines that have both apocrine and paracrine effects on adjacent myocardial cells leading to chronic local and systemic inflammation [42].

Few recent studies have found an association of systemic oxidative stress with HFpEF. In their study [43–45], Vitiello et al. measured plasma levels of C-reactive protein, interleukin-6, 8-epi-prostaglandin F2α, and thiobarbituric acid reactive substances (TBARS) in eighteen HFpEF patients and 14 controls. The authors concluded that HFpEF exhibits an elevation in a broad spectrum of biomarkers indicative of an inflammatory and a prooxidative state [43]. Of note, however, in their study, the HFpEF population was older by a decade than the controls. Aging, by itself, can increase oxidative stress and its markers [46]. Our study, on the other hand, has shown that oxidative stress and angiotensin activity were not elevated in HFpEF. The results of our study support the lack of clinical benefit seen with therapies to reduce angiotensin activity and oxidative stress such as RAAS inhibitors in HFpEF in large clinical trials [8–12].

4.1. Limitations and Future Directions. There are limitations to our study. Foremost, despite consistency with previous clinical trials, it is possible that our study did not have sufficient power to detect associations between RAS or oxidative stress and HFpEF. On the other hand, a similar sized study easily detected a difference in oxidative stress markers in patients with and without AF [18]. Blood samples were drawn from nonfasting subjects and at variable times during the day. Thiol reduced state is affected by meals in animals [47]. Nevertheless, variations are relatively small and plasma levels of DROMs and IsoPs are not affected by the prandial state [48]. Also, it is known that plasma levels of oxidized and reduced thiols undergo a small diurnal variation [49]. Again, DROMs and IsoPs are not known to show any diurnal variation [18]. Moreover, four different markers, measuring oxidative stress in the hydrophilic and hydrophobic phases, showed similar results, making technical errors unlikely. Finally, echocardiography could have misclassified patients, despite using standard criteria [50].

In conclusion, we did not find evidence of systemic RAS activation or oxidative stress in patients with early HFpEF. This finding is consistent with the lack of efficacy of RAS inhibitors in the treatment of HFpEF. The lack of RAS activation and systemic oxidation seems to differentiate HFrEF from HFpEF. This suggests different mechanisms in genesis or propagation of these two forms of HF, which would explain the difference in benefit with treatment modalities.

Disclosure

Samuel C. Dudley Jr. is the inventor of patent applications: (1) 11/895,883 Methods and Compositions for Treating Diastolic Dysfunction, (2) 13/503,812 Methods of Diagnosing Diastolic Dysfunction, (3) 13/397,622 Methods for Treating Diastolic Dysfunction and Related Conditions, (4) 13/658,943 Method of Improving Diastolic Dysfunction, (5) 13/841,843 Myosin Binding Protein-C for Use in Methods Relating to Diastolic Heart Failure, and (6) 61/728,302 Mitochondrial Antioxidants and Diabetes.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Is Lipoprotein-Associated Phospholipase A2 a Link between Inflammation and Subclinical Atherosclerosis in Rheumatoid Arthritis?

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Objective. Lipoprotein-associated phospholipase A2 (Lp-PLA2), a marker of vascular inflammation, is associated with cardiovascular disease. This prospective study of an inception cohort aimed to investigate whether the level of Lp-PLA2 is associated with subclinical atherosclerosis in patients with rheumatoid arthritis (RA). *Methods*. Patients from northern Sweden diagnosed with early RA were consecutively recruited into an ongoing prospective study. From these, all patients ≤60 years ($n = 71$) were included for measurements of subclinical atherosclerosis at inclusion (T0) and five years later (T5). Forty age- and sex-matched controls were included. The patients were clinically assessed, SCORE, Reynolds Risk Score, and Larsen score were calculated, and blood samples were drawn from all individuals at T0 and T5. *Results*. There was no significant difference in the level of Lp-PLA2 between patients with RA and controls ($p > 0.05$). In simple linear regression models among patients with RA, Lp-PLA2 at T0 was significantly associated with intima media thickness (IMT) at T0 and T5, flow mediated dilation (FMD) at T0 and T5, ever smoking, male sex, HDL-cholesterol (inversely), non-HDL-cholesterol, SCORE, Reynolds Risk Score, and Larsen score ($p < 0.05$). *Conclusion*. In this cohort of patients with early RA, the concentration of Lp-PLA2 was associated with both subclinical atherosclerosis and disease severity.

1. Introduction

Patients with rheumatoid arthritis (RA) have increased atherosclerosis compared with the general population [1–4]. Atherosclerosis is now recognised as an inflammatory disease *per se* [5] and the two diseases, atherosclerosis and RA, are considered to share many similarities [6], albeit the link between them is, as yet, not evident.

Subclinical atherosclerosis precedes cardiovascular disease (CVD) and an increased intima media thickness (IMT), measured by ultrasonography, is regarded as an early indicator of a generalized atherosclerosis [7]. Several studies in the general population, as well as in patients with RA, have shown a relationship between an increased IMT and a future cardiovascular event [8–12]. We, and others, have previously shown that patients with established RA have a premature atherosclerosis as measured by an increased IMT of the common carotid artery (CCA) compared with controls [13, 14]. An even earlier sign of atherosclerosis, that is, endothelial dysfunction, indicated by an impaired flow mediated vascular dilation (FMD) of peripheral arteries, can also be measured using ultrasonography [15]. In the general population, FMD has been associated with other established risk factors for CVD and shown to be predictive of a future CV event [15–17].

Lipoprotein-associated phospholipase A2 (Lp-PLA2), formerly also known as platelet-activating factor acetylhydrolase (PAF-AH), is an enzyme expressed, among others,

by inflammatory cells in atherosclerotic plaques [18, 19]. Lp-PLA2 hydrolyses phospholipids in low-density lipoprotein (LDL) to yield proinflammatory products such as oxidized free fatty acids. These proinflammatory products play a critical role in the endothelial chemotactic response by stimulating the expression of adhesion molecules and cytokines as well as recruiting inflammatory cells. Hence, Lp-PLA2 is suggested to be a useful and potent biomarker of the vascular inflammation involved in the pathophysiology of atherosclerosis [19–21].

In the circulation, Lp-PLA2 is carried bound mainly to LDL, and several epidemiological studies in the general population have shown a correlation between Lp-PLA2 levels and traditional cardiovascular risk factors [18, 22, 23]. In the general population, higher concentrations of Lp-PLA2 have also been shown to be associated with an increased risk of CVD [18]. Previous studies of patients with thalassemia, metabolic syndrome, or human immunodeficiency virus (HIV) infection, each diagnosis being characterised by an increased inflammation, have shown an association between Lp-PLA2 and subclinical atherosclerosis measured by IMT [20, 23, 24]. In populations without a known inflammation, however, the results are contradictory [21, 25–27]. To the best of our knowledge there are no studies on the relationship between Lp-PLA2 and atherosclerosis in patients with RA.

In an ongoing prospective case-control study of patients with very early RA [28], we have found a significant increase in the subclinical atherosclerosis, measured by IMT and FMD, during the first five years of rheumatic disease [29]. In the present study, we hypothesized that vascular inflammation, reflected by the concentration of Lp-PLA2, contributes to the atherosclerotic disease in patients with RA. Thus, our primary aim was to investigate whether the level of Lp-PLA2 was associated with subclinical atherosclerosis at baseline (T0) or after the first five years following a diagnosis of RA (T5). A secondary aim was to identify markers of inflammation and traditional CVD risk factors associated with Lp-PLA2 and hence with a possible vascular inflammation preceding CVD.

2. Material and Methods

2.1. Patients and Controls. The present study is a part of a continuing structured programme on early RA for prospective analysis of CVD development in patients from northern Sweden using the nationwide Swedish Rheumatoid Arthritis Registry. All eligible patients with newly diagnosed RA (according to ACR criteria) [30] and symptomatic for no longer than 12 months are continuously enrolled into the register as soon as possible following diagnosis (T0). The inclusion criteria for the patients with RA and controls have previously been described in detail [28]. Five years after inclusion into the study (T5), 71 of the 79 patients with RA originally included were willing to participate in the follow-up study, and 40 of the original 44 controls were reassessed. The controls (one control for two patients except for in 13 cases one control per patient) were matched for age (±5 years) and sex. Only those individuals participating in the follow-up assessment, that is, T5, were included in this study. All individuals gave their written consent in accordance with the Declaration of Helsinki. The study was approved by the Regional Ethics Committee of Umeå University, Umeå, Sweden.

2.2. Physical Examination and Surveys. All patients were examined clinically at inclusion into the study and regularly thereafter at 3, 6, 12, 18, 24, and 60 months. The number of swollen and tender joints (28-joint count) and the patient's global assessment were registered, and a disease activity score (DAS28), including the erythrocyte sedimentation rate (ESR), was calculated [31]. Posterior-anterior radiographs of the hands, wrists, and feet were obtained at baseline and after five years and were graded according to the Larsen score by two rheumatologists (EB and Solbritt Rantapää-Dahlqvist) [32]. All participants completed a survey on comorbidity. Any previous CVD events were verified by reference to medical records. Up to the five-year follow-up assessment, eight (11%) of the patients with RA had suffered a CVD event (3 acute myocardial infarction, 3 stroke, and 2 thromboembolic event) whilst two (5%) of the controls had suffered a CVD event (both coronary artery bypass graft surgery). Blood pressure was measured at the time of ultrasound measurements. Body mass index (BMI), European Systematic Coronary Risk Evaluation (SCORE) [33], and Reynolds Risk Score [34] were calculated at both T0 and T5. These compound measures of CVD risk factors estimate the risk of death due to a CVD event during the next 10 years. In addition to traditional CVD risk factors, the Reynolds Risk Score includes C-reactive protein (CRP) concentrations. When calculating the Reynolds Risk Score, all patients were regarded as being nondiabetic due to a lack of information regarding levels of haemoglobin A1c for all individuals; this assumption may have resulted in an underestimation of the risk score.

2.3. Ultrasound Investigations. The patients were examined by ultrasound as soon as possible following diagnosis (at T0); the mean (±SD) time after the primary symptom of RA was 16.2 (±6.6) months. The ultrasound investigations at the follow-up (T5) were performed 5 years after the initial examinations (mean and median being 60 months, with a range 59–63, after the first examination). All ultrasonography examinations of patients with RA and controls were performed by the same experienced investigator (EL); the individuals were in a supine position in a quiet, temperature controlled room. A Sequoia 512 ultrasound system (Siemens (Acuson) Corp) was used with a 15L8 transducer for measurement of the brachial artery and an 8L5 transducer for carotid artery studies. All investigations were digitally stored for analyses to be performed by the single observer (EL; intraobserver variability for IMT $r = 0.988$). The protocol for these investigations has previously been described in detail [28].

2.4. Blood Sampling. In the present study all patients and controls donated a blood sample at the time of both ultrasound measurements, that is, at T0 and T5, and serum was stored at −80°C. After thawing, serum concentrations of Lp-PLA2 (ng/mL) were measured using an ELISA (R&D

Table 1: Measurement of Lp-PLA2, intima media thickness (IMT), flow mediated dilatation (FMD), traditional risk factors for cardiovascular disease (CVD), and disease activity in patients with early rheumatoid arthritis (RA) and in age- and sex-matched controls evaluated both at baseline (T0) and after 5 years (T5). Data are expressed as mean value (standard deviation).

	RA ($n = 71$)		Controls ($n = 40$)	
	At T0	At T5	At T0	At T5
Lp-PLA2, ng/mL	144.0 (41.7)	154.6 (38.9)*	132.0 (37.8)	148.1 (41.7)**
Intima media thickness, mm	0.52 (0.13)	0.58 (0.13)***	0.54 (0.13)	0.60 (0.12)***
Endothelium dependent flow mediated vasodilatation, %	109.2 (4.7)	107.0 (4.7)***	107.2 (4.5)	106.0 (4.6)
Systolic blood pressure, mmHg	123.5 (14.4)	126.3 (13.9)*	117.7 (11.3)	124.1 (12.1)***
Cholesterol, mmol/L	5.5 (0.9)	5.3 (1.0)	5.3 (1.1)	5.6 (1.1)
HDL, mmol/L	1.6 (0.5)	1.6 (0.5)	1.5 (0.4)	1.7 (0.5)
Non-HDL-cholesterol, mmol/L	3.9 (0.9)	3.7 (1.0)	3.9 (1.1)	3.9 (1.1)
Triglycerides, mmol/L	1.3 (0.5)	1.2 (0.5)	1.1 (0.3)	1.0 (0.5)
BMI, kg/m^2	25.8 (4.0)	25.7 (4.5)	25.1 (4.9)	25.2 (4.2)
CRP, mg/L	11.9 (10.8)	7.7 (7.2)**	n/a	n/a
DAS28	3.5 (1.4)	3.1 (1.5)†	n/a	n/a

* p value < 0.05; ** p value < 0.01; *** p value < 0.0001; † p value = 0.061; all values compared with T0.
Lp-PLA2: lipoprotein-associated phospholipase A2; HDL: high-density lipoproteins; BMI: body mass index; CRP: C-reactive protein; DAS28: disease activity score for 28-joint count.

Systems, Abingdon, UK). Rheumatoid factor (RF; 67% of the patients were seropositive), CRP (mg/L), and erythrocyte sedimentation rate (ESR; mm/h) were measured according to routine methodology. Whenever several analyses of DAS28, CRP, or ESR were performed on any given individual, the assessment closest to the ultrasound measurement was used in any subsequent statistical analysis. Blood was also drawn after an overnight fast for analysis of blood lipids: cholesterol (mmol/L), high-density lipoproteins (HDL; mmol/L), and triglycerides (mmol/L) using routine methods at each of the participating hospitals. Lp-PLA2 concentration results were available for 70 and 66 patients with RA at T0 and T5, respectively. Correspondingly, results for Lp-PLA2 were available for 38 and 40 controls at T0 and T5, respectively.

2.5. Statistics. Comparisons over time within the RA patient group and within the control group were performed using the Wilcoxon paired test. Simple and multiple linear regression analyses were used to identify variables associated with Lp-PLA2. Results from simple linear regression (variables with $p < 0.05$), together with clinical assumptions (variables with $p > 0.05$), determined which covariates were included in the multiple linear regression models. Differences in variables between patients with RA and matched controls were analysed using simple conditional logistic regression analyses. Occasional missing values, due to missing information, were regarded as random. Non-HDL-cholesterol was calculated as total cholesterol minus HDL-cholesterol. Based on previously published data [20], calculations showed that a sample size of 71 would render 99% power to detect a correlation between IMT and Lp-PLA2 with a correlation coefficient of 0.46. p values < 0.05 were considered statistically significant. All calculations were made using SPSS 20.0 (SPSS Inc., Chicago, USA).

3. Results

For this study, 71 patients with RA (61 (86%) women) and 40 controls (32 (80%) women) were included. The mean age (SD) of the patients with RA was 51.5 (10.7) years and 48.1 (10.9) years for the controls. Among the patients with RA, 35 (58%) declared themselves to ever being a smoker, corresponding with 14 (39%) among the controls. Six (9%) patients with RA and 5 (12%) controls had ever used statins.

The concentration of Lp-PLA2 increased significantly during the 5-year follow-up period, both for the patients with RA and the controls (Table 1). At both time points the concentrations of Lp-PLA2 were numerically higher in patients with RA compared with controls (p values > 0.05) (Table 1).

Among the patients with RA, the concentration of Lp-PLA2 at T0 was significantly associated with IMT as well as with FMD at both baseline and follow-up (Table 2). After adjustment for sex and age, Lp-PLA2 was still significantly associated with IMT at T0 and T5 (Table 2). At T0, the Lp-PLA2 concentration was also significantly associated with non-HDL-cholesterol, HDL (inversely), diastolic blood pressure, smoking, SCORE, and Reynolds Risk Score as well as the Larsen score (Table 2). Adjustment for disease activity, measured by DAS28 at T0, did not change significantly the association between the Lp-PLA2 levels at T0 and disease severity measured by the Larsen score at T0 (Table 3).

At T5 Lp-PLA2 was significantly associated with non-HDL-cholesterol, HDL (inversely), cholesterol, BMI, and Reynolds Risk Score among the patients with RA (Table 4). There were no associations between Lp-PLA2 and any of the measures of disease activity, that is, CRP, ESR, and DAS28, at either T0 or T5 (data not shown). Furthermore, there was no significant association between Lp-PLA2 concentration and the Larsen score at T5, nor were there any significant

Table 2: Results of simple regression models among the 71 patients with early RA with the concentration of Lp-PLA2 at T0 as the dependent variable.

	Lp-PLA2 at T0		
	β	95% CI	p value
IMT T0 ($n = 70$)	9.7/mm	2.1; 17.2	0.013[†]
IMT T5 ($n = 70$)	8.8/mm	1.4; 16.2	0.02[†]
FMD T0 ($n = 70$)	−2.4/%	−4.5; −0.4	0.02
FMD T5 ($n = 70$)	−2.5/%	−4.6; −0.5	0.02
Non-HDL-cholesterol T0 ($n = 54$)	16.9/mmolL^{-1}	5.8; 28.0	0.004
HDL T0 ($n = 55$)	−22.6/mmolL^{-1}	−42.0; −3.1	0.02
Diastolic blood pressure T0 ($n = 66$)	1.3/mmHg	0.1; 2.5	0.04
Ever smoking ($n = 70$)	0.8/year	0.08; 1.4	0.03
SCORE T0 ($n = 53$)	11.4/unit	3.3; 19.5	0.007
Reynolds Risk Score T0 ($n = 38$)	5.2/unit	1.5; 8.8	0.007
Larsen score T0 ($n = 50$)	2.9/unit	0.013; 5.90	0.05

[†]Still significant after adjustment for sex and age.
RA: rheumatoid arthritis; Lp-PLA2: lipoprotein-associated phospholipase A2; IMT: intima media thickness; FMD: flow mediated dilation; HDL: high-density lipoproteins.

Table 3: Multiple regression models among 71 patients with early RA with the concentration of Lp-PLA2 at T0 as dependent variable.

	Lp-PLA2 at T0		
	β	95% CI	p value
Larsen score T0	2.8/unit	−0.3; 5.8	0.06
DAS28 T0	−4.6/unit	−2.0; 0.3	0.03

RA: rheumatoid arthritis; Lp-PLA2: lipoprotein-associated phospholipase A2; DAS28: disease activity score for 28-joint count.

associations between Lp-PLA2 and age, or any medication either at T0 or at T5 (data not shown).

Among the controls, Lp-PLA2 at T0 was significantly associated with IMT at T0 (β 3.2, $p = 0.05$) and Lp-PLA2 at T5 was significantly associated with several variables measured at T0: IMT (β 3.9, $p < 0.05$), cholesterol (β 6.1, $p < 0.001$), triglycerides (β 3.3, $p < 0.05$), diastolic blood pressure (β 4.1, $p = 0.01$), age (β 5.2, $p < 0.001$), and SCORE (β 4.1, $p < 0.05$). Among the same individuals Lp-PLA2 at T5 was significantly associated with IMT (β 3.3, $p < 0.05$) and cholesterol (β 5.8, $p < 0.001$) at T5. After adjustment for sex and age no significant association between Lp-PLA2 and IMT was found among the controls, neither at T0 nor at T5 (data not shown).

4. Discussion

In this study, the serum concentration of Lp-PLA2 was associated with measures both of subclinical atherosclerosis over time and of disease severity at disease onset in patients with early RA.

Table 4: Results of simple regression models among 71 patients with early RA with the concentration of Lp-PLA2 at T5 as the dependent variable.

	Lp-PLA2 at T5		
	β	95% CI	p value
IMT T5 ($n = 66$)	4.4/mm	−2.7; 11.5	0.22
FMD T5 ($n = 66$)	−1.7/%	−3.8; 0.4	0.10
Non-HDL-cholesterol T5 ($n = 61$)	19.0/mmolL^{-1}	10.5; 27.5	0.001
HDL T5 ($n = 61$)	−31.8/mmolL^{-1}	−50.3; −13.3	0.001
Cholesterol T5 ($n = 61$)	11.5/mmolL^{-1}	2.1; 20.8	0.02
Ever smoking ($n = 66$)	0.4/year	−0.3; 1.1	0.26
BMI T5 ($n = 66$)	2.5/unit	0.3; 4.7	0.03
SCORE T5 ($n = 61$)	3.6/unit	−2.0; 9.2	0.21
Reynolds Risk Score T5 ($n = 39$)	2.8/unit	−0.2; 5.7	0.06

RA: rheumatoid arthritis; Lp-PLA2: lipoprotein-associated phospholipase A2; IMT: intima media thickness; FMD: flow mediated dilation; HDL: high-density lipoproteins; BMI: body mass index.

From previously published reports, it is evident that patients with RA have an increased development of atherosclerosis compared with the general population, with different underlying causes being proposed to explain this observation [3, 4, 6, 13]. A strong theory to date is that the inflammatory load among the patients with RA affects the arteries and gives rise to a subclinical vascular inflammation. An increased level of Lp-PLA2 among patients with RA was shown several years ago, when it was presented as a marker of disease activity among such patients [35, 36]. However, more recently published studies on other inflammatory diseases, as well as on the general population, suggest that Lp-PLA2 cannot be regarded as a marker of a systemic inflammation but as a mere biomarker of atherosclerosis [19]. With this background in mind, we measured the concentration of Lp-PLA2 as a marker of vascular inflammation. However no significant difference in the levels of Lp-PLA2 in patients with RA and controls was found, neither early in the disease nor after 5 years, albeit the concentrations of Lp-PAL2 were numerically higher among the patients with RA at all time points.

It is now recognized that atherosclerosis is the result of an inflammatory process in the vessel wall [5], and early atherosclerosis can be identified as an endothelial dysfunction by FMD or arterial wall thickening by IMT. The Lp-PLA2 concentration at inclusion of patients with early RA into this study, as well as at the five-year follow-up assessment, was found to be associated with both measurements of early atherosclerosis. Lp-PLA2 is regarded as a highly specific biomarker for vascular inflammation and burden of atherosclerosis [19]. The correlation between this biomarker and atherosclerosis is well studied in the general population, as well as in other inflammatory diseases; however there are also contradictory results [20, 21, 23–27]. After five years following a diagnosis of RA, the levels of Lp-PLA2 found at inclusion could still explain the extent

of atherosclerosis measured by IMT and FMD in patients with RA. There are, to the best of our knowledge, only a few prospective studies on Lp-PLA2 and the development of subclinical atherosclerosis and none regarding patients with an inflammatory disease. Two studies on patients with diabetes mellitus found measurements of Lp-PLA2 to be associated with the progression of atherosclerosis over time [37, 38] and Liu et al. verified these results in the general population [27]. In this study a similar result was found, with an association between Lp-PLA2 and prospectively registered measures of atherosclerosis in patients with early RA.

In previous studies involving this cohort of patients, the extent of atherosclerosis was associated with traditional cardiovascular risk factors [28, 29]. In the present study we found, consistent with other published studies, the levels of Lp-PLA2 to be associated with several traditional risk factors [18, 22, 23]. Moreover, the compound measure of CVD risk that included inflammation, that is, the Reynolds Risk Score, was strongly associated with Lp-PLA2 at both T0 and T5. The Lp-PLA2 molecule is carried in the circulation mainly bound to LDL [18, 19] and, as was to be expected, the level of Lp-PLA2 was found to be evidently associated with the concentrations of blood lipids. Non-HDL-cholesterol, in some regards a better measurement of the risk of CVD than LDL [39], was strongly associated with Lp-PLA2 both at T0 and at T5.

Radiological progression is a measurement of disease severity over time in patients with RA. We found a significant relationship between Lp-PLA2 concentration and the Larsen score at the time of diagnosis of RA. In these patients, the same inflammatory process that leads to joint damage may also affect the vascular walls causing a vascular inflammation, as reflected by elevated concentrations of Lp-PLA2. This association was not altered significantly by adjustment for disease activity at inclusion, again indicating that Lp-PLA2 is not just a marker of disease activity. In a multiple regression model, both IMT and Larsen score at inclusion were significantly associated with the concentration of Lp-PLA2, indicating that the processes leading to joint damage and vascular damage are, in some part, interlinked.

The main strength of the present study is the prospective design from the onset of disease. In northern Sweden almost all individuals newly diagnosed with RA are included in a structured follow-up programme. Of these patients, all of those aged ≤60 years were invited to participate in this study within 12 months of their diagnosis. Data on biomarkers and traditional CVD risk factors, as well as variables related to the RA disease, were collected from the onset of disease and then continuously during the five years of follow-up. Another strength of this study is that the same person (KE) undertook all of the laboratory-based analyses and that Lp-PLA2 in samples collected at both time points (i.e., T0 and T5) was measured simultaneously. Furthermore, all ultrasound measurements at both time points, and their analysis, were undertaken by the same person (EL).

Conversely, a limitation of this study is that it is strictly observational; in other words, no influence could be made on medications and other variables observed. Another limitation is the number of control subjects; however this study was directed primarily at studying the serum concentrations of Lp-PLA2 among the patients with RA. Another limitation is the lack of data on LDL-cholesterol levels. However, non-HDL-cholesterol was calculated, based on findings reported in some studies that it is more strongly associated with a risk of CVD than LDL-cholesterol [39]. The relationship between Lp-PLA2 and LDL-cholesterol must be regarded as well studied [18, 22]. Furthermore, we were not able to explore the association between Lp-PLA2 and CVD since there were too few CV events during the follow-up. Still, this study is the first of its kind, and further studies, including a follow-up of the individuals in the present study, will be able to clarify this association.

5. Conclusions

In this study, the level of Lp-PLA2 among patients with RA was associated with subclinical atherosclerosis, prospectively measured by IMT and FMD. Among these patients with early RA, this biomarker of vascular inflammation was also associated with Larsen score, indicating that over time the deleterious disease process may also affect the vascular walls. Taken together, our findings indicate a continuous vascular inflammation among patients with RA possibly leading to the development of atherosclerosis and hence to CVD. This possibility adds to the knowledge of the mechanisms responsible for the observed increased risk of CVD among patients with RA.

Conflict of Interests

None of the authors declare any potential conflict of interests.

Acknowledgments

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Research Article

Protective Effects of Cilastatin against Vancomycin-Induced Nephrotoxicity

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Vancomycin is a very effective antibiotic for treatment of severe infections. However, its use in clinical practice is limited by nephrotoxicity. Cilastatin is a dehydropeptidase I inhibitor that acts on the brush border membrane of the proximal tubule to prevent accumulation of imipenem and toxicity. The aim of this study was to investigate the potential protective effect of cilastatin on vancomycin-induced apoptosis and toxicity in cultured renal proximal tubular epithelial cells (RPTECs). Porcine RPTECs were cultured in the presence of vancomycin with and without cilastatin. Vancomycin induced dose-dependent apoptosis in cultured RPTECs, with DNA fragmentation, cell detachment, and a significant decrease in mitochondrial activity. Cilastatin prevented apoptotic events and diminished the antiproliferative effect and severe morphological changes induced by vancomycin. Cilastatin also improved the long-term recovery and survival of RPTECs exposed to vancomycin and partially attenuated vancomycin uptake by RPTECs. On the other hand, cilastatin had no effects on vancomycin-induced necrosis or the bactericidal effect of the antibiotic. This study indicates that cilastatin protects against vancomycin-induced proximal tubule apoptosis and increases cell viability, without compromising the antimicrobial effect of vancomycin. The beneficial effect could be attributed, at least in part, to decreased accumulation of vancomycin in RPTECs.

1. Introduction

Vancomycin (VAN) is a glycopeptide antibiotic that is widely used for the treatment of severe Gram-positive infections such as those caused by methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis* [1, 2].

Patients hospitalized in the cardiac care or cardiovascular surgery units frequently require an intravenous or intra-arterial catheter. Approximately 3% of these patients develop catheter-related bloodstream infection (CRBSI), although the incidence may be as high as 16% [3]. In clinical cases of prolonged *S. aureus* CRBSI, VAN is the most commonly used antimicrobial treatment [4]. Nevertheless, VAN has potentially fatal side effects [1, 2, 5, 6]. Nephrotoxicity is the side effect that most limits the dose of VAN, particularly in patients receiving high doses or combinations with other antibiotics, such as aminoglycosides [7]. VAN-induced nephrotoxicity has been reported to occur in 5–25% of patients [2, 8], although this incidence can rise to 20–35%, with a consequent increase in the severity of renal failure when VAN is administered concomitantly with aminoglycosides [9].

The mechanism underlying VAN-induced nephrotoxicity remains unclear despite numerous studies performed over several decades, although some authors have suggested that it is similar to that of gentamicin [10]. Recent animal and

cellular studies have shown that oxidative stress, inflammatory events, and apoptotic cell death might play a role in the pathogenesis of VAN-induced nephrotoxicity [1, 2, 7], which directly affects renal proximal tubular epithelial cells (RPTECs) and leads to renal tubular ischemia and acute tubulointerstitial damage [2, 8, 11]. In fact, increased urinary excretion of proximal tubule cells after administration of VAN has been demonstrated in animal studies [12]. VAN directly triggers depolarization of mitochondrial membrane potential, release of cytochrome c, and activation of caspase 9, which in turn activates caspase 3, a key component in the execution stage of apoptosis [7].

Prevention of VAN-induced nephrotoxicity without decreasing efficacy is a highly desirable objective in treatment of MRSA-induced CRBSI. Although several *in vitro* and *in vivo* approaches have been proposed to reduce VAN-induced renal toxicity, such as antioxidants or erythropoietin [1, 7, 11, 13, 14], it is unclear whether such approaches would limit the bactericidal capacity of VAN. Therefore applicability in humans is questionable and has yet to be established [15]. Therapeutic drug monitoring is one of the few effective options for prevention of VAN-induced nephrotoxicity, although it is clearly insufficient [2, 7], and the search for alternative protective strategies against toxic damage to the proximal tubule is a key research area today.

We previously reported the usefulness of cilastatin in the prevention of acute kidney injury (AKI) induced by common nephrotoxic agents (e.g., cisplatin) without reducing therapeutic activity [16–20]. Cilastatin is an inhibitor of dehydropeptidase I (DHP-I), which is found in the cholesterol rafts of the brush border of RPTECs [18]. Our experimental evidence suggests that binding of cilastatin to DHP-I interacts with apical cholesterol lipid rafts [16, 18, 19] to protect (*in vivo* and *in vitro)* against the apoptosis and oxidative stress induced by nephrotoxic agents. Clinical studies also support this protective role of cilastatin (imipenem-cilastatin) against cyclosporine A- (CsA-) induced nephrotoxicity [21–24].

Studies have shown that cilastatin (or imipenem-cilastatin) has the potential to protect against VAN-induced nephrotoxicity [25–27]; however, evidence for the antiapoptotic effects of cilastatin on VAN-induced AKI is insufficient. Thus, the aims of the present study were to evaluate the role of cell death as the main pathogenic mechanism in VAN-mediated renal cell injury and to evaluate whether cilastatin can reduce or prevent VAN-induced proximal tubule cell death without compromising bactericidal power.

2. Material and Methods

2.1. Chemicals. VAN was obtained from Normon (Madrid, Spain) and dissolved in cell culture medium at the specified concentrations.

Crystalline cilastatin was kindly provided by Merck Sharp & Dohme S.A. (Madrid, Spain). A dose of 200 μg/mL was chosen because it is cytoprotective and falls within the reference range for clinical use [18, 19].

2.2. Proximal Tubular Primary Cell Culture. Porcine RPTECs were obtained as previously described [18]. Briefly, cortex was obtained by slicing a kidney and disaggregated by incubation in Ham's F-12 medium containing collagenase A (Boehringer Mannheim, Germany) at a final concentration of 0.6 mg/mL. Digested tissue was then filtered, washed, and centrifuged by resuspension in isotonic, sterile Percoll gradient (45% [v/v]) at 20,000 g for 30 minutes. Proximal tubules were collected from the deepest fraction, washed, and resuspended in supplemented DMEM/Ham's F-12 in a 1 : 1 ratio (with 25 mM HEPES, 3.7 mg/mL sodium bicarbonate, 2.5 mM glutamine, 1% nonessential amino acids, 100 U/mL penicillin, 100 mg/mL streptomycin, 5×10^{-8} M hydrocortisone, 5 mg/mL ITS, and 2% fetal bovine serum). Proximal tubules were seeded at a density of 0.66 mg/mL and incubated at 37°C in a 95% air/5% CO_2 atmosphere. RPTECs were used when they reached confluence (~80%).

2.3. Cell Morphology Analysis. Pictures of cell morphology were obtained using 4x objective of Olympus IX70 microscope (Olympus, Hamburg, Germany) in phase-contrast imaging 24 hours after treatment with VAN (0.6, 3, and 6 mg/mL) or VAN plus cilastatin (200 μg/mL).

2.4. Quantification of Cell Detachment. RPTECs were cultured and treated with VAN (0.6, 3, and 6 mg/mL) in the presence or absence of cilastatin (200 μg/mL) for 24 h. Detached cells were collected, resuspended in 300 μL of phosphate-buffered saline (PBS), and quantified by flow cytometry (Gallios Beckman Coulter, Barcelona, Spain). Results were obtained as cell counting for 60 s and we selected the gate according to FS (forward scatter) and SS (side scatter). These data were analyzed using Kaluza for Gallios Software (Beckman Coulter).

2.5. Measurement of Apoptosis and Necrosis. Cell nuclei were visualized after DNA staining with the fluorescent dye 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, Missouri, USA) in order to detect evidence of apoptosis. In brief, cells on coverslips were treated with VAN (0.6, 3, or 6 mg/mL) with or without cilastatin 200 μg/mL for 24 h. Thereafter, cells were fixed in 4% formaldehyde for 10 min, rinsed with PBS, and permeabilized with 0.5% Triton X-100 for 5 min. Cells were then rinsed with PBS and incubated with DAPI (12.5 μg/mL) at room temperature for 15 min. Finally after removing excess dye, coverslips were mounted in microscope slides and imaging was performed as previously described [18, 20].

DNA fragmentation was measured in RPTECs treated with VAN (0.6, 3, or 6 mg/mL) in the presence or not of cilastatin 200 μg/mL using Cell Death Detection ELISA^{PLUS} Kit (Boehringer Mannheim, Roche, Mannheim, Germany) according to the manufacturer's protocol.

To detect any evidence of necrosis, release of lactate dehydrogenase (LDH) from RPTECs was measured in the culture medium 24 and 48 h after exposure to VAN (3 and 6 mg/mL) in the presence or not of cilastatin (200 μg/mL), as previously described [18]. Release of LDH was expressed relative to total LDH released by treatment with 0.1% Triton X-100 (100% release).

2.6. Measurement of Early/Late Apoptosis Using Flow Cytometry. Early and late VAN-induced apoptosis were measured using annexin V (BD Pharmingen, Madrid, Spain) and propidium iodide (PI, Sigma-Aldrich). Cells were pretreated with VAN (0.6, 3, and 6 mg/mL) alone or in combination with cilastatin (200 μg/mL) before being trypsinized, washed twice with PBS, and incubated for 30 min in the dark in 100 μL buffer containing 5 μL fluorescein isothiocyanate- (FITC-) labeled annexin V and 5 μL PI for flow cytometry (Gallios, Beckman Coulter). At least 10 000 cells were analyzed in each case. Data were analyzed using Kaluza for Gallios Software (Beckman Coulter).

2.7. Cell Viability Assay. Cell survival was measured by MTT assay as described previously [18, 20]. In brief, after 24 h treatment with VAN 0.6, 3, or 6 mg/mL alone or in combination with cilastatin (200 μg/mL), RPTECs were incubated with 0.5 mg/mL of MTT for 3 h in darkness at 37°C. Thereafter, the volume was removed and 100 μL of 50% dimethylformamide in 20% SDS (pH 4.7) was added, incubating plates at 37°C overnight. The amount of colored formazan formed was measured at 595 nm.

Alternatively, an Olympus IX70 inverted microscope fitted to a spectrofluorometer SLM AMINCO 2000 was used to measure MTT reduction in real time on single cells at 570 nm, as previously described [20]. Recordings of the first seconds after addition of MTT show the initial kinetics of MTT reduction and formazan production, thus offering a first approach to the activity and function of the mitochondrial chain in intact cells.

2.8. Quantification of Colony-Forming Units. RPTECs were plated on six-well plates and treated for 24 h with VAN 3 or 6 mg/mL alone or in combination with cilastatin (200 μg/mL), to measure the long-term protective effects of cilastatin as described previously [18, 20]. Briefly, supernatants were discarded and adherent cells were washed in saline serum, trypsinized, seeded in Petri dishes (100 mm), and allowed to grow for 7 days in drug-free complete medium. Adherent cells colonies were fixed for 5 minutes with 5% paraformaldehyde/PBS and stained with 0.5% crystal violet/20% methanol for 2 minutes. Excess dye was removed by washing with PBS. Finally, crystal violet was eluted with 50% ethanol/50% sodium citrate 0.1 M (pH 4.2) and quantified at 595 nm.

2.9. Cellular VAN Transport and Accumulation. Accumulation of VAN in RPTECs was measured using a Fluorescence Polarization Immunoassay technology on a TDX Chemistry Analyzer (Abbot Laboratories, USA) in accordance with the manufacturer's instructions, in the same way that it was described previously [20]. The results were expressed as follows: [μg VAN/μg protein].

2.10. Microorganism Susceptibility Assays. We tested 8 unique clinical isolates collected from blood, abscesses, and urine from patients in our hospital in 2012. The isolates corresponded to 4 *Staphylococcus aureus* strains (2 methicillin-susceptible and 2 methicillin-resistant), 3 *Enterococcus faecalis* strains, and 1 *Enterococcus faecium* strain. Previous minimum inhibitory concentration (MIC) based on microdilution testing (MicroScan panels, Siemens, Sacramento, USA) revealed that all Gram-positive isolates were susceptible to VAN.

Susceptibility Testing. To determine MICs broth microdilution method was performed with standard cation-adjusted Mueller-Hinton broth (CAMHB) as previously described in the guidelines of the Clinical and Laboratory Standards Institute [28]. VAN was tested at dilutions ranging from 0.06 to 64 μg/mL with or without cilastatin (200 μg/mL).

Minimum bactericidal concentrations (MBCs) were determined as previously described [29, 30]. Briefly, 0.1 mL from the MIC well and 4 further dilutions were cultured in blood agar plates and incubated at 37°C for 24 to 48 h. The values of MBCs were recorded as the lowest dilution decreasing ≥99.9 in growth (≥3-log 10 reduction in colony-forming units (CFU)/mL) in comparison with control.

We compared the results obtained with VAN alone or in combination with cilastatin.

2.11. Statistical Methods. Quantitative variables were summarized as the mean ± standard error of the mean (SEM). Differences were considered statistically significant for bilateral alpha values under 0.05. Factorial ANOVA was used when more than 1 factor was considered. When a single factor presented more than 2 levels, a post hoc analysis (least significant difference) was performed, if the model showed significant differences between factors. When demonstrative results are shown, they represent a minimum of at least 3 repeats. When possible, a quantification technique was used to illustrate reproducibility.

3. Results

3.1. Cilastatin Reduces VAN-Induced Proximal Tubular Cell Damage. VAN induces dose-dependent cell death in primary culture of RPTECs. When RPTECs are exposed to increasing concentrations of VAN for 24 hours, direct observation by phase microscopy shows cell rounding and detachment from the plate. Cilastatin significantly reduced the impact observed at every VAN concentration (Figure 1(a)).

However, VAN-induced cell death causes early detachment of damaged cells from the plate. Figure 1(b) shows the quantification of nonadherent cells from control plates and VAN-treated plates (0.6, 3, and 6 mg/mL) in combination or not with cilastatin. Cilastatin significantly reduced cell detachment in cells treated with 3 and 6 mg/mL.

3.2. Cilastatin Protects against VAN-Induced Apoptosis but Not Necrosis. Estimation of apoptotic cell death was obtained in adherent cells stained with DAPI (Figures 2(a)–2(d)). Incubation with 0.6, 3, and 6 mg/mL led to cell shrinkage with significant nuclear condensation, fragmentation, and formation of apoptotic-like bodies (arrows). Figure 2(e) shows quantification of apoptotic nuclei in adherent cells. Treatment with cilastatin significantly ameliorates VAN-induced nuclear apoptosis.

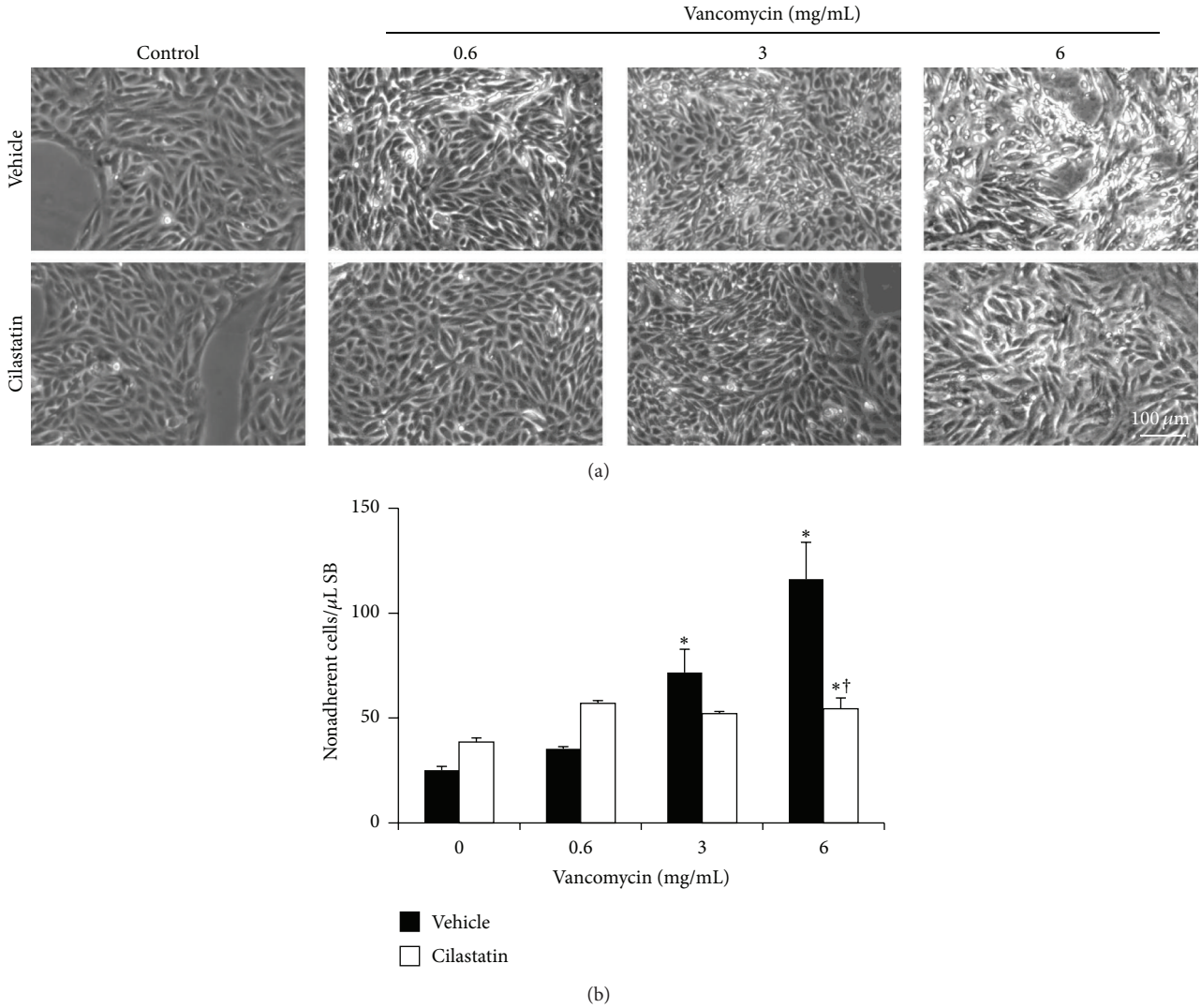


Figure 1: Effects of cilastatin on vancomycin-treated renal proximal tubular epithelial cells (RPTECs) morphology. RPTECs were cultured in the presence of vancomycin (0.6, 3, and 6 mg/mL) and vancomycin plus cilastatin (200 μg/mL) for 24 hours. (a) Phase-contrast photomicrographs are shown (representative example of at least three independent experiments; original magnification 40x). (b) Effect of cilastatin on vancomycin-induced detachment of RPTECs, measured by flow cytometry and determined by counting the number of cells in an equal volume of buffer. Data are represented as the mean ± SEM of at least three separate experiments. $^{*}p \leq 0.05$ versus control and control plus cilastatin, $^{\dagger}p \leq 0.0001$ versus the same data without cilastatin.

After 24 hours of exposure to VAN 0.6, 3, and 6 mg/mL, apoptosis of RPTECs measured as nucleosomal DNA fragmentation and migration from nuclei to cytosol was quantified and compared with apoptosis under the same conditions but in the presence of cilastatin (Figure 2(f)). RPTECs exposed to 3 and 6 mg/mL VAN present an increase in nucleosomes recovered from cytosol. Cilastatin significantly prevented these changes in nucleosomal enrichment.

To evaluate the effect of cilastatin on VAN-induced necrosis, release of LDH from RPTECs to the culture medium was measured after treatment with VAN 3 and 6 mg/mL in combination or not with cilastatin at different time periods. After 24 hours no changes were found in LDH values at any concentration of VAN, and slight changes were found after 48 h only with VAN 6 mg/mL (≤5% of maximal release of LDH). Interestingly, coincubation with cilastatin did not modify this small increase in necrotic cell death. Thus, reduction of VAN-induced cell death with cilastatin seems to be specific for apoptosis (Figure 2(g)).

3.3. Cilastatin Protects against VAN-Induced Early and Late Apoptosis. To evaluate the effect of cilastatin on VAN-induced early and late apoptosis, RPTECs stained with annexin V and PI were analyzed after treatment with VAN (0.6, 3, and 6 mg/mL) with or without cilastatin (200 μg/mL) for 24 h.

The amount of early-apoptotic cells was expressed as the percentage of PI-negative/annexin V-positive cells

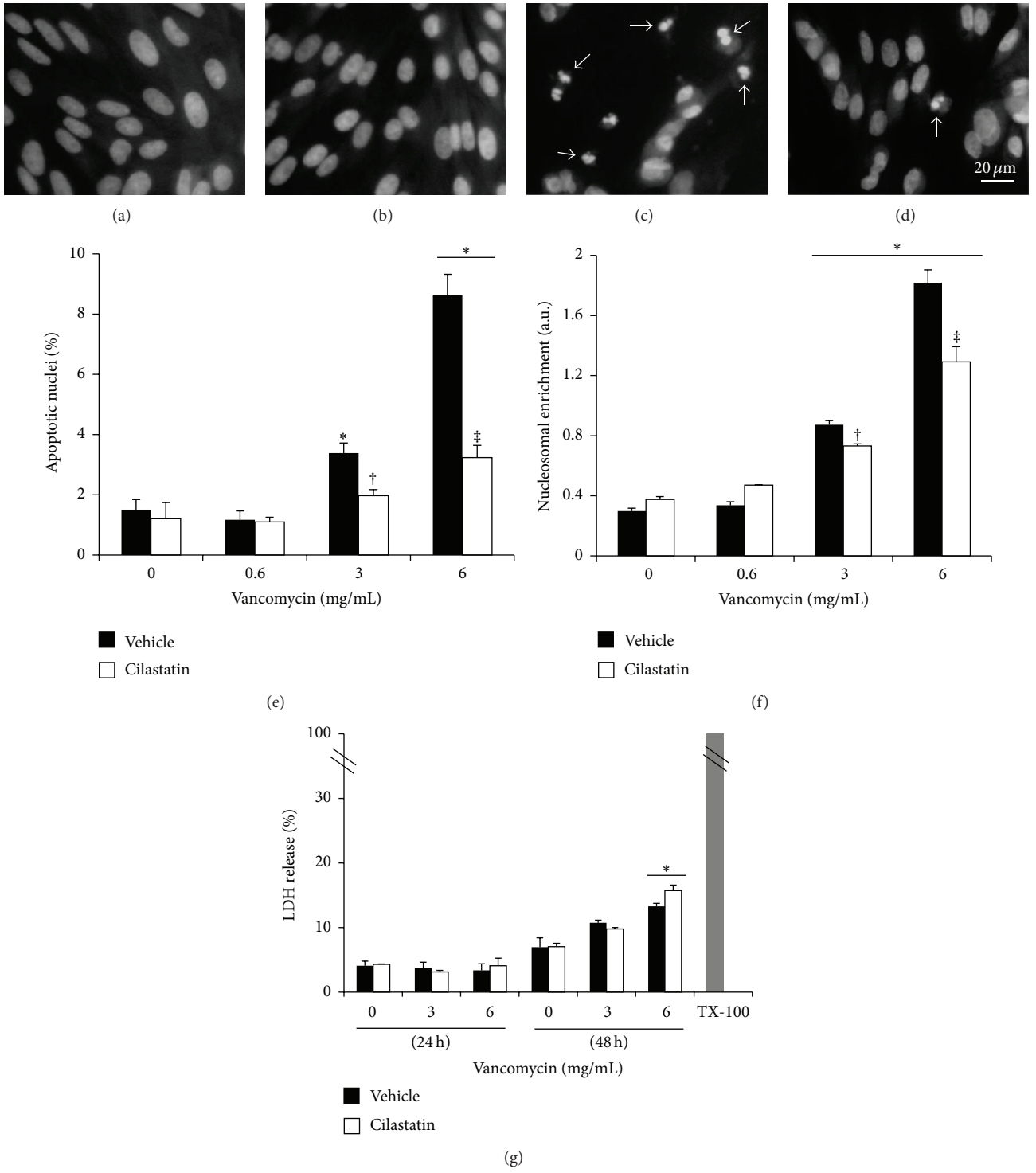


Figure 2: Cilastatin protects against vancomycin-induced apoptosis but not necrosis. Proximal tubular epithelial cells (RPTECs) were cultured in the presence of vancomycin (0.6 and/or 3 and 6 mg/mL) and vancomycin plus cilastatin (200 μg/mL) for 24 and/or 48 hours. (a–d) Nuclear staining with DAPI. Adherent RPTECs were stained with DAPI to study if apoptotic-like nuclear morphology was present. Arrows point to fragmented apoptotic nuclei. (e) Quantitative approach to the images presented in (a–d). (f) Oligonucleosomes at 24 hours were quantified in the cell soluble fraction and detected with an ELISA kit. (g) Effect of cilastatin in vancomycin-induced release of LDH. Data are presented as % of total release of LDH obtained by Triton X 100 (TX-100) cell treatment. Data are represented as the mean ± SEM of at least three separate experiments. $^{*}p < 0.007$ versus control and control plus cilastatin, $^{\dagger}p \leq 0.05$ versus the same data without cilastatin, $^{\ddagger}p < 0.0001$ versus the same data without cilastatin.

(Figure 3(a), lower right quadrant of each plot), and the amount of late-apoptotic cells was expressed as the percentage of PI-positive/annexin V-positive cells (Figure 3(a), upper right quadrant of each plot). VAN (3 and 6 mg/mL) caused an increase in the percentage of both early-apoptotic and late-apoptotic cells (Figures 3(b) and 3(c)). Cilastatin significantly reduced this increase in both early and late-apoptotic cells (Figures 3(b) and 3(c)).

3.4. Cilastatin Downgrades VAN-Induced Mitochondrial Damage. We quantified the functional impact of treatment with VAN on cell survival by measuring the percentage of adherent cells still able to reduce MTT to formazan after exposure to increasing doses of VAN. Coincubation with cilastatin increases cell survival in every condition analyzed. Differences that were statistically significant were only found for incubations with cilastatin in VAN 3 and 6 mg/mL for 24 h (Figure 4(a)).

Moreover, the effect of VAN on mitochondria was observed very early after addition of VAN to cell culture plates. In Figure 4(b), an inverted IX-80 microscope was fitted to obtain absorbance readings at specific wavelengths on single (or small groups of) cells in culture.

A quick and deep depression in MTT reduction activity was observed in RPTECs exposed to VAN 6 mg/mL compared with controls (Figure 4(b)). Coincubation with cilastatin partially recovers this effect. Differences are observed even during the first 5 minutes after addition of VAN.

3.5. Cilastatin Improves Long-Term Recovery and Cell Viability in RPTECs after Exposure to VAN. To know the long-term viability of surviving RPTECs after 24 hours of exposure to VAN, we tested the ability of those cells to proliferate into new cell colonies. CFUs were quantified as specified in Section 2. The CFU count decreased after 24 hours of treatment with VAN, and this decrease was clearly dose-dependent (Figure 5(a)). When VAN was exposed in the presence of cilastatin, the number of CFUs was significantly higher after 7 days of recovery for every VAN concentration studied. The intracellular dye was extracted, and absorbance was quantified at 595 nm (Figure 5(b)).

3.6. Cilastatin Reduces Intracellular Accumulation of VAN. In many cases, nephrotoxicity is in part dependent on the intracellular concentration of drug. As cilastatin is a ligand of the brush border membrane, we investigated whether it could affect VAN uptake by RPTECs. To test this hypothesis, we measured intracellular VAN content by TDX analysis, as described in Section 2. Figure 6 shows that cellular VAN content increased progressively in a dose-dependent manner when RPTECs were incubated for 24 hours in the presence of different concentrations of drug. Coincubation with cilastatin significantly reduced accumulation of VAN into the cells for every concentration studied (Figure 6). These results confirm that incubation with cilastatin in primary cultures of RPTECs decreases cellular accumulation of VAN. This effect may be involved in the observed reduction of VAN impact on RPTECs damage death and survival.

3.7. Cilastatin Has No Effect on the Antimicrobial Action of VAN. The MICs and MBC values of VAN obtained for each isolate in the absence or with the addition of cilastatin were either the same or varied within $\pm 1 \log 2$ dilution (Table 1), thus implying that cilastatin does not inhibit the activity of VAN against any of the isolates tested.

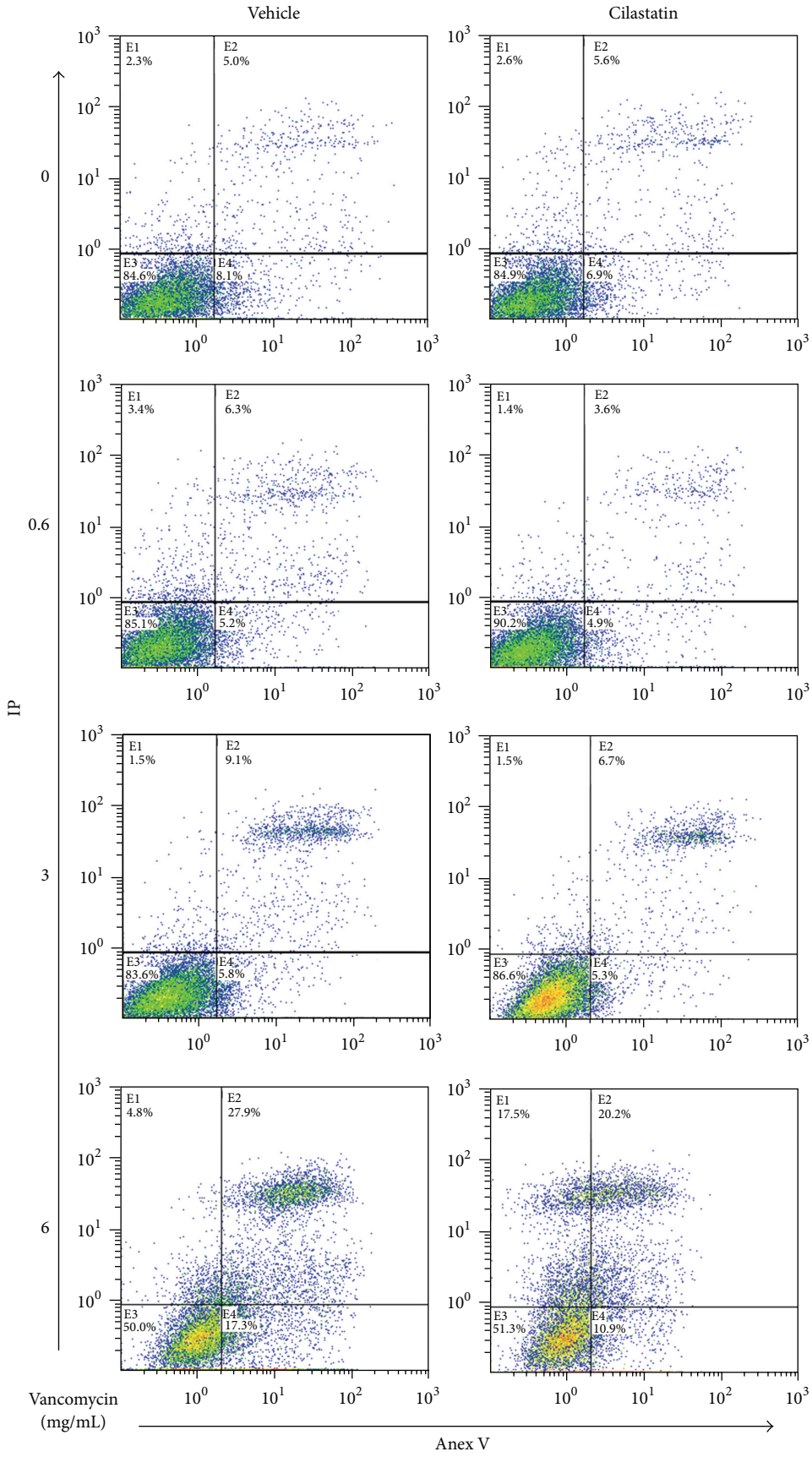
4. Discussion

CRBSI are a common complication of coronary and intensive care units. When CRBSI is caused by MRSA, then polypeptide antibiotics are the only alternative to methicillin. VAN is one of the most commonly used antibiotics in the clinical management of MRSA-induced CRBSI. Clinical and preclinical studies have shown that nephrotoxicity is the main side effect of VAN and that this in turn induces AKI, thus limiting dose and duration of administration [2, 31]. Renal impairment can also influence the prognosis of patients with cardiovascular disease, thus increasing cardiovascular risk. In fact, renal dysfunction is a major risk factor for the development of nonrenal complications and a marker of lesions elsewhere in the vascular tree [32, 33]. It is associated with increased morbidity and mortality, prolonged hospital stays, and higher healthcare costs [2, 34]. Therefore, prevention of renal dysfunction and preservation of the proximal tubule are key components in strategies aimed at preventing renal damage and potential cardiovascular complications.

Several studies have shown RPTECs to be a key target of VAN-induced toxicity [8, 11, 25, 35]. Although the pathogenesis of VAN-induced nephrotoxicity is not fully understood, several mechanisms are known to cause and amplify renal damage [1, 2, 8, 11].

In proximal tubule cell cultures, VAN concentrations similar to the observed plasma levels with therapeutic doses have shown that VAN induced apoptosis but not necrosis cell death [7, 36]. Consistent with these results, we found that direct observation of VAN-treated primary cell cultures revealed characteristic apoptotic morphological changes in a dose-dependent way. Necrosis was only observed after 48 hours of treatment, never higher than a 5%. RPTECs treated with VAN presented early and severely diminished capacity to reduce MTT to formazan, directly related to mitochondrial damage. Several authors consider alteration of mitochondrial function in RPTECs to be a major factor in VAN-induced nephrotoxicity [35, 37], leading to DNA degradation and cell death, as recently demonstrated elsewhere [7].

Previous studies have shown that VAN-induced nephrotoxicity may be alleviated *in vivo* by cilastatin (imipenem/cilastatin) simultaneous treatment. Toyoguchi et al. [25] showed that cilastatin may reduce VAN-induced nephrotoxicity in rabbits by decreasing serum BUN and creatinine levels. Nakamura et al. [26, 38] presented similar results in rats. Both authors conclude that the protection observed after treatment with cilastatin is associated with reduced accumulation of VAN in renal tubules [25, 26, 38]. In fact, accumulation of VAN in renal cells has been proposed as a major cause of toxicity [2, 35, 37]. Our results are consistent with these findings as we recorded significant reductions in the accumulation of VAN in the presence of cilastatin,



(a)

Figure 3: Continued.

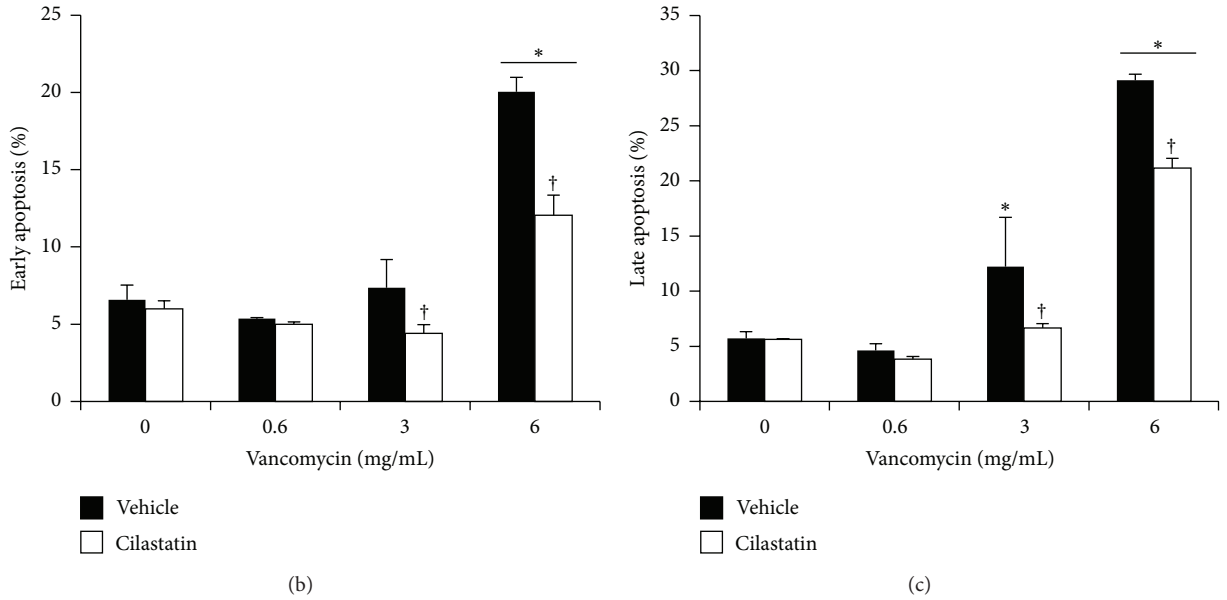


Figure 3: Effect of cilastatin on vancomycin-induced early and late apoptosis. Vancomycin-induced early and late-apoptotic cell death in proximal tubular epithelial cells and the effect of cilastatin were determined by flow cytometry with annexin V/propidium iodide assay after 24 hours of treatments. (a) Representative scatter plots of propidium iodide (y axis) versus annexin V (x axis). The lower right quadrants represent the early-apoptotic cells (annexin V-positive/propidium iodide-negative) and the upper right quadrants represent the late-apoptotic cells (annexin V-positive/propidium iodide-positive). (b) Quantification of early-apoptotic cells in all conditions (lower right quadrants). (c) Quantification of late-apoptotic cells in all conditions (upper right quadrants). Results are expressed as % of total cells quantified. Data are represented as the mean ± SEM of at least three separate experiments. $^{*}p < 0.05$ versus control and control plus cilastatin, $^{\dagger}p < 0.05$ versus the same data without cilastatin. IP, propidium iodide. Anex V, annexin V.

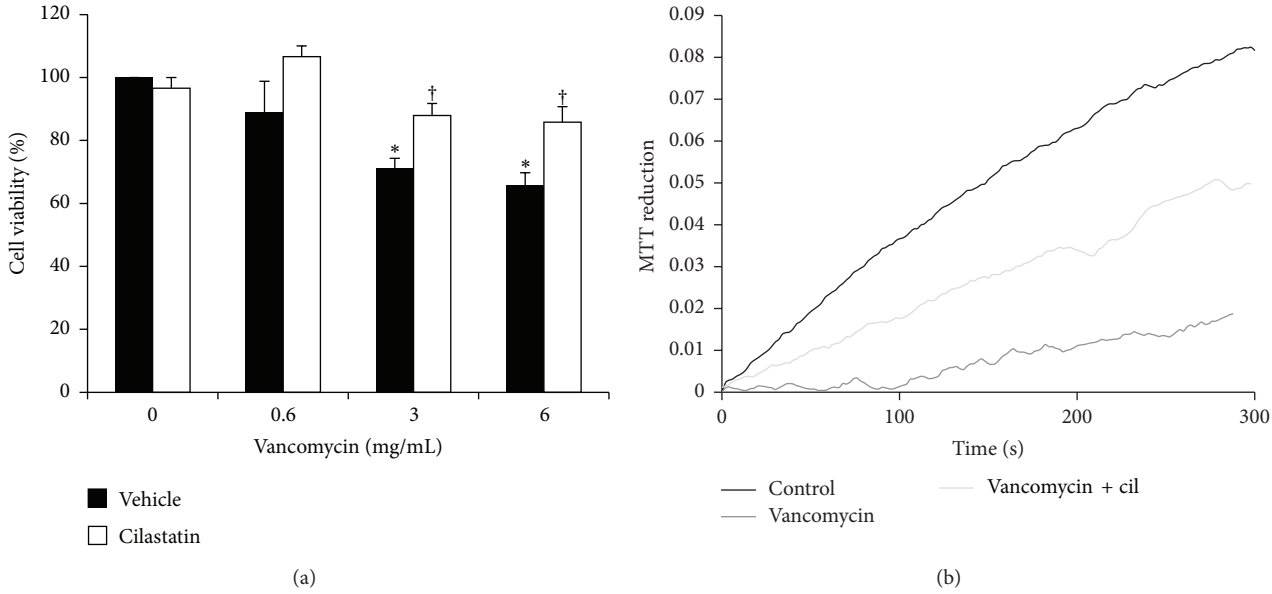


Figure 4: Effect of cilastatin on vancomycin-induced mitochondrial damage. Renal proximal tubular epithelial cells (RPTECs) were exposed to vancomycin and vancomycin plus cilastatin (200 μg/mL) for 24 hours. (a) Cell viability was determined by the ability to reduce MTT. Results are expressed as the percentage of the value obtained relative to control (without vancomycin and cilastatin) of at least three separate experiments. (b) Changes in the mitochondrial oxidative capacity of RPTECs were assessed by MTT reduction at 570 nm. The graph shows formation of formazan as detected in isolated cells in real time with no treatment (control) and vancomycin 6 mg/mL with or without 200 μg/mL cilastatin, after the incubation times in seconds given on the x-axis. $^{*}p < 0.05$ versus control and control plus cilastatin, $^{\dagger}p < 0.05$ versus the same data without cilastatin.

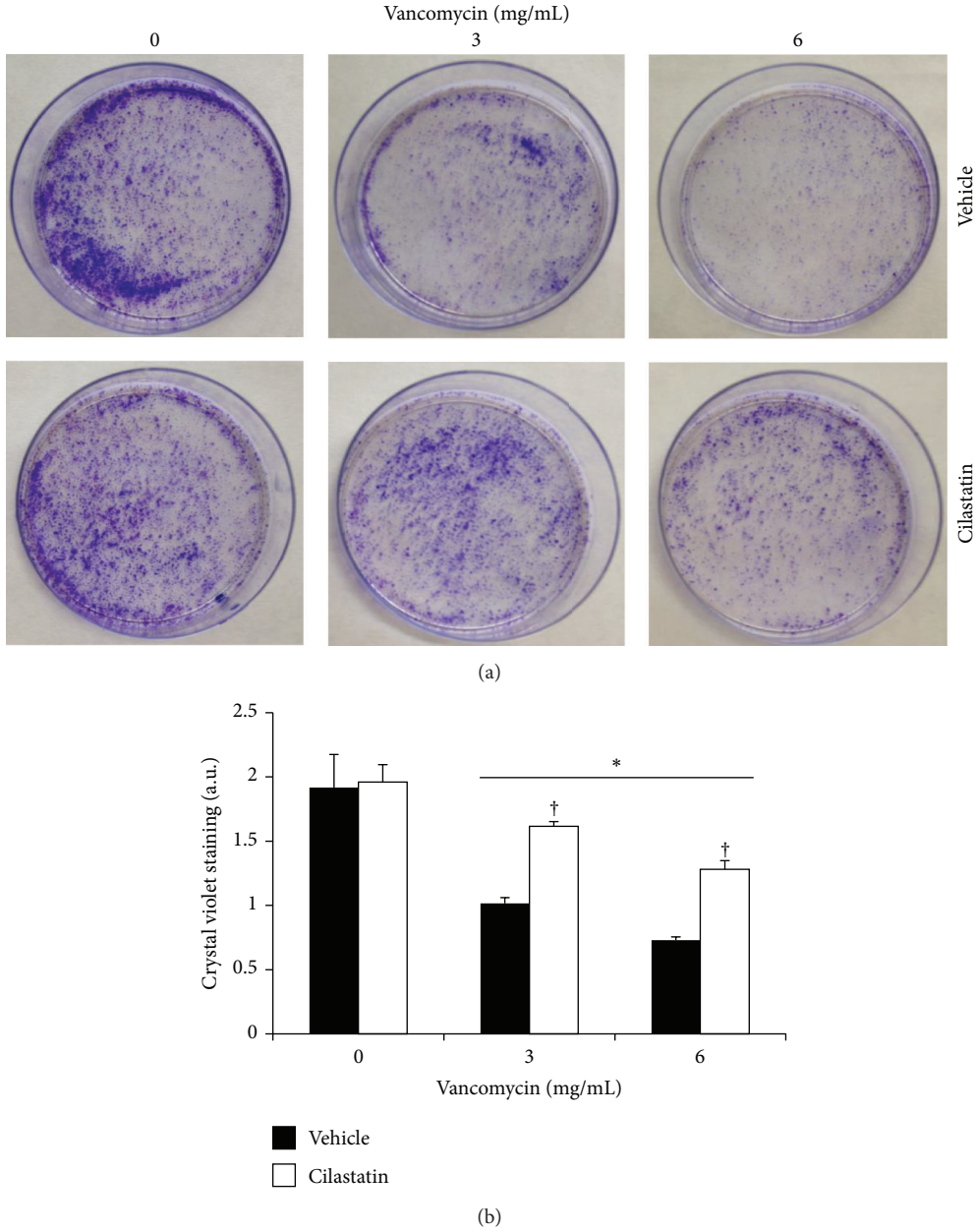


Figure 5: Cilastatin preserves long-term recovery of vancomycin-treated proximal tubular epithelial cells (RPTECs). (a) RPTECs were incubated with vancomycin 3 and 6 mg/mL in the presence or absence of 200 μg/mL cilastatin for 24 hours. The number of colony-forming units was determined by staining with crystal violet after 7 days. (b) Quantification of crystal violet staining. Data are expressed as mean ± SEM of three separate experiments. $^{*}p < 0.05$ versus control and control plus cilastatin, $^{\dagger}p < 0.05$ versus the same data without cilastatin.

although, to our knowledge, ours is the first study to demonstrate that cilastatin is able to reduce apoptosis and mitochondrial injury in RPTECs.

Some authors suggested that the mechanism behind VAN-induced renal damage was similar to that of gentamicin [10, 26, 39], which is induced by accumulation of the drug from the brush border membrane to the renal proximal tubules [40, 41]. Gentamicin is transported inside the cell by endocytosis involving megalin, a brush border lipid raft ligand [41]. VAN and gentamicin colocalize in endosomes in the renal proximal tubular cells [42] and activate cathepsins triggering apoptosis [41]. If gentamicin accumulation is reduced by inhibition of its transport mechanisms, nephrotoxicity is alleviated [41].

We have published that binding of cilastatin to lipid raft bound DHP-I inhibits any vesicle based transport or signalization requiring internalization of the brush border lipid raft in proximal tubules [19–21]. In fact, cilastatin seems to be able to reduce luminal entry of drugs across the membranes (e.g., CsA, tacrolimus, and cisplatin) even if they are not substrates for DHP-I activity [18, 19]. Although the exact mechanism of VAN accumulation in proximal cells has not been elucidated

Table 1: *In vitro* activity of vancomycin alone and with cilastatin against clinical isolates of *Staphylococcus aureus* and *Enterococcus* spp.

	Strain 1		Strain 2		Strain 3		Strain 4	
Staphylococcus aureus	Vehicle	Cil	Vehicle	Cil	Vehicle	Cil	Vehicle	Cil
MIC	0.5	1	0.5	1	1	0.5	0.5	0.5
MBC	16	32	2	2	1	0.5	0.5	0.5
	Strain 5		Strain 6		Strain 7		Strain 8	
Enterococcus spp.	Vehicle	Cil	Vehicle	Cil	Vehicle	Cil	Vehicle	Cil
MIC	0.5	0.5	0.25	0.5	1	2	0.5	0.5
MBC	>16	>16	>4	>8	>16	>32	>8	>8

Table shows the effect of cilastatin (200 μg/mL) against inhibitory and bactericidal activity of vancomycin (0–64 μg/mL) in clinical bacteria isolated. *Staphylococcus aureus*: strains numbers 1 and 4, methicillin-resistant; strain numbers 2 and 3, methicillin-susceptible. *Enterococcus* spp.: strains numbers 5, 7, and 8, *E. faecalis*; strain number 6, *E. faecium*.
MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; vehicle, cation-adjusted Mueller-Hinton broth; cil, cilastatin.

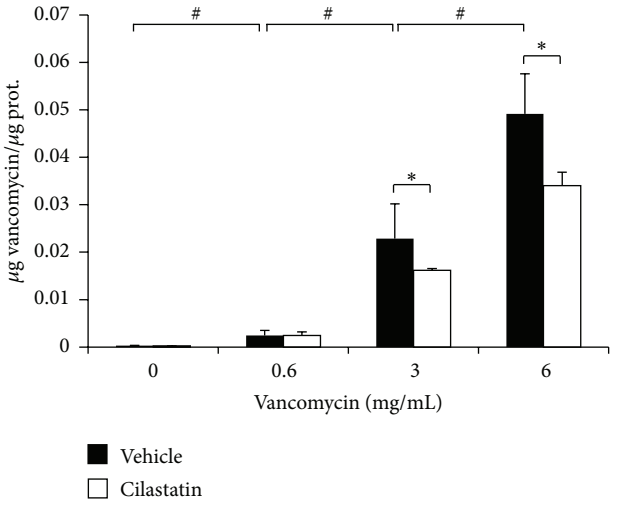


Figure 6: Effects of cilastatin on vancomycin accumulation in proximal tubular epithelial cells (RPTECs). Intracellular accumulation was measured in lysates of PTECs treated with vancomycin 0.6, 3, and 6 mg/mL for 24 hours, in the presence or absence of cilastatin (200 μg/mL), using fluorescence polarization immunoassay (TDX) specific assays. Cilastatin was shown to prevent entry of vancomycin into RPTECs. Values were expressed as means ± SEM of vancomycin concentration ($n = 4$ different experiments). ANOVA model $p < 0.0001$. Factors: cilastatin effect $^{*}p < 0.05$; dose effect $^{\#}p < 0.05$.

yet [36] and remains open to debate [26, 43, 44], Fujiwara et al. [42] recently revealed that significant amounts of VAN were present in the apical pole, specifically in the S1 and S2 segments of the proximal tubules. The presence of VAN near the brush border could also suggest the presence of an unknown transporter(s) in this area [42], a hypothesis that was also reported by Nakamura et al. [36]. Cilastatin seems to be able to interfere with VAN transport, as previously described for other toxins [18, 19]. Thus, interference by cilastatin with VAN uptake and accumulation on RPTECs could also explain the fast protection observed in real-time experiments performed to analyze mitochondrial oxidative capacity and integrity. VAN immediately inhibits reduction of MTT to formazan, although coincubation with cilastatin partially restores this process. The very short time course of the cilastatin blocking effect strongly suggests that cilastatin inhibits uptake of VAN by RPTECs, a process that was already described by Toyoguchi et al. [25] and Nakamura et al. [26, 38]. Interference with entry of VAN could also explain the renal protection associated with a decrease in cell death by apoptosis.

Other mechanisms could also be involved in the ability of cilastatin to protect against VAN-induced nephrotoxicity. Previous results obtained by our group showed the ability of cilastatin to inhibit apoptosis induced by other nephrotoxic agents, such as CsA, tacrolimus [19], and cisplatin *in vitro* and *in vivo* [16–18] without interfering with their effectiveness on their respective target cells. Cilastatin was able to inhibit cellular and nuclear morphological changes, mitochondrial depolarization and release of cytochrome c, caspase activation, DNA fragmentation, and cell death caused by apoptosis but not necrosis in RPTECs [18].

In our model of cisplatin-induced nephrotoxicity, cilastatin inhibits internalization of the Fas-Fas ligand system bound to cell membrane lipid rafts blocking apoptosis amplification and protecting the cells [15, 18]. We do not know if the same mechanism applies in the VAN-induced renal apoptosis, but it is clear that DHP-I binding to brush border lipid rafts on RPTECs gives cilastatin the chance to interfere with the process of apoptosis.

Interestingly, our analysis of the effect of cilastatin on VAN-sensitive bacteria showed that cilastatin did not modify the MIC or MBC of VAN against any of the isolates tested. These results were expected owing to the absence of brush border and DHP-I in bacteria, thus demonstrating a specific effect on RPTECs. We show that cilastatin has a promising therapeutic role in humans. Moreover, some authors have previously reported that treatment with imipenem/cilastatin has nephroprotective effects on CsA-induced AKI in kidney recipients [21], bone marrow recipients [22], and heart recipients [23]. Therefore, protection against kidney damage caused by VAN used to treat MRSA-induced CRBSI is possible, specifically in patients with AKI.

In conclusion, our results show that cilastatin attenuates VAN-induced acute renal failure *in vitro* by decreasing apoptosis without affecting antibacterial activity. This effect could be related, at least in part, to the reduction in accumulation of the drug in cells. Therefore, cilastatin could represent a

novel therapeutic approach in reducing VAN-induced renal damage without compromising bactericidal efficacy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Blanca Humanes, Juan Carlos Jado, and Sonia Camaño contributed equally to this work.

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Research Article

Characteristics and Outcomes of Patients with Acute Myocardial Infarction at Non-PCI Capable Hospitals in 2007 and in 2014

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Background. There is little known about whether characteristics and outcomes of patients with acute myocardial infarction (AMI) have changed over the years in non-PCI capable hospitals in real-life. Our aim was to assess them between 2007 and 2014. *Methods*. It was a retrospective cohort study. Characteristics and in-hospital mortality (standardized in cases of different characteristics between the groups by original simple method) were assessed for all patients with non-ST elevation myocardial infarction (NSTEMI) and ST elevation myocardial infarction (STEMI) at two non-PCI capable hospitals: one in 2007 ($n = 104$) and another in 2014 ($n = 58$). *Results*. In 2014, females were older than in 2007 (80.18 ± 7.54 versus 76.15 ± 8.77, $p = 0.011$), males were younger (71.61 ± 11.22 versus 79.20 ± 7.63, $p = 0.019$), less had renal failure (RF) (19% versus 34.6%, $p < 0.0001$) and reinfarction (13.8% versus 35.6%, $p < 0.0001$), and the proportion of males (31% versus 43.3%, $p = 0.001$) and the proportion of NSTEMI (60.3 versus 69.2, $p < 0.0001$) decreased. In cases of STEMI there were no differences in patient characteristics. STEMI (18.8% versus 21.7%) and standardized mortalities by gender, RF, and reinfarction NSTEMI (19.47%, 15.34%, and 17.5%, resp., versus 17.1%) showed no differences between 2007 and 2014. *Conclusions*. There were some differences in patient characteristics but not in mortality for AMI at non-PCI capable hospitals between 2007 and 2014.

1. Introduction

There is little known about whether the clinical and demographical characteristics and in-hospital mortality of patients with acute myocardial infarction (AMI) have changed over the years in non-PCI capable hospitals (no team of interventional cardiologists) in real-life.

It was shown that life expectancy continues to increase [1, 2]. Consequently, the prevalence of age-related conditions, such as cardiovascular disease, is continuously increasing, and probably the age of patients with cardiovascular diseases is increasing, especially at non-PCI capable hospitals. Our previous study showed that patients treated conservatively in a non-PCI capable hospital and patients treated interventionally in a PCI capable hospital were significantly different: patients treated conservatively were much older, there were more women than men, and more often it was a non-ST elevation myocardial infarction (NSTEMI) than a ST elevation myocardial infarction (STEMI) [3]. Over the past several decades, the mortality rate for AMI has been decreasing with the development of reperfusion therapy and adjunctive pharmacological therapies [4]. However, most studies are done in the PCI capable hospitals and usually they are randomized clinical trials with their narrow inclusion criteria and wide exclusion criteria. The recent study showed discrepancies between trials and real-life: despite all current efforts, in-hospital mortality of patients with AMI was stagnating on a high level compared with data of randomized clinical trials [5].

Our aim was to assess the characteristics and outcomes of patients with acute myocardial infarction at non-PCI capable hospitals at two different periods: in 2007 and in 2014.

2. Material and Methods

It was a retrospective cohort study. Data of all patients hospitalized at the Kaunas Clinical Hospital (KCH) in 2007 and at the Republican Hospital of Kaunas (RHK) in 2014 with the diagnosis of an acute myocardial infarction (confirmed by

the troponin test) were analysed. Both of these hospitals are non-PCI capable hospitals. The KCH was the main non-PCI capable hospital for conservative treatment of AMI in Kaunas city in 2007, and the RHK was the main non-PCI capable hospital for conservative treatment of AMI in Kaunas city in 2014. In both years patients received the same medications: heparin subcutaneously, dual antiplatelets therapy (aspirin and clopidogrel), B blockers, ACE inhibitors, and statins. No fibrinolytic therapy was administered, because in the same city there is a PCI capable hospital which has a skilled PCI laboratory with experienced interventional cardiologists on duty 24 h a day. A transfer for primary angioplasty or conservative treatment was chosen by the judgment of the attending cardiologist based on presenting characteristics and duration of symptoms. The enrolment of patients was consecutive and continued throughout the year. The study was approved by the Ethics Committee of the Lithuanian University of Health Sciences.

Patients of each hospital were divided into two groups according to electrocardiographic (ECG) changes on arrival to hospital: NSTEMI and STEMI [1]. Patients with conduction defects or electronic ventricular pacing were excluded. Patients were assessed by age, gender, comorbidities (diabetes mellitus (DM), renal failure (RF), or both of them (DM + RF)), reinfarction, echocardiographic left ventricular ejection fraction (LVEF), and the type of AMI (NSTEMI and STEMI). Their characteristics and in-hospital mortality were compared between the two hospitals, for example, between the two different periods of treatment at a non-PCI capable hospital in Kaunas city. Patients who arrived to RHK in 2014 also were divided into more detailed four subgroups according to ECG changes on arrival to the hospital: STEMI with positive T wave, STEMI with negative T wave, NSTEMI with positive T wave, and NSTEMI with negative T wave. Their age, gender, comorbidities, reinfarction, and LVEF also were compared and additional data as duration of chest pain before arriving to the hospital (<12 hours, 12–24 hours, and >24 hours) and high-sensitive troponin T level were compared between these subgroups.

In-hospital mortalities standardized by the frequency of some factors (which showed significant differences between the hospitals) were calculated by this original mathematical formula:

x = mortality of specific group, for example, RF in cases of NSTEMI in KCH $*$ frequency of RF in NSTEMI in RHK + $y *$ frequency of patients without RF with NSTEMI in RHK. x, in this example, the mortality of patients with NSTEMI in KCH, is standardized by RF (such mortality would be if the frequency of RF in KCH would be the same as in RHK in cases of NSTEMI); y, in this example, the mortality of patients without RF in cases of NSTEMI in KCH, is as follows:

$$y = \frac{\text{NSTEMI mortality in KCH} - \left(\text{NSTEMI mortality in cases of RF in KCH} * \text{frequency of RF in NSTEMI in KCH}\right)}{\text{frequency of patients without RF with NSTEMI in KCH}}. \quad (1)$$

2.1. Statistical Analysis. Values were expressed as the mean ± standard deviation and as a percentage. Statistical significance was accepted when the probability value was $p < 0.05$. Differences in continuous variables between the two groups were assessed using unpaired Student's t-test and Mann-Whitney U test. Differences in continuous variables between more groups were assessed using One-Way ANOVA; comparisons of discrete variables were performed using Pearson's Chi-square test. Statistical analysis was performed using statistical package SPSS 21.0 and MS Excel.

3. Results

Patients hospitalized at the RHK in 2014 with the diagnosis of an AMI in comparison with the patients hospitalized at the KCH in 2007 were different in some characteristics: females were older, but males were younger, and less of the patients had RF and reinfarction (Table 1). And the proportion of males and the proportion of patients with NSTEMI decreased between them in comparison with the KCH in 2007. In 2007 in KCH NSTEMI was more frequent than STEMI, 69.2% versus 30.8%, $p < 0.0001$, while in 2014 in RHK the prevalence of NSTEMI was insignificant, 60.3% versus 39.7%, $p > 0.05$.

Progressive heart failure was the main cause of death in both hospitals: 12 cases (66.7%) at KCH in 2007 and 7 cases (63.6%) at RHK in 2014. Other causes were cardiogenic shock (4 cases) and arrhythmia (2 cases) at KCH in 2007, and cardiogenic shock (1 case), arrhythmia (1 case), cerebral stroke (1 case), pulmonic embolism (1 case) at RHK in 2014.

There were no significant differences in patient age, comorbidities, LVEF, and in-hospital mortality between STEMI and NSTEMI in each hospital, except that more males had NSTEMI than STEMI at KCH in 2007 (Table 2). However, there were no significant differences in male and female mortalities between STEMI and NSTEMI at each hospital.

In STEMI group there were no significant differences in patient characteristics and in-hospital mortalities at non-PCI capable hospitals between 2007 and 2014 (Table 3).

In NSTEMI group, significant differences were found between hospitals (years) in gender and frequency of patients with RF and reinfarction (Table 3). Therefore, sex-standardized in-hospital mortality and in-hospital mortalities standardized by the frequency of RF and reinfarction were calculated by our original mathematical formula.

Mortality of patients with NSTEMI in KCH standardized by RF (such mortality would be if the frequency of RF in KCH would be the same as in RHK in cases of NSTEMI) is as follows:

x = mortality of patients with RF in cases of NSTEMI in KCH $*$ frequency of RF in NSTEMI in RHK + $y *$ frequency of

Table 1: Characteristics and in-hospital mortality of patients with acute myocardial infarction at non-PCI capable hospitals in 2007 and in 2014.

Variable	KCH 2007 y. ($n = 104$)	RHK 2014 y. ($n = 58$)	p
Age (year)	77.47 ± 8.39	77.52 ± 9.6	0.564
Female age (year)	76.15 ± 8.77	80.18 ± 7.54	0.011
Male age (year)	79.20 ± 7.63	71.61 ± 11.22	0.019
Male *n* (%)	45 (43.3)	18 (31)	0.001
STEMI (%)	32 (30.8)	23 (39.7)	0.281
NSTEMI (%)	72 (69.2)	35 (60.3)	<0.0001
DM *n* (%)	7 (6.7)	5 (8.6)	0.774
RF *n* (%)	36 (34.6)	11 (19)	<0.0001
DM + RF *n* (%)	10 (9.6)	1 (1.7)	0.012
Reinfarction *n* (%)	37 (35.6)	8 (13.8)	<0.0001
LVEF (%)	37.91 ± 12.95	39.18 ± 11.68	0.598
In-hospital mortality *n* (%)	18 (17.3)	11 (19)	0.792
Mortality female *n* (%)	13 (22)	8 (20)	0.808
Mortality male *n* (%)	5 (11.1)	3 (16.7)	0.55

Data presented are mean value ± SD or number (percentage) of patients. Age and LVEF were compared using Mann-Whitney *U* test; other data were compared using Pearson's Chi-square test. KCH: Kaunas Clinical Hospital, RHK: Republican Hospital of Kaunas, STEMI: ST elevation myocardial infarction, NSTEMI: non-ST elevation myocardial infarction, DM: diabetes mellitus, RF: renal failure, DM + RM: diabetes and renal failure, and LVEF: left ventricular ejection fraction.

Table 2: Comparison of characteristics and in-hospital mortality between patients with STEMI and NSTEMI at non-PCI capable hospitals in 2007 and in 2014.

Variable	KCH 2007 y. ($n = 104$)			RHK 2014 y. ($n = 58$)		
	STEMI ($n = 32$)	NSTEMI ($n = 72$)	p	STEMI ($n = 23$)	NSTEMI ($n = 35$)	p
Age (years)	76.7 ± 8.85	77.8 ± 8.2	0.56	75.8 ± 8.86	78.6 ± 10.15	0.152
Female age (year)	77.4 ± 6.69	79.1 ± 7.16	0.371	78 ± 6.27	81.5 ± 8.04	0.059
Male age (year)	74.6 ± 13.9	76.59 ± 9.05	0.616	71.75 ± 11.32	71.5 ± 11.74	0.824
Female *n* (%)	24 (75)	35 (48.6)	0.193	15 (65.2)	25 (71.4)	0.154
Male *n* (%)	8 (25)	37 (51.4)	<0.0001	8 (34.8)	10 (28.6)	0.815
DM *n* (%)	2 (6.2)	5 (6.9)	0.896	2 (8.7)	3 (8.6)	0.987
RF *n* (%)	7 (21.9)	29 (40.3)	0.069	4 (17.4)	7 (20)	0.804
DM + RF *n* (%)	4 (12.5)	6 (8.3)	0.506	0	1 (2.9)	0.414
Reinfarction *n* (%)	10 (31.2)	27 (37.5)	0.539	4 (17.4)	4 (11.4)	0.519
LVEF (%)	34.1 ± 11.86	39.5 ± 13.14	0.054	36.6 ± 12.06	40.5 ± 11.81	0.243
In-hospital mortality *n* (%)	6 (18.8)	12 (16.7)	0.795	5 (21.7)	6 (17.1)	0.662
Female mortality *n* (%)	5 (20.8)	8 (22.9)	0.854	3 (20)	5 (20)	1.000
Male mortality *n* (%)	1 (12.5)	4 (10.8)	0.89	2 (25)	1 (10)	0.396

Data presented are mean value ± SD or number (percentage) of patients. Age was compared using Student's *t*-test in the Republican Hospital of Kaunas (RHK) and using Mann-Whitney *U* test in the Kaunas Clinical Hospital (KCH), also LVEF was compared using Mann-Whitney *U* test, other data were compared using Pearson's Chi-square test. STEMI: ST elevation myocardial infarction, NSTEMI: non-ST elevation myocardial infarction, DM: diabetes mellitus, RF: renal failure, DM + RM: diabetes and renal failure, and LVEF: left ventricular ejection fraction.

patients without RF with NSTEMI in RHK. y is the mortality of patients without RF in cases of NSTEMI in KCH. So, $x = 20.7 * 0.2 + y * 0.8$,

$$y = \frac{16.7 - (20.7 * 0.403)}{0.597} = 14.0. \quad (2)$$

So, mortality of 20% of patients is 20.7% and mortality of 80% of patients is 14.0%. Total mortality is $x = 20.7 * 0.2 + 14.0 * 0.8 = 15.34$. So, mortality of patients with NSTEMI in KCH in 2007 standardized by RF is 15.34%.

Mortality of patients with NSTEMI in KCH standardized by reinfarction is as follows:

$$x = 14.8 * 0.114 + y * 0.886,$$
$$y = \frac{16.7 - (14.8 * 0.375)}{0.625} = 17.84, \quad (3)$$

so $x = 17.5$. So, mortality of patients with NSTEMI in KCH in 2007 standardized by reinfarction is 17.5%.

Table 3: Comparison of characteristics and in-hospital mortality of patients with STEMI and NSTEMI at non-PCI capable hospitals between 2007 and 2014.

Variable	STEMI			NSTEMI		
	KCH 2007 y. ($n = 32$)	RHK 2014 y. ($n = 23$)	p	KCH 2007 y. ($n = 72$)	RHK 2014 y. ($n = 35$)	p
Age (year)	76.7 ± 8.85	75.8 ± 8.86	0.71	77.8 ± 8.2	78.6 ± 10.15	0.678
Female age (year)	77.4 ± 6.69	78 ± 6.27	0.788	79.1 ± 7.16	81.5 ± 8.04	0.23
Male age (year)	74.6 ± 13.9	71.75 ± 11.32	0.657	76.59 ± 9.05	71.5 ± 11.74	0.146
Male *n* (%)	8 (25)	8 (34.8)	1.000	37 (51.4)	10 (28.6)	<0.0001
DM *n* (%)	2 (6.2)	2 (8.7)	0.73	5 (6.9)	3 (8.6)	0.746
RF *n* (%)	7 (21.9)	4 (17.4)	0.682	29 (40.3)	7 (20)	0.037
DM + RF *n* (%)	4 (12.5)	0	0.078	6 (8.3)	1 (2.9)	0.282
Reinfarction *n* (%)	10 (31.2)	4 (17.4)	0.244	27 (37.5)	4 (11.4)	0.005
LVEF (%)	34.1 ± 11.86	36.6 ± 12.06	0.454	39.5 ± 13.14	40.5 ± 11.81	0.72
In-hospital mortality *n* (%)	6 (18.8)	5 (21.7)	0.785	12 (16.7)	6 (17.1)	0.951
Mortality female *n* (%)	5 (20.8)	3 (20)	0.95	8 (22.9)	5 (20)	0.791
Mortality male *n* (%)	1 (12.5)	2 (25)	0.522	4 (10.8)	1 (10)	0.941

Data presented are mean value ± SD or number (percentage) of patients. Age in STEMI and NSTEMI group was compared using Student's t-test and LVEF using Mann-Whitney U test; other data were compared using Pearson's Chi-square test. KCH: Kaunas Clinical Hospital, RHK: Republican Hospital of Kaunas, STEMI: ST elevation myocardial infarction, NSTEMI: non-ST elevation myocardial infarction, DM: diabetes mellitus, RF: renal failure, DM + RM: diabetes and renal failure, and LVEF: left ventricular ejection fraction.

Table 4: Characteristics and in-hospital mortality of patients in electrocardiographic subgroups at the Republican Hospital of Kaunas (RHK) in 2014.

Variable	RHK 2014 y. ($n = 58$)				p
	STEMI ($n = 23$)		NSTEMI ($n = 35$)		
	Positive T wave ($n = 10$)	Negative T wave ($n = 13$)	Positive T wave ($n = 11$)	Negative T wave ($n = 24$)	
Age (years)	75.2 ± 10.29	76.31 ± 7.59	78.64 ± 6.99	78.63 ± 11.45	0.75
Female *n* (%)	5 (50)	10 (76.9)	10 (90.9)	15 (62.5)	0.171
Male *n* (%)	5 (50)	3 (23.1)	1 (9.1)	9 (37.5)	
Pain time <12 h *n* (%)	7 (70)	9 (69.2)	6 (54.5)	12 (50)	0.806
Pain time 12–24 h *n* (%)	1 (10)	0	1 (9.1)	2 (8.3)	
Pain time >24 h *n* (%)	2 (20)	4 (30.8)	4 (36.4)	10 (41.7)	
Troponin T hs (ng/L)	216 ± 49.07	1188.9 ± 584.4	829 ± 253.17	1134.5 ± 265.67	0.29
LVEF (%)	36 ± 10.59	37.08 ± 13.39	39.56 ± 12.9	41 ± 11.61	0.72
In-hospital mortality *n* (%)	2 (20)	3 (23.1)	3 (27.3)	3 (12.5)	0.73

Data presented are mean value ± SD or number (percentage) of patients. Age, troponin, and LVEF were compared using One-Way ANOVA; other data were compared using Pearson's Chi-square test. STEMI: ST elevation myocardial infarction, NSTEMI: non-ST elevation myocardial infarction, DM: diabetes mellitus, RF: renal failure, DM + RM: diabetes and renal failure, troponin T hs: troponin T high-sensitive, LVEF: left ventricular ejection fraction.

Sex-standardized mortality of patients with NSTEMI in KCH is as follows:

$$x = 10.8 * 0.286 + y * 0.714,$$
$$y = \frac{16.7 - (10.8 * 0.514)}{0.486} = 22.94, \quad (4)$$

so $x = 19.47$. So, mortality of patients with NSTEMI in KCH in 2007 standardized by gender is 19.47%.

Sex-standardized and standardized by RF and reinfarction in-hospital mortality of patients with NSTEMI and not standardized in-hospital mortality of patients with STEMI are shown in Figure 1. In-hospital mortality of patients with STEMI was not standardized, because there were no significant differences in patient characteristics between both hospitals (years).

Comparison of more detailed ECG groups at RHK in 2014 did not show significant differences. There were no differences between all four subgroups of patients in age, gender, pain time, troponin level, LVEF, and in-hospital mortality (Table 4). However, some tendencies can be noted in this table. Troponin level tended to be greater in cases of STEMI and NSTEMI with negative T wave than in cases with positive T wave. In-hospital mortality tended to be lowest in cases of NSTEMI with negative T wave.

4. Discussion

Our study showed that there were some differences in patient characteristics at studied non-PCI capable hospitals between 2007 and 2014. In 2014, females were older, but males were younger, less of the patients had RF and reinfarction, and the

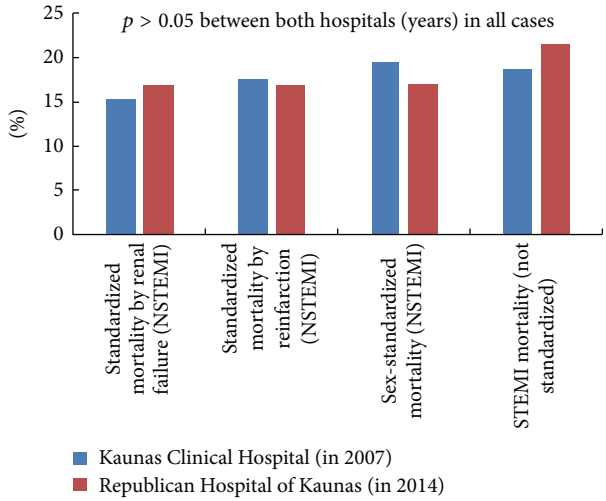

Figure 1: Sex-standardized and standardized by RF and reinfarction in-hospital mortality of patients with NSTEMI and not standardized in-hospital mortality of patients with STEMI at non-PCI capable hospitals between 2007 and 2014. In-hospital mortality of patients with STEMI was not standardized, because there were no differences in patient characteristics between both hospitals (years).

proportion of males and patients with NSTEMI decreased. In 2007, NSTEMI was more frequent than STEMI, while in 2014 the prevalence of NSTEMI was insignificant. In cases of STEMI there were no differences in patient characteristics at non-PCI capable hospitals between 2007 and 2014. However, in cases of NSTEMI in 2014 less often than in 2007 patients were male and had RF and reinfarction.

Trends in AMI in 4 US states between 1992 and 2001 also showed an increase of patient age [6]. However, other studies showed that the proportion of patients with NSTEMI increased from 1990 to 2006 and from 2002 to 2011 [7, 8], in contrast to our study from 2007 to 2014. Also, McManus et al. reported that the incidence rates (per 100.000) of STEMI declined appreciably (121 to 77), whereas the incidence rates of NSTEMI increased slightly (126 to 132), between 1997 and 2005. Although in-hospital and 30-day case-fatality rates remained stable in both groups in that study, 1-year postdischarge death rates declined between 1997 and 2005 for patients with STEMI and NSTEMI [9]. But all these studies were done not at non-PCI capable hospitals in contrast to our study. More recent hospitalization-based analysis revealed a marked increase of NSTEMI among constant AMI frequency and showed discrepancies between trials and real-life: in-hospital mortality of patients with AMI was stagnating on a high level compared with data of randomized clinical trials [5]. Our study was a retrospective analysis of real-life data.

Characteristic of STEMI group was similar between the two hospitals. However, in NSTEMI group differences between hospitals in gender and frequency of patients with RF and reinfarction were found. Therefore, we calculated sex-standardized mortality and mortalities standardized by the frequency of RF and reinfarction by our simple method. Only in these three characteristics the difference was found, so we did not perform full standardization and did not calculate a risk-adjusted mortality by more complex models as it should be done in cases of comparison between many different hospitals with very different patients [10, 11]. Krumholz et al. showed that a simple 7-variable risk model performed as well as more complex models in comparing hospital outcomes for AMI. They concluded that although there is a continuing need to improve methods of risk adjustment, their results provide a basis for hospitals to develop a simple approach to compare outcomes [12]. The right choice of the comparison method is very important. One study, which compared four different methods across 83 hospitals in America, found that of 28 identified as the "worst" mortality hospitals by one company, 12 appeared in the "best" category when other methods were used [13]. However, our standardized mortalities showed no significant differences between in-hospital mortalities of neither patients with STEMI nor patients with NSTEMI in studied non-PCI capable hospitals between 2007 and 2014, as well as not standardized mortalities.

Analysis of more detailed ECG subgroups at a non-PCI capable hospital in 2014 showed no significant differences between all four subgroups of patients in age, gender, pain time, troponin level, LVEF, and in-hospital mortality. However, troponin level tended to be greater in cases of STEMI and NSTEMI with negative T wave than in cases with positive T wave in our study. It is possible that troponin tended to be greater for patients who arrived to hospital later with already inverted T wave, because troponin is increasing until the first 2–4 days of AMI. The patient-reported ischemic time (pain time in Table 4) showed no differences between the subgroups, but it is a subjective criterion. The recent study showed that terminal T wave inversion is a better predictor of outcomes in ST elevation MI than the patient-reported ischemic time and for patients undergoing urgent percutaneous coronary intervention it predicted worse outcomes [14]. However, in-hospital mortality tended to be lowest in cases of NSTEMI with negative T wave in our study performed at non-PCI capable hospitals. This subgroup may have included patients with not only NSTEMI, but also STEMI of late ECG stage with resolved ST segment elevation also, for example, who arrived to a hospital too late, and urgent percutaneous coronary intervention in such cases is not recommended, because it will not improve outcomes [15]. Therefore, the treatment at a non-PCI capable hospital is reasonable to such patients.

Limitations of our study are that two different hospitals were compared with the small cohort. However, the number of patients with AMI is decreasing in non-PCI capable hospitals. The main non-PCI capable hospital for conservative treatment of AMI in the same city was chosen in both years. This study was conducted in two similar non-PCI capable hospitals of the same city, but in two different periods.

5. Conclusions

In 2014 at a non-PCI capable hospital, females were older than in 2007, but males were younger, less of the patients had RF and reinfarction, and the proportion of males and the proportion of patients with NSTEMI decreased. In cases

of STEMI there were no differences in patient characteristics at non-PCI capable hospitals between 2007 and 2014. In cases of NSTEMI in 2014 less often than in 2007 patients were male and had RF and reinfarction. There were no differences in sex-standardized in-hospital mortality and in-hospital mortalities standardized by the frequency of RF and reinfarction in cases of NSTEMI nor not standardized mortality in cases of STEMI between 2007 and 2014. We propose our used simple method of standardization for other comparisons of in-hospital mortalities in cases when groups are different only in few characteristics.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Space-Time Analysis to Identify Areas at Risk of Mortality from Cardiovascular Disease

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This study aimed at identifying areas that were at risk of mortality due to cardiovascular disease in residents aged 45 years or older of the cities of Cuiabá and Várzea Grande between 2009 and 2011. We conducted an ecological study of mortality rates related to cardiovascular disease. Mortality rates were calculated for each census tract by the Local Empirical Bayes estimator. High- and low-risk clusters were identified by retrospective space-time scans for each year using the Poisson probability model. We defined the year and month as the temporal analysis unit and the census tracts as the spatial analysis units adjusted by age and sex. The Mann-Whitney *U* test was used to compare the socioeconomic and environmental variables by risk classification. High-risk clusters showed higher income ratios than low-risk clusters, as did temperature range and atmospheric particulate matter. Low-risk clusters showed higher humidity than high-risk clusters. The Eastern region of Várzea Grande and the central region of Cuiabá were identified as areas at risk of mortality due to cardiovascular disease in individuals aged 45 years or older. High mortality risk was associated with socioeconomic and environmental factors. More high-risk clusters were observed at the end of the dry season.

1. Introduction

About 17 million cardiovascular disease (CVD) related deaths occur annually worldwide [1]. In Brazil, pathologies related to CVD are among the leading causes of death in individuals older than 45 years [2]. In Mato Grosso, heart disease is the second leading cause of death, and its prevalence has shown a trend for an increase, particularly among the elderly [3, 4]. In the cities of Cuiabá and Várzea Grande, CVD has been the major cause of death in the past 5 years, accounting for about 1,000 deaths per year [2].

Middle-aged individuals and the elderly are population at risk for CVD because many CVD risk factors are more prevalent during these stages of life. Moreover, increasing age is associated with a gradual decrease of the physiological resilience of the human body [5].

Several epidemiological studies have shown that almost 80% of patients with heart disease have classic risk factors. These include hypertension, dyslipidemia, obesity, diabetes, advanced age, male sex, family history, and certain lifestyle-related behaviors [6, 7]. Socioeconomic vulnerability, quality of and access to health services, and exposure to heat waves, high temperatures, and air pollution can also be important risk factors for CVD [8–11].

The relationships between the various factors related to the development of a disease can be observed through the distribution of these factors in the geographic space. Mapping health problems and identifying populations at risk of certain diseases and risk factors can be useful management action tools for planning, monitoring, and surveillance in public health [12].

The designation of priorities based on the space-time distribution of a disease enables better resource allocation, implementation of prevention strategies, or even emergency treatment for some diseases [13]. This study aimed to identify areas with a high and low risk of mortality from CVD among middle-aged and elderly individuals living in the urban areas of Cuiabá and Várzea Grande between 2009 and 2011.

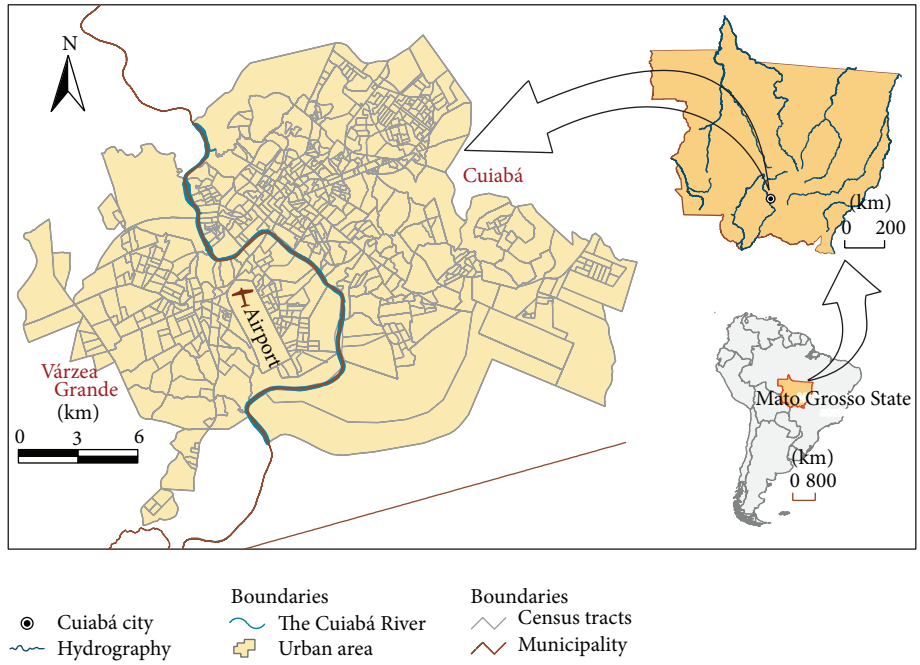

Figure 1: Study area: the urban areas of Cuiabá and Várzea Grande.

2. Methods

2.1. Study Design. This is an ecological spatiotemporal study of CVD mortality rates within clusters that were identified by a risk classification methodology.

2.2. Population and Study Area. The study population included individuals aged 45 years and older living in the urban areas of Cuiabá and Várzea Grande who died from CVD (as classified by Chapter IX of the 10th Revision of the International Classification of Diseases-ICD-10-codes I00 to I99) between 2009 and 2011.

Cuiabá and Várzea Grande (Figure 1) are the most important cities within the metropolitan area of the State of Mato Grosso. These cities, separated physically by the Cuiabá River, form a conurbation with a population of about 820,000 inhabitants, corresponding to 90% of the total population of the metropolitan area. Approximately 98% of the population is concentrated within the urban areas. Furthermore, inhabitants aged 45 years and older represent approximately 60% of the total population of the 2 cities. The last census reported an increase in the rate of aging and life expectancy of this population [14].

2.3. Data Source. Mortality data were obtained from the Mortality Information System of the Brazil Unified Health System (SIM/SUS), given by the Health Department of the State of Mato Grosso. Information on the population, socioeconomics data *shapefiles* of the municipalities, and an aggregate of census tracts of 2010 census data were derived from the Brazilian Institute of Geography and Statistics.

Temperature and humidity records were obtained from the National Institute of Meteorology (INMET) [15], and aerosol optical depth (AOD) data, through which the concentrations of fine particulate matter ($PM_{2.5}$) were estimated, were derived from the Aerosol Robotic Network (AERONET) [16].

2.4. Variables. We selected income ratio, education, and availability of basic services as proxy indicators of social and economic status of the residents.

The income ratio variable indicates situations of inequality in the census tracts. It was obtained by dividing the proportion of individuals with an income below the minimum wage by the proportion of individuals with an income 5 times above the minimum wage. A higher income ratio indicates a bad economic situation.

The education variable indicates the educational level of the population within the census tracts. It was obtained by assessing the ratio of the numbers of literate individuals aged 15 years or older in the census tract over the total population of the census tract, multiplied by 100. A high value for this variable indicates a higher educational level.

The "availability of basic services" variable refers to the availability of regular garbage collection and sanitation services within the neighborhood. This variable was obtained by dividing the number of households within "open sewer" and/or accumulated garbage in the surroundings by the total number of households in the census tract, multiplied by 100. A high value for basic services indicates poor sanitation conditions.

Temperature range, humidity, and $PM_{2.5}$ were selected as environmental variables as they have well-known associations with CVD mortality.

The temperature range variable indicates variations in temperatures. It was obtained by subtracting the minimum

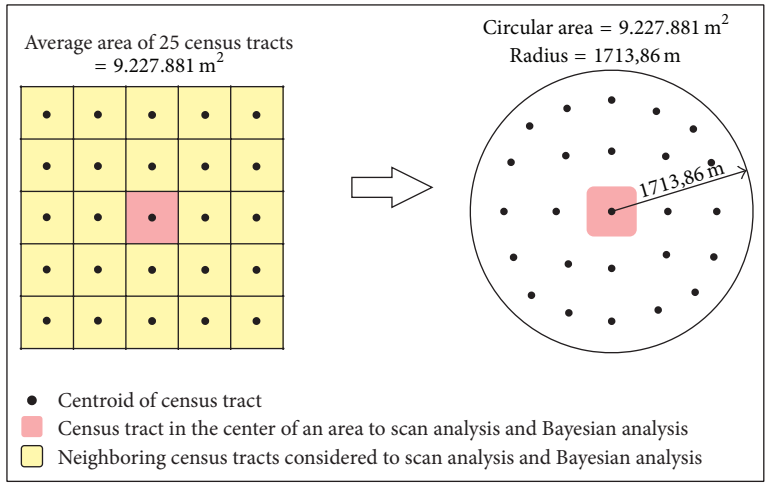

Figure 2: Radius setting scheme for Bayesian analysis and scan statistics.

from the maximum temperature. The humidity variable refers to the proportion of humidity in the environment during a certain period. $PM_{2.5}$ is a mixture of liquid and solid particles suspended in the air derived from combustion [17]. It was estimated by converting the values of AOD (500 nm) using the equation $y = 5 + (\text{AOD} \times 40)$ [18].

2.5. Ethical Considerations. The Ethics Committee of the National School of Public Health (CAAE 18634613.0. 0000.5240) approved this study.

2.6. Data Analysis. We analyzed deaths caused by CVD among individuals aged 45 years or older residing in the urban areas of the cities of Cuiabá and Várzea Grande from 2009 to 2011, which corresponded to 92% of the entire population including rural area. We geocoded the individuals' home addresses and grouped them by census tract for further analysis.

We calculated mortality rates for each census tract from 2009 to 2011 to characterize the spatial distribution of CVD related deaths in the study area. The Local Empirical Bayes estimator using the weighted mean of the crude mortality rate within a location and the mortality rate of neighbors within each sector was performed to analyze these rates. This offered significant stability for the mortality rates, taking into consideration the random fluctuations in data derived from small areas [19]. The neighborhood matrix for the Bayesian estimation contained all neighbors within a distance of 1,713.86 meters to the centroid of each census tract. This distance is equal to the radius of a circle with an area equal to the average of 25 census tracts (9,227,871 m^2) or 2 neighbors on each side of each census tract (Figure 2).

To identify the spatiotemporal clusters for a high and low risk of death due to CVD, statistical tests of spatial scans were performed using SaTScan 9.3 software (http://www.satscan.org/). This technique depends on the likelihood ratio between areas. It performs the spatial scan by moving a cylindrical window at the centroid of each census tract, where the base is the circular geographical area around the centroid and the cylinder height is time. Inside each cylinder, the observed and expected number of CVD deaths by the chosen probability model is calculated, resulting in the RR for each area [20]. Thus, it is possible to identify spatiotemporal clusters using a value that represents how an area is more or less susceptible to having the presence of the event (e.g., deaths related to CVD) when compared to the other studied areas.

The variables included in the space-time scan analysis were population data, number of cases (adjusted for age and sex), and the Cartesian coordinates (UTM Projection-Zone 21 South, metric units) of the census tract centroids. We performed a retrospective analysis for each year, using a Poisson probability model with a circular radius of 1,713.86 meters (Figure 2) and considering a cluster with up to 50% of the population at risk. The time unit of the analysis was the year and month of the occurrence, and the formation of clusters was limited to a minimum of 1 and a maximum of 4 months annually. Monte Carlo simulations were replicated 999 times to create confidence intervals and envelopes.

After the space-time scan, we created a database containing only the census tracts that were within a cluster of high or low risk in 2 or 3 of the analyzed years. We grouped these census tracts into 2 categories, those belonging to a low-risk cluster and those belonging to a high-risk cluster. A nonparametric Mann-Whitney U test was used to compare the means of the socioeconomic and environmental variables between the different risk categories using the SPSS 20.0 software.

The socioeconomic variables were only analyzed spatially because available data represented the average of the 3 years (2009–2011) for the census tracts. In contrast, the environmental variables were only analyzed temporally, since the available data represented the monthly averages of the entire study area. The values assumed for the environmental variables were the averages of data that correlated with each cluster's year and month of occurrence.

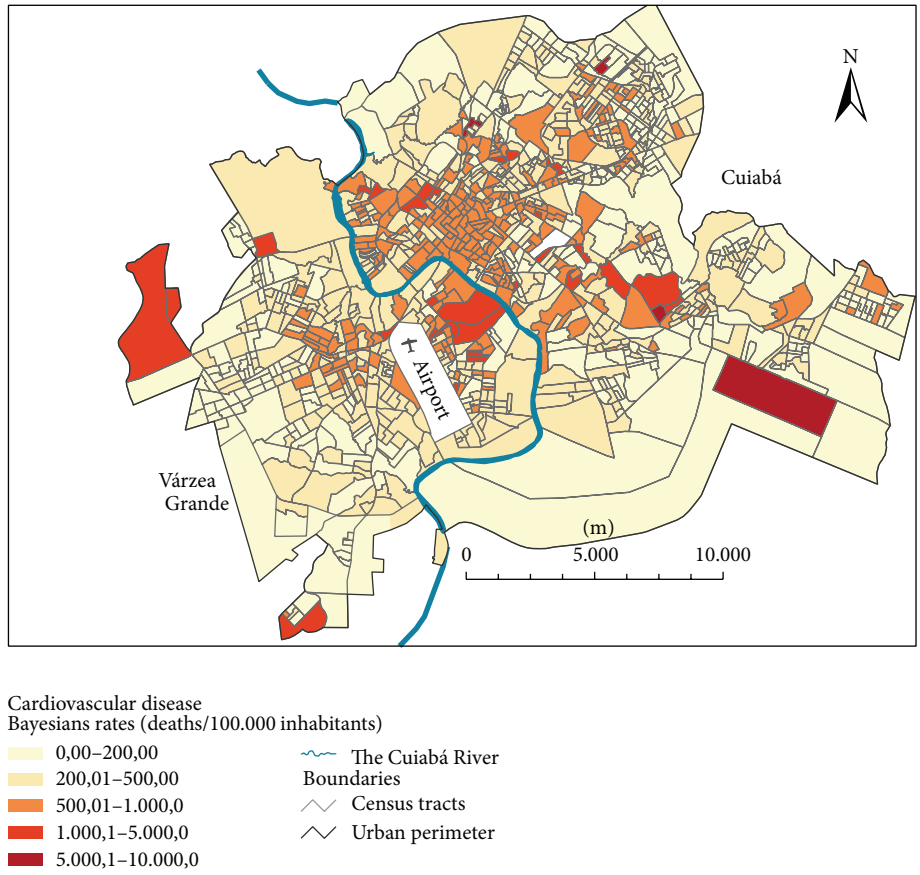

Figure 3: Spatial distribution of CVD mortality rates in adults aged 45 years or older.

3. Results

Between 2009 and 2011, 2,762 deaths from CVD in individuals aged 45 years or older and residing in the urban areas of the cities of Cuiabá and Várzea Grande were observed. The distributions of deaths by age group were 13%, 21%, 27%, and 35% among those aged 45–54 years, 55–64 years, 65–74 years, and ≥75 years, respectively. Ischemic heart disease and cerebrovascular disease were the main causes of death, accounting for 25% and 32% of CVD deaths, respectively.

The average mortality rate obtained by the Local Empirical Bayes estimator was 370 deaths per 100,000 inhabitants for the 2 cities from 2009 to 2011. We observed high CVD mortality areas (1,000–10,000 deaths per 100,000 inhabitants) in the Eastern and Western regions of Várzea Grande and in the Southeastern and Midwestern regions of Cuiabá. In both municipalities, we found areas with intermediate rates of CVD mortality (500–1,000 deaths per 100,000 inhabitants) in the central regions (Figure 3).

The Cuiabá and Várzea Grande conurbation contained 1166 census tracts within its urban area; of these, 62.7% (731 census tracts) had a relative risk (RR) for CVD mortality of <1 and 37.3% (435 census tracts) had an RR > 1. The average RR was 0.99 (standard deviation 1.6), and 25% of the census tracts had RRs > 1.4.

A total of 13, 10, and 20 high-risk clusters and low-risk clusters were detected in 2009, 2010, and 2011, respectively. In 2009, we identified 11 high-risk clusters encompassing 81 census tracts and 2 low-risk clusters encompassing 66 census tracts. In 2010, 8 high-risk clusters encompassing 101 census tracts and 2 low-risk clusters encompassing 47 census tracts were identified. Finally, in 2011, we identified 14 high-risk clusters encompassing 67 census tracts and 6 low-risk clusters encompassing 211 census tracts. High-risk areas for CVD mortality were observed in central and Eastern Várzea Grande and in central and Southeastern Cuiabá (Figure 4).

During the entire study period, only 78 census tracts (distributed in 23 clusters) were within the high-risk clusters or low-risk clusters for at least 2 to 3 years. A total of 19 clusters encompassing 50 census tracts were classified as high risk and 4 clusters encompassing 28 census tracts as low risk.

About 79% of high-risk clusters were detected during the dry season (between June and November), while 75% of low-risk clusters were observed in the rainy season (February to May).

High-risk clusters were found in areas where the mean income ratios were higher and where higher average temperatures and higher levels of fine particulate matter ($PM_{2.5}$) were observed. In contrast, low-risk clusters were areas where the mean humidity levels were high (Table 1).

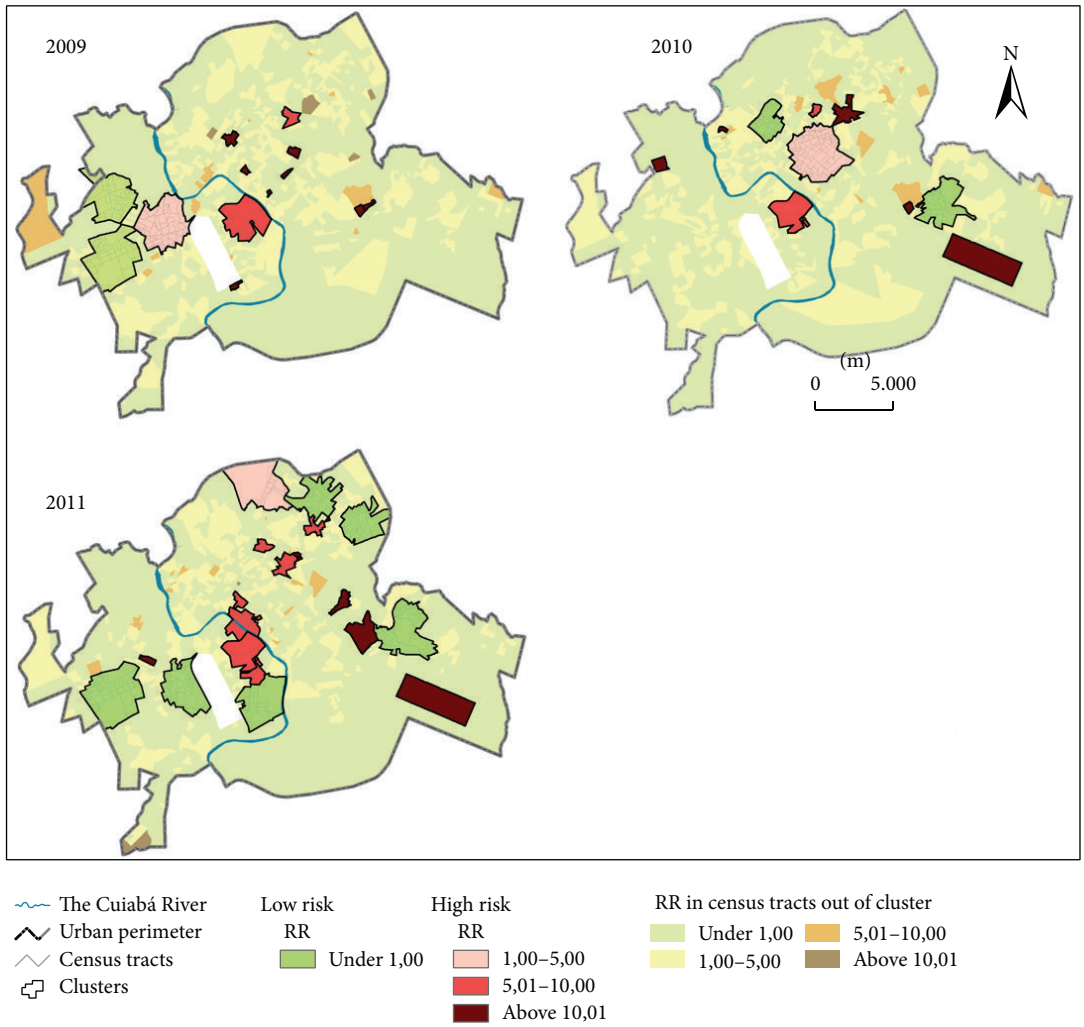

Figure 4: Spatial distribution of high and low relative risk (RR) clusters of CVD mortality.

4. Discussion

The areas at risk of CVD mortality in Cuiabá and Várzea Grande differed based on the socioeconomic factors of their residents and environmental factors. Barcellos and Sabroza [13] attribute the existence of diseases concentration areas primarily to local living conditions. This hypothesis assumes that the geographical space is associated with factors related to the development of diseases and its distribution among various social groups.

We found high-risk clusters for CVD mortality during periods with a higher temperature range (12 to 13°C), lower humidity (68 to 70%), and higher concentrations of $PM_{2.5}$ (16 to 19 $\mu g/m^3$). Climate variability influences human health directly or indirectly [17]. Local environmental conditions related to temperature, humidity, air pollutants, and land use can accentuate the weakness of the body in fighting diseases by increasing inflammation and creating favorable conditions for the development of a disease [21, 22].

Rapid temperature changes and low humidity are associated with disease exacerbation and CVD mortality [17, 23]. This can be explained by the physiological exhaustion caused by prolonged exposure to thermal stress over many consecutive days [24]. Cheng and Su [25] and Huang et al. [24] argue that there is a strong relationship between thermoregulation and the circulatory regulation of an individual. This means that, in environmental situations with normal or moderate changes, the human organism can adapt more easily; however, when abrupt and intense changes occur, there is an overload, which affects the individual's resilience, eventually leading to illness and sometimes even death.

In the cities of Cuiabá and Várzea Grande, the effects of climatic events overlap with the intensification of fires. During the dry season (from June to November), several localities suffer from increased air pollution caused by wildfires and the burning of household waste in backyards and vacant lots [26]. Furthermore, the geographical location within a depression, combined with the lack of rain, reduced wind speed, and temperatures during this period make the region a target of recurrent episodes of heat exchange. In this process, a layer of cold air is retained in the region near the earth's surface, reducing the local temperature and hindering the dispersion

Table 1: Comparison of socioeconomic and environmental factors by risk categories, Cuiabá and Várzea Grande (2009–2011).

	Mean	Confidence interval		Mann-Whitney U test	
		Lower limit	Upper limit	Value	p value
Socioeconomics variables					
Income ratio (%)					
Low risk	0.03	0.02	0.05		
High risk	0.13	0.01	0.26	5.10	0.03
Total	0.07	0.02	0.11		
Availability of basic services (%)					
Low risk	12.38	5.45	19.30		
High risk	11.86	3.77	19.94	0.01	0.93
Total	12.20	6.99	17.40		
Education (%)					
Low risk	70.69	67.37	74.01		
High risk	68.67	60.95	76.39	0.32	0.57
Total	69.96	66.57	73.36		
Environmental variables					
Temperature range (°C)					
Low risk	10.03	9.64	10.41		
High risk	12.49	11.98	13.01	60.44	<0.001
Total	10.91	10.51	11.32		
Humidity (%)					
Low risk	73.58	72.49	74.67		
High risk	69.10	67.79	70.41	26.64	<0.001
Total	71.97	71.01	72.93		
$PM_{2.5}$ ($\mu g/m^3$)					
Low risk	14.24	12.79	15.69		
High risk	17.39	15.75	19.03	7.67	0.01
Total	15.37	14.24	16.50		

of pollutants. This results in the formation of a haze consisting of many pollutants (smog) from industrial emissions, cars, and wildfires [27].

$PM_{2.5}$ has high toxicity. Fine particles (smaller than 2.5 μm) can reach the deeper parts of the respiratory system and even cross the epithelial barrier, triggering an inflammatory response [28, 29]. Several studies have noted increased morbidity and mortality from CVD related to this pollutant. Evidence suggests that short-term $PM_{2.5}$ exposure increases the risk of arterial thrombosis, including myocardial infarction and stroke, while chronic exposure increases the formation of atherosclerotic plaques, reducing life expectancy by a few years [30]. In Brazil, an ecological study in the Brazilian Amazon found an association between $PM_{2.5}$, described as the percentage of hours of exposure to $PM_{2.5}$ with a concentration of $>25\,mg/m^3$, and CVD mortality in the elderly (β adjusted = 0.05; $p = 0.002$) [4].

Census tracts with higher income ratios had high-risk clusters for CVD mortality in our study. There are similar results in other cities of Brazil. Some authors, in Porto Alegre [31], in Pernambuco [32], and in Rio de Janeiro [33–36], observed higher CVD mortality risks associated with socioeconomic inequalities and precarious sanitation services.

Income inequality generates a geographic concentration of poverty that is associated with many social disadvantages (e.g., low education level, low income, and precarious sanitation services) and increases the exposure of residents to various diseases [37, 38]. According to Fiscella and Tancredi [39], certain lifestyle-related behaviors (e.g., smoking cessation, diet improvement, and physical activity) are not very prevalent in low socioeconomic status; that is why the population can be more vulnerable to diseases. Silva and Ribeiro [40] add that the health risks related to extreme weather conditions also increase in these areas, both because of the houses built with materials and techniques that hinder thermal insulation and because of financial constraints that affect the ability of residents to obtain suitable devices for regulating unfavorable microclimate conditions (e.g., air conditioning, fans, and heaters).

The area with the greatest risk of CVD mortality was located in the central area of the Eastern region of Várzea Grande. This region consists of several suburban neighborhoods. It has a high population density and various social

problems related to water supply, public transport, access to health services, and violence, while having a residential population with unequal economic profiles [41].

Limitations. The main limitation of this study was the use of secondary data and, therefore, the accuracy and validity of the variables that were used. These limitations might have affected our results by underestimating or overestimating associations; however, information from secondary databases has been widely used in epidemiological studies worldwide [42].

The reliability of mortality data in the Midwest region of Brazil is satisfactory, since only an average of 4% of cases had a poorly defined cause of death from 2009 to 2011 [2]. In the cities of Cuiabá and Várzea Grande, the proportion of deaths with ill-defined causes followed the decreasing national trend, suggesting an improvement in mortality information in this region. In Cuiabá and Várzea Grande city, the proportions of deaths with poorly defined causes during the study period were on average 1.4% and 0.9%, respectively [2]. Data from the Brazilian Institute of Geography and Statistics and National Institute of Meteorology have been described as reliable by several Brazilian studies [43, 44].

Diseases, especially chronic diseases, reflect a summation of individual and biological factors that manifest themselves for different reasons and times throughout an individual's life. It is important to note that this study took into account a limited number of indicators and that there are other CVD risk factors such as hypercholesterolemia, hypertension, smoking, alcohol consumption, physical inactivity, poor diet, and obesity, which were not accounted for [45]. Various studies have shown that these factors tend to occur simultaneously [46–48], increasing the risk of CVD, especially when associated with poor living conditions, variations in environmental parameters, and exposure to air pollution [49].

5. Conclusions

In conclusion, individuals aged 45 years or older from the Eastern region of Várzea Grande and the Cuiabá central region had the highest risk of CVD mortality. Socioeconomic conditions and environmental characteristics were strongly associated with this increased risk. Moreover, more high-risk clusters were observed during the end of the dry season and the beginning of the rainy season (August to November).

A geographic element's incorporation in this epidemiological study enabled the identification of areas at risk, which enables the redirection of epidemiological surveillance and environmental health measures.

We therefore suggest that it would be beneficial to establish policies for economic development and social empowerment that value health promotion and education, primarily the adoption of healthy habits as well as the monitoring of indicators related to air pollution and other individual risk factors related to lifestyle.

Conflict of Interests

The authors declare that they have no competing interests.

Authors' Contribution

Poliany C. O. Rodrigues conducted conception and design of the study, acquisition of the data, analysis and interpretation of the data, and drafting of the paper. Emerson S. Santos did analysis and interpretation of the data. Eliane Ignotti did conception and design, interpretation of the data, and critical revision of the paper. Sandra S. Hacon did interpretation of the data and critical revision of the paper. All authors read and approved the final paper.

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Review Article

Phytochemical Compounds and Protection from Cardiovascular Diseases: A State of the Art

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Cardiovascular diseases represent a worldwide relevant socioeconomical problem. Cardiovascular disease prevention relies also on lifestyle changes, including dietary habits. The cardioprotective effects of several foods and dietary supplements in both animal models and in humans have been explored. It was found that beneficial effects are mainly dependent on antioxidant and anti-inflammatory properties, also involving modulation of mitochondrial function. Resveratrol is one of the most studied phytochemical compounds and it is provided with several benefits in cardiovascular diseases as well as in other pathological conditions (such as cancer). Other relevant compounds are *Brassica oleracea*, curcumin, and berberine, and they all exert beneficial effects in several diseases. In the attempt to provide a comprehensive reference tool for both researchers and clinicians, we summarized in the present paper the existing literature on both preclinical and clinical cardioprotective effects of each mentioned phytochemical. We structured the discussion of each compound by analyzing, first, its cellular molecular targets of action, subsequently focusing on results from applications in both ex vivo and in vivo models, finally discussing the relevance of the compound in the context of human diseases.

1. Introduction

Cardiovascular diseases (CVDs) still remain the primary cause of death worldwide according to the World Health Organization and American Heart Association statistics [1]. Different approaches have been proposed to reduce the high global incidence of CVDs and to improve human health. The consumption of functional foods or dietary supplements for lowering the risk of CVDs has gained attention over the last few years from both scientific and clinical communities [2, 3]. Several antioxidant compounds can be found in vegetables (e.g., vitamins and phenolic compounds). They are partly responsible for the health benefits by scavenging reactive oxygen radicals (ROS) and by inhibiting cellular damage at different levels. Although the literature contains several review articles describing either general health benefits of phytochemical supplements or the cardioprotective effects of a single phytochemical compound, no comprehensive review article has been so far reported focusing on both preclinical and clinical cardiovascular beneficial effects of the most known compounds (resveratrol, *Brassica oleracea*, curcumin, and berberine).

In the present paper we attempted to fill up this literature gap. In order to reach our goal, we discussed each phytochemical compound by analyzing its molecular targets of action, discussing all existing in vitro, ex vivo, and in vivo data related to its cardiovascular beneficial properties, finally highlighting the evidence available in human CVDs.

2. Resveratrol

Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a natural polyphenolic compound that exists in *Polygonum cuspidatum*, grapes, peanuts, and berries, as well as in their

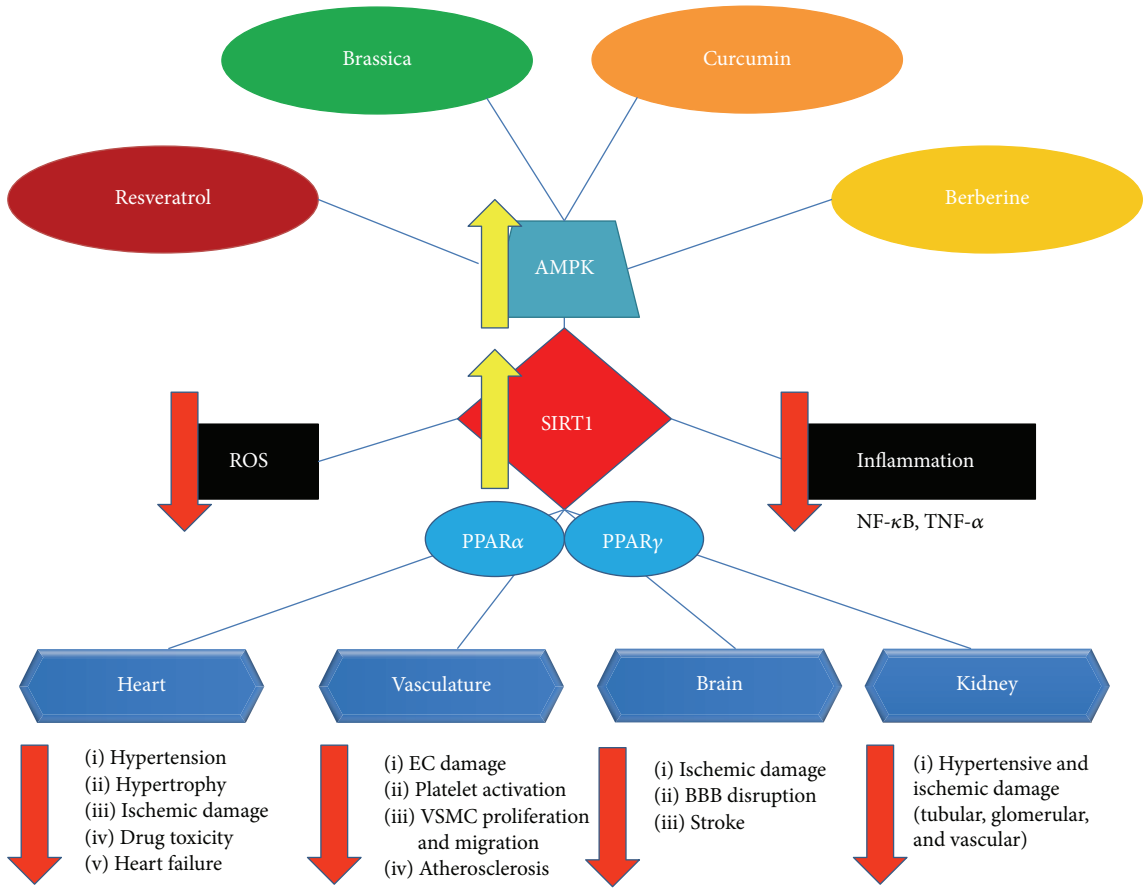

Figure 1: Schematic representation of beneficial effects exerted in the cardiovascular system by resveratrol, *Brassica oleracea*, curcumin, and berberine. The AMPK/SIRT-1/PPARα/γ molecular pathway, underlying most of the effects of all vegetable compounds, is illustrated. AMPK: 5′-adenosine monophosphate-activated protein kinase; SIRT-1: silent mating type information regulation-1; PPAR: peroxisome proliferator-activated receptor; ROS: reactive oxygen species; NF-κB: nuclear factor-κB; TNF-α: tumor necrosis factor-α; EC: endothelial cell; VSMC: vascular smooth muscle cell; BBB: blood brain barrier.

manufactured products, especially red wine [4]. It exists in both *cis*- and *trans*-configurations, of which *trans*-resveratrol is the principal biologically active form [5]. Interestingly, red wine (therefore resveratrol) was supposed to be one of the factors responsible for the "French Paradox," together with several lifestyle and dietary factors. The term is used to describe the low incidence of CVDs in French population despite its high intake of saturated fats. However, the median daily dose of resveratrol to be protective is estimated to be 20 mg/kg/day, whereas its concentration in red wine is roughly 1.98–7.13 mg/L [6]. Therefore, the assumption of a high quantity of red wine per day would be needed for a man to obtain a protective dose of resveratrol, causing serious health problems [7, 8]. However, an inverse association between moderate alcohol consumption (30–50 gr/day) and CVDs has been assessed in several epidemiological studies. The physiological mechanism of the protective effect of alcohol seems to be at least in part related to its effect in reducing platelet action.

2.1. Molecular Targets and Properties. Resveratrol interacts with multiple targets in cardio- and cerebrovascular diseases, age-related diseases, cancer, and so forth, [9, 10]. The main molecular mechanism mediating resveratrol biological effects is the 5′-adenosine monophosphate-activated protein kinase (AMPK)/silent mating type information regulation-1 (SIRT-1) pathway (Figure 1) [11, 12]. The precise mechanism through which resveratrol activates SIRT-1 is not completely understood [13]. Other minor pathways mediate some of the resveratrol effects and they will be briefly mentioned below.

The most important properties of resveratrol are connected with oxidative stress, vascular inflammation, and platelet aggregation. In fact, resveratrol upregulates the endogenous antioxidant systems, such as superoxide dismutase (SOD) enzymes, in endothelial cells (ECs) and in cardiac myoblasts and it reduces ROS production [14, 15]. Moreover, it reduces arachidonic acid and prostaglandin E2 synthesis. It inhibits phospholipase A2 and ciclooxygenase-2 activity; it antagonizes the function of the most important molecules involved in inflammation, such as nuclear factor-κB (NF-κB), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), inducible nitric oxide synthase (iNOS) activity, and monocyte chemotactic protein-1 (MCP-1) [16–19]. Resveratrol also prevents platelet activation by modulating platelet adhesion,

secretion and activation signaling, ROS production, and apoptosis and by enhancing nitric oxide (NO) production [20–22]. Furthermore, several studies demonstrated that resveratrol inhibits protein kinase C (PKC) activation and intracellular calcium release, thus blocking phosphoinositide metabolism upstream platelet activation signaling [23].

2.2. Hypertension: Preclinical Studies. The "antihypertensive" effect of resveratrol is thought to be mediated by both endothelium-dependent and endothelium-independent mechanisms [24, 25], by inhibition of vascular smooth muscle cell (VSMC) contractility, by reduction of vasoconstrictor molecules expression (angiotensin (Ang) II and endothelin (ET)-1), and by inhibition of strain-induced ET-1 gene expression through the extracellular-signal regulated kinase (ERK) 1/2 pathway [11, 26, 27]. Finally, its effect on the sympathetic nervous system (SNS) can also contribute to blood pressure (BP) lowering [28]. The antihypertensive effect of resveratrol (administered at the dose of 10–320 mg/kg body weight/day) has been demonstrated, although with some controversial results, in several hypertensive animal models, including spontaneously hypertensive (SHR), two kidney one-clip hypertensive, partially nephrectomized, and deoxycorticosterone acetate- (DOCA-) salt hypertensive rat models, and in the Ang II-infused mouse [11, 29–32]. Controversies appear mainly related to the specific model under study. In particular, resveratrol was more effective in lowering BP in animals with either diabetes or metabolic syndrome in which variable doses of resveratrol (20 mg/kg/day) were administered [33].

Along with the antihypertensive effect, an improvement of endothelial function was described, being largely attributable to endothelial NO synthase (eNOS) activation [34, 35]. This effect can certainly contribute to protecting vasculature from hypertensive damage [36].

2.2.1. Hypertension: Clinical Studies. Scarce information is available regarding the antihypertensive effect of resveratrol in humans. A recent meta-analysis has shown that treatment with ≥150 mg/day of resveratrol, considered as a very low dose, decreases systolic BP (SBP) without affecting diastolic BP (DBP) [37]. Interestingly, doses of 12,5–100 μL of resveratrol were shown to enhance Acetylcholine-mediated vasorelaxation in blood vessels from patients with hypertension and dyslipidemia but not in vessels from healthy subjects [38]. Although resveratrol supplementation did not exert any effect on BP in healthy obese adults and in patients with metabolic syndrome, it significantly improved flow-mediated dilatation (FMD) in these subjects [37, 39]. Similar results were obtained in patients with previous myocardial infarction (MI) receiving 10 mg of resveratrol daily for 3 months [40]. However, the duration of these clinical trials was too short in order to assess the long-term consequences of the dietary intervention.

No clinical trials are available yet exploring the BP lowering effect of resveratrol in hypertensive patients.

2.3. Atherosclerosis and Dyslipidemia: Preclinical Studies. Resveratrol acts at the very early stages of atherosclerosis by increasing the hepatic uptake of low-density lipoprotein (LDL) through an AMPK independent mechanism and by reducing the expression of *intercellular adhesion molecule-1* (ICAM-1) and of vascular cell adhesion molecule-1 (VCAM-1) on endothelium [41, 42]. Additional in vitro studies demonstrated that resveratrol, likely via the phosphatidylinositol 3′-kinase (PI3K)/protein kinase B (PKB or Akt) pathway, blunts MCP-1 and chemokine receptor type 2 expression in monocytes [43, 44]. Also, it reduces foam cell formation by upregulating the expression of cholesterol transporters and by downregulating the uptake of oxidized LDL (Ox-LDL) [45]. The anti-inflammatory and antioxidant properties of resveratrol may be responsible for inhibition of LDL oxidation, of macrophage migration and transformation into foam cells, as well as of VSMCs migration and proliferation [46, 47].

Several in vivo studies have shown the hypocholesterolemic effect of a standard dose of resveratrol (20 mg/kg/day) [48, 49]. In the apolipoprotein (APO) $E^{-/-}$ mice, resveratrol downregulated the hepatic 3-hydroxy 3-methylglutaryl coenzyme A (HMG-CoA) reductase enzyme, a key enzyme involved in cholesterol biosynthesis, thus reducing total and LDL cholesterol and increasing high-density lipoprotein (HDL) cholesterol [50]. In the high fat fed mice, resveratrol increased liver expression of cholesterol 7α-hydroxylase (CYP7A1) which led to increased bile acid synthesis and secretion, thus lowering the plasma level of total and LDL cholesterol [51].

2.3.1. Atherosclerosis and Dyslipidemia: Clinical Studies. A meta-analysis evaluating the benefits of resveratrol supplementation on plasma lipids revealed no significant effect on any of the lipid parameters (e.g., total LDL and HD-cholesterol and triglycerides) independently of the dose, duration of the study, and cardiovascular risk of the considered population [52]. However, few single studies included in this meta-analysis reported that a relatively low dose of resveratrol treatment (250 mg per day for 3 months) led to a significant decrease of total cholesterol, total and ox-LDL, and ApoB levels in patients with type 2 diabetes mellitus (T2DM), coronary artery disease (CAD), hyperlipidemia, and other cardiovascular risk factors [53]. Similarly, total cholesterol and triglyceride levels were reduced by a very low dose of resveratrol (20 mg/per day for 2 months) in patients with stable angina [54].

2.4. Obesity and T2DM: Preclinical Studies. Resveratrol reduced lipid accumulation both in vivo and in vitro by inhibiting lipogenesis, increasing apoptosis, and promoting lipolysis [55–57]. In Sprague-Dawley rats the body fat-lowering effect of 30 mg resveratrol/kg body weight/day was mediated, at least, in part, by reduction in fatty acid uptake from circulating triacylglycerols, as well as by a de novo lipogenesis in adipose tissue [58]. In addition, it modulated insulin signaling pathway and improved insulin sensitivity in adipose and muscle tissue, as well as glucose uptake and insulin secretion [59, 60]. In human muscle cells derived from T2DM patients, resveratrol may improve glucose utilization and resistance to hyperglycemia by inhibiting phosphorylation of Insulin Receptor Substrate-1 [61].

In vivo, resveratrol restores vascular function through antioxidant, anti-inflammatory, and antiapoptotic properties, as it was observed treating rats with very low doses (0,75 mg/kg/three times a day) [62]. At a standard dose of 20 mg/kg/day, it improved cardiac function in both type 1 and type 2 DM [63–65].

2.4.1. Obesity and T2DM: Clinical Studies. Resveratrol, at the standard dosage of 500 mg three times a day, improved insulin sensitivity in both obese and metabolic syndrome patients [66, 67]. However, other studies failed to confirm these findings [68, 69]. Anti-inflammatory effects of resveratrol were reported in several clinical studies performed in patients with high cardiovascular risk profile [54, 70]. Administration of resveratrol using different chemical formulae at several dosages was associated with decreased oxidative stress in patients with metabolic syndrome [71].

2.5. Ischemic Heart Disease: Preclinical Studies. Resveratrol protects against ischemic heart disease through multiple mechanisms. The mechanisms underlying the preconditioning effect of resveratrol (0,5 mg/kg/day) appear to be mainly mediated by NO and the antioxidant enzyme heme oxygenase-1 (HO-1) [72].

In vitro studies showed that resveratrol upregulated vascular endothelial growth factor (VEGF) expression in cardiomyocytes and in ECs through an increased oxidative-stress related proteins Thioredoxin-1 (Trx-1) and HO-1 expression [73]. It also protected cardiac tissue from cell death through multiple mechanisms including antiapoptotic effects and autophagy [74, 75].

Pretreatment of rats with resveratrol resulted in cardioprotection when the isolated heart was subjected to 30 min global ischemia followed by 2 hr reperfusion, or following permanent left anterior descending coronary artery (LAD) occlusion [76]. Resveratrol can potentiate regeneration of infarcted myocardium in a LAD occlusion rat model by stimulating neovascularization and cardiac stem cells [76, 77]. Interestingly, pretreatment with resveratrol largely restored the altered microRNAs expression in the ischemic heart [78].

The protective effects of resveratrol in the ischemic myocardium were confirmed in vivo [72, 79]. An interesting study conducted by Kanamori et al. suggested that only high dose (50 mg/kg/day) of resveratrol may be an effective treatment for ischemic heart failure (HF) by preventing necrotic area expansion and by improving cardiac function. Authors tested two doses of resveratrol (5 mg/kg and 50 mg/kg) demonstrating the dose-dependent effect of this compound [80].

2.5.1. Ischemic Heart Disease: Clinical Studies. Few clinical trials investigated the effects of both standard and low doses of resveratrol in stable angina, acute coronary syndromes, and previous MI with positive results [40, 54, 70]. Additional studies suggested that resveratrol may be cardioprotective through increase of adiponectin and reduction of thrombogenic plasminogen activator inhibitor type 1 (PAI-1) [81, 82].

2.6. Cardiac Hypertrophy and Heart Failure: Preclinical Studies. Resveratrol was shown to prevent cardiac hypertrophy and dysfunction through reduction of oxidative stress, inhibition of hypertrophic gene expression, and increase of Ca^{2+} handling [83]. The antihypertrophic effect of resveratrol may be BP independent. For instance, low doses of resveratrol (2.5 mg/kg/day) prevented cardiac hypertrophy without reducing BP in SHR and Dahl-salt sensitive rats [84, 85]. The cardioprotective properties were demonstrated in several animal models, including pressure-overload, volume overload, SHR, doxorubicin-induced cardiotoxicity, myocarditis, MI, and ischemia-reperfusion (I/R) injury [15, 84, 86–90]. Recently, Sung et al. demonstrated that high doses of resveratrol (320 mg/kg/day) promote beneficial remodeling and improve both diastolic function and cardiac energy metabolism in a mice model of pressure-overload HF, thus increasing animal survival [91].

2.6.1. Cardiac Hypertrophy and Heart Failure: Clinical Studies. In one study, performed in patients with HF of ischemic origin, treatment with resveratrol significantly improved diastolic function and induced a modest increase of systolic performance, despite the low dose administered (10 mg of resveratrol capsule/day) [40].

2.7. Cerebrovascular Disease: Preclinical Studies. The previously described beneficial vascular properties of resveratrol can also explain protection from ischemic stroke [92]. In vitro resveratrol promoted angiogenesis in cerebral ECs and prevented impairment of eNOS-dependent vasorelaxation of cerebral arterioles in diabetes [93, 94]. It also reduced infarct size in a rat model of focal cerebral ischemia and preserved blood brain barrier function by interfering with occludin and zonula occludens- (ZO-)1 tight junctions [92, 95, 96]. The stroke protective effects of resveratrol were also attributed to its specific neuroprotective properties [97, 98].

2.7.1. Cerebrovascular Disease: Clinical Studies. There are no clinical studies investigating the protective effects of resveratrol in stroke patients. Interestingly, a single dose (250 mg) of *trans*-resveratrol increased cerebral blood flow during a mental stress (cognitive tasks) in healthy adult subjects [99].

2.8. Other Cardiovascular Diseases: Preclinical Studies. Resveratrol protected from doxorubicin-induced cardiotoxicity in a variety of animal models through the above discussed mechanisms [100–102]. However, there is scarce information on the cardioprotective effects of resveratrol in cancer patients treated with either doxorubicin or other cardiotoxic chemotherapeutic agents.

Few studies suggested an antiarrhythmic property. In fact, resveratrol caused a significant antiarrhythmic effect in three models of arrhythmia: aconitine-induced, ouabain-induced, and coronary ligation-induced arrhythmias [103]. Furthermore, chronic oral low-dose resveratrol treatment (5 mg/kg/day for 4 weeks starting one week before MI) significantly suppressed MI-induced ventricular tachycardia and ventricular fibrillation [104]. Recently, Baczko et al.

designed and characterized a multifunctional resveratrol-derived small molecule, compound 1, targeting a number of key pathways involved in atrial fibrillation (AF), able to reduce the average and total AF duration in a model of inducible AF in conscious dogs [105].

3. *Brassica oleracea*

Brassica oleracea (BO) is a commonly used phytochemical. The species include broccoli, cauliflowers, Brussel sprouts, and kale. BO is highly enriched with bioactive molecules, whose effects on health have been partly explored [106–108]. It is known that the content of vitamin C varies significantly between the different subspecies of *Brassica*. These differences mainly depend on genotype and also on industrial storing, processing, and domestic cooking that reduce the final levels of available antioxidant compounds [109].

BO, in particular broccoli sprouts, is rich in glucosinolates: they are large molecules composed by a β-D-thioglucose group, a sulphonated oxime group and an amino-acidic side chain [110]. Sulforaphane is the active metabolite of glucoraphanin and is produced after hydrolyzation by myrosinase enzyme [111]. Cooking the vegetables partially denatures myrosinase; however, when glucoraphanin reaches the intestinal flora myrosinase-producing bacteria release the active metabolite that is then absorbed [112]. After absorption, sulforaphane is partly conjugated with glutathione in the liver, forming sulforaphane-glutathione. After reaching the kidneys, where it becomes sulforaphane-N-acetylcysteine, it is finally excreted in the urine [113]. Other active compounds of *Brassica* plants are anthocyanins, carotenoids, vitamin C, tocopherol, folic acid, and minerals. We will focus our discussion on sulforaphane and anthocyanins, as the main components of BO.

3.1. Molecular Targets and Properties. Molecular targets of BO include NF-κB, nuclear factor-2 (Nrf2), mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), Akt/PKB, and AMPK/SIRT-1/peroxisome proliferator-activated receptor-α (PPARα)/uncoupling protein-2 (UCP2) [114–117]. By interacting with these molecular signaling pathways, BO plays antioxidant, anti-inflammatory, and antithrombotic effects.

3.2. Preclinical Studies. Broccoli sprouts exert several cardiovascular beneficial effects [106, 107].

With regard to glucosinolates, sulforaphane has been proven to be the most beneficial. In vitro, it induced expression of detoxification enzymes, the so called "ARE" targets (Antioxidant Response Elements: nicotinamide adenine dinucleotide (NADH) quinone reductase, HO-1, and glutathione transferase), and several nuclear factors, such as Nrf2, involved in ROS elimination and xenobiotic excretion [114]. Sulforaphane suppressed the expression of MAPK p38 in ECs through activation of Nrf2, thus leading to reduction of VCAM-1 synthesis [118]. By reducing ROS, sulforaphane lowered ox-LDL level in blood [119]. In a study conducted in rats fed for 14 weeks with 200 mg/day of dried broccoli sprouts, a significant increase in glutathione content was observed along with increased glutathione reductase and peroxidase (GPx) activities in both heart and kidneys [120]. Interestingly, administration of broccoli sprouts in pregnant female stroke prone-SHR (SHRSP) decreased oxidative stress and BP levels, compared to females fed with control diet. Furthermore, offspring of females maintained on broccoli diet during pregnancy had also lower BP and tissue inflammation in adulthood, regardless of diet [121].

Anthocyanins are known to promote optimal platelet function and antithrombotic effects [122]. These compounds can act on different types of cells involved in atherosclerosis development. In fact, they exert a protective effect toward TNF-α induced MCP-1 secretion in primary human ECs [123]. Anthocyanins prevented the expression of VEGF stimulated by platelet derived growth factor (PDGF) AB in VSMCs and by MAPK p38 and c-JNK inhibition [115]. Moreover, anthocyanins extract induced endothelium-dependent relaxation in porcine coronary arteries [124]. Increased cardiac glutathione concentrations in rats receiving long-term administration of anthocyanins contributed to the antioxidant effects [125]. The protective effect on heart also depends on reduction of hypertrophy-associated increased phosphorylation of PKC and on activation of Akt/PKB [116]. Moreover, anthocyanins prevented CD40-activated proinflammatory signaling in ECs by regulating cholesterol distribution [126]. They also inhibit the activation of NF-κB and lipopolysaccharides induced NO biosynthesis in macrophages [127].

Broccoli sprouts protect from myocardial oxidative damage and cell death in ischemia/reperfusion (I/R) rat models. In particular, anthocyanins decreased the extent of cell death in cultured cardiomyocytes and reduced infarct size by inhibiting signal transducer and activator of transcription 1 (STAT1) stimulation [106, 128]. Notably, BO improved diabetic nephropathy in rats [107] and prevented renal damage in salt-loaded SHRSP, independently from SBP, through AMPK/SIRT1/PPARα/UCP2 axis activation [117]. In fact, selective inhibition of PPARα antagonized the nephroprotective effects of BO sprouts, consistently with previous evidence on the role of cyanidin as PPARα agonist [129].

3.3. Clinical Studies. The beneficial effects in humans were enhanced when broccoli supplements were combined with fresh broccoli sprouts administration in healthy subjects who consumed either 68 gr of broccoli sprouts or 6 Brocco-Max pills (about 3 gr of freeze-dried broccoli sprouts in 6 pills) for 7 days [130, 131]. In a small clinical trial conducted in 6 men and 6 women, all smokers, eating 100 gr of broccoli sprouts daily for 7 days, a significant reduction of both total and LDL cholesterol along with urinary 8-isoprostanes and other markers of oxidative stress was observed [119]. The administration of 150 mL/day kale juice for 12 weeks in 32 men with hypercholesterolemia significantly reduced plasma LDL cholesterol and increased both HDL cholesterol and GPx activity, thus lowering CAD risk [108]. In addition, broccoli sprouts supplement could play favorable effects on lipid profiles and OX-LDL/LDL cholesterol ratio in T2DM [132]. Recently, anthocyanins intake (8,4–23,6 mg/day) was shown to associate with lower arterial stiffness and central BP in women [133].

Results from human trials are controversial. In fact, Curtis et al. showed no effect on markers of CVDs (including inflammatory biomarkers, platelet reactivity, lipids, and glucose), on liver and kidney function, as well as on anthropometric parameters, BP, and heart rate, following 12-week intervention with 500 mg/day cyanidin in postmenopausal women [134].

4. Curcumin

Curcumin (diferuloylmethane) is a naturally occurring phenolic compound isolated as a yellow pigment from spice turmeric (Curcuma Longa). This compound has received attention due to its various biological and pharmacological activities. Its therapeutic effects have been extensively investigated, particularly in the treatment of cancer and inflammatory diseases [135].

There is growing evidence that curcumin has a potential role in protection from several CVDs [135, 136].

4.1. Molecular Targets and Properties. Curcumin interacts with different molecular targets, such as Janus Kinase 2 (JAK2)/STAT3, AMPK/UCP2, Akt/Nrf2, ERK, MAPK p38, JNK, ICAM-1, MCP-1, and IL-8 [137–141]. As a consequence, it exerts anti-inflammatory, antiplatelet, and antioxidant properties [141–144]. Concerning the latter, a single dose of 15 mg/kg of curcumin appears to decrease levels of xanthine oxidase, superoxide anion, lipid peroxides, and myeloperoxidase and to increase levels of SOD, catalase, GPx, and glutathione-S-transferase (GST) [145]. Moreover, this phytochemical reduces level of eNOS and iNOS through the activation of NF-κB and protein-1 (AP-1) [146]. Curcumin is also a potent inducer of HO-1 in ECs through activation of ARE in several cardiovascular cells exposed to curcumin 5–15 μM [147]. Moreover, curcumin appears to attenuate mitochondrial alterations and respiratory cellular dysfunction [148].

4.2. Preclinical Studies. Curcumin plays a protective role on endothelium by inducing HO-1 in bovine aortic ECs [147]. It exerts antiproliferative and antiapoptotic effects on VSMCs, exposed to 1–25 μM of curcumin, thus attenuating carotid artery neointima formation [149–151]. It plays a relevant role on calcium homeostasis in both skeletal muscle and cardiac sarcoplasmic reticulum [152].

The role of curcumin in CVDs has been investigated in several animal models. For instance, 1,66 mg curcumin/kg showed a hypolipidemic effect and protection from aortic fatty streak development [153, 154]. The cardioprotective role of curcumin was shown in myocardial ischemia rat models [145, 155]. In I/R models, curcumin reduced collagen synthesis and fibrosis and significantly improved left ventricular end-diastolic volume, stroke volume, and ejection fraction [156]. In addition, it reduced MI size and depressed lactate dehydrogenase release in the coronary blood flow through activation of JAK2/STAT3 [137]. These beneficial effects could be related to a decrease of proinflammatory cytokines and of cardiomyocyte apoptosis [157].

In two different HF models, 50 mg curcumin/kg/day ameliorated systolic function and prevented myocardial hypertrophy by inhibiting p300-HAT (histone acetyltransferases) [158]. Additionally, a larger amount of curcumin (200 mg/kg/day) showed a protective role in adriamycin-induced cardiac damage [159] and it also prevented cardiovascular complications in diabetes [146]. In fact, it reduced high glucose-induced overexpression of inflammatory cytokines in macrophages [144]. A beneficial role toward myocardial injury was reported in renal I/R injury rat models [160].

Finally, a standard dose of curcumin (25–50 mg/kg/day) protected against cerebral ischemic insult [161], as well as aging-related cerebrovascular dysfunction via AMPK/UCP2 pathway. It protected neurons against ischemic injury through Akt/Nrf2 pathway [138, 139]. In different stroke models curcumin not only decreased oxidative stress but also attenuated reperfusion injury by preventing neutrophil adhesion to the cerebrovascular microcirculation [162, 163].

4.3. Clinical Studies. Controversial results exist with regard to the effect of curcumin on plasma lipids in healthy subjects. In fact, in healthy volunteers, a dosage of 500 mg curcumin/day decreased both serum lipid peroxides and total cholesterol and increased HDL cholesterol [164]. Hypolipidemic effects were also observed in patients affected by atherosclerosis, acute coronary syndrome, and T2DM. Moreover, the effect of curcumin administration on lipid profile was evaluated in acute coronary syndrome (ACS) patients at escalating doses (low dose, 3 times 15 mg/day; moderate dose, 3 times 30 mg/day; high dose, 3 times 60 mg/day). Unexpectedly, this study showed that the low dose of curcumin was associated with higher reduction of total, HDL and LDL cholesterol levels [165, 166]. On the other hand, a meta-analysis failed to show protective effects of curcumin on both cholesterol and triglycerides in a heterogeneous population [167]. Curcumin administration and aerobic exercise training increased FMD in postmenopausal women [168]. Interestingly, curcumin may improve the blood compatibility of rapamycin-eluting stents through its antiplatelet properties [169].

5. Berberine

Berberine (BBR), an alkaloid isolated from *Hydrastis canadensis*, the Chinese herb Huanglian, and many other plants, such as the Berberidaceae and Ranunculaceae families, has a long history in traditional Chinese medicine. BBR is present in roots, rhizomes, and stem bulk of the plants. Various pharmacological actions, including antibiotic, immunostimulant, antitumor, and antimotility properties have been described for BBR [170, 171].

Recent studies have indicated that BBR may be also effective in treating chronic, multifactorial diseases, including diabetes, hyperlipidemia, heart diseases, cancer, neurological disorders, and inflammatory diseases [172, 173].

5.1. Molecular Targets and Properties. Molecular mechanisms mediating antioxidant effects appear to be mainly related

to upregulation of both SOD and UCP2 and to downregulation of NADPH oxidase expression [174, 175] with particular regard to NADPH oxidase 2/4 subunits [175]. BBR administration activates Nrf2 pathway, which is crucial for antioxidant and anti-inflammatory activities [176]. BBR could suppress inflammation by blocking the MAPK pathways in a AMPK-dependent manner, by inhibiting the NF-κB signaling pathway and the Rho GTPase pathway and by attenuating transcription activity of AP-1, which is possibly mediated by PPARα activation [177–179].

5.2. Preclinical Evidences. In vitro studies demonstrated the role of BBR in counteracting endothelial progenitor cells (EPCs) dysfunction. In fact, BBR improved the proliferative ability of EPCs impaired by TNF-α via activation of PI3K/Akt/eNOS signaling pathway [180]. Moreover, BBR induced endothelium-dependent vasorelaxation and enhanced endothelium-independent VSMC dilatation through a partial reduction of oxidative stress [181].

In VSMCs, isolated from thoracic aorta of Sprague-Dawley rats, BBR inhibited Ang II- and heparin binding epidermal growth factor- (HB-EGF-) induced VSMC proliferation and migration. In vivo results showed a reduction of neointima formation after balloon injury, thus lowering risk of restenosis [182]. Zimetti et al. demonstrated a double protective effect of BBR on cholesterol homeostasis underlying foam cells formation and on the inflammatory phenotype in mouse and human macrophages [183].

BBR affected glucose metabolism by increasing insulin secretion, stimulating glycolysis, suppressing adipogenesis, and increasing glucokinase activity and both glucose transporter-4 (GLUT-4) and glucagon-like peptide (GLP-1) levels in glucose-consuming tissues [184].

Furthermore, BBR was shown to have lipid-lowering properties in animals as well as in hyperlipidemic patients through mechanisms different from those of statins, involving activation of ERK pathway and increase of LDLR expression on the hepatocytes surface [185]. Interestingly, contrasting results were reported with regard to modulation of the gene encoding proprotein convertase subtilisin kexin 9 (PCSK9), a natural inhibitor of LDLR. In HepG2 cells 20 μM BBR downregulated the transcription of the gene [186], whereas 400 mg BBR/kg/day significantly reduced body weight and improved lipid profile by increasing the PCSK9 expression levels through Sterol Regulatory Element-Binding Proteins activation in the high fat diet (HFD) rat model [187].

BBR, at the dosage of 100 mg/kg/day, plays positive inotropic, antiarrhythmic, and vasodilator properties related to the cardiovascular system [188, 189]. The antiarrhythmic effects are due, at least in part, to preferential blockade of the components of the delayed rectifying potassium current, I(Kr), and I(Ks) and to increased effective refractory period of Purkinje fibers [190, 191].

The beneficial effects of BBR were demonstrated in several animal models such as SHR, HFD rats, pressure-overload HF, and myocardial ischemia [187, 192–194]. Notably, 50 Sprague-Dawley rats were treated with BBR (30 or 60 mg/kg) demonstrating that BBR had cardioprotective effects against acute ischemic myocardial injury in a dose-dependent manner [194]. BBR counteracted several pathological features of hypertension, including suppression of endoplasmic reticulum stress, inhibition of ROS accumulation, and attenuation of endothelium-dependent contractions in SHR [195]. The antihypertensive effect of BBR derivative 6-protoberberine (PTB-6) was shown in conscious SHR and Wistar-Kyoto (WKY) rats, and it was mediated by reduced SNS activity through a negative inotropic and chronotropic effect [192].

A recent in vivo study reported that BBR can prevent cardiac hypertrophy and attenuate cardiomyocyte apoptosis in the transverse aortic contraction treated rat model [193].

In a rat model of MI, BBR administration significantly enhanced autophagic activity, attenuated adverse left ventricular remodelling, and preserved left ventricular systolic function. Interestingly, low-dose BBR (10 mg/kg per day) was associated with greater improvement in cardiac function compared with high-dose BBR (50 mg/kg per day) [196]. In diabetic rat models, BBR protected the heart against I/R injury, improved cardiac function, and reduced myocardial apoptosis via activation of AMPK and PI3K/Akt and eNOS signaling [197]. In addition, cardioprotective effects of BBR in myocardial ischemia are due to its antioxidant and anti-inflammatory properties [194].

Chronic administration of BBR significantly reduced oxidative stress and vascular inflammation and suppressed atherogenesis in ApoE$^{-/-}$ mice by AMPK-dependent UCP2 expression [174].

In a middle cerebral artery occlusion (MCAO) model, BBR improved neurological outcome and reduced I/R-induced cerebral infarction 48 hrs after MCAO. The protective effect of BBR was confirmed in vitro [198].

5.3. Clinical Evidences. BBR has shown good safety results in human studies [199]. A randomized clinical trial tested its effects in 156 patients with chronic congestive HF. The BBR-treated group (1,2–2 gr/day) showed significantly greater increases in left ventricular ejection fraction and exercise capacity, significant improvements on the dyspnea-fatigue index, and decreased rates of ventricular premature complexes and long-term mortality [200].

Treatment of 100 arrhythmic patients with BBR resulted in a >89% reduction in premature beating in the majority of patients and >50% reduction in the remaining patients [201]. These results were independently reproduced [202]. A recent meta-analysis, including 11 randomized controlled studies (874 Chinese participants affected by hyperlipidemia, T2DM, or both diseases), has shown a significant reduction in total cholesterol, triglycerides, and LDL cholesterol levels and a small but significant increase in HDL cholesterol [203].

In T2DM patients, high-dose BBR administration (100–200 mg/kg/day) was associated with a significant reduction in glycated hemoglobin, fasting plasma insulin, postprandial glucose, and fasting plasma glucose [204].

BBR beneficial effects were also observed in hypercholesterolemic European patients [205].

A recent meta-analysis emphasized the role of BBR in the treatment of hypertension. In fact, BBR associated with

Table 1: Preclinical effects of vegetable compounds.

Vegetable	Preclinical effects	Reference
Resveratrol	(i) Upregulates the **antioxidant system** and reduces **ROS** production	[14, 15]
	(ii) Inhibits **vascular inflammation** and prevents **platelet activation**	[16–22]
	(iii) Lowers **BP** in animals with either diabetes or metabolic syndrome	[33]
	(iv) Inhibits very early stages of **atherosclerosis**	[41, 42]
	(v) Reduces **lipid accumulation** by inhibiting lipogenesis, increasing apoptosis, and promoting lipolysis	[55–57]
	(vi) Protects against **ischemic heart disease**	[72]
	(vii) Protects cardiac tissue from **cell death** though apoptosis and autophagy	[74, 75]
	(viii) Potentiates **regeneration of infarcted myocardium**	[76, 77]
	(ix) Prevents cardiac hypertrophy and dysfunction	[83]
	(x) Promotes **angiogenesis** in cerebral ECs and prevents impairment of eNOS-dependent **vasorelaxation** of cerebral arterioles	[93, 94]
	(xi) Protects from doxorubicin-induced **cardiotoxicity**	[100–102]
	(xii) **Antiarrhythmic effects**	[103]
Brassica oleracea	(i) Induces expression of **detoxification enzymes** (ARE targets)	[114]
	(ii) Lowers **ox-LDL** blood levels	[119]
	(iii) Decreases **oxidative stress and BP levels** in pregnant female SHRSP	[121]
	(iv) Promotes **optimal platelet function** and **antithrombotic** effects	[122]
	(v) Acts on different types of cells involved in **atherosclerosis** development	[123]
	(vi) Regulates **cholesterol** distribution	[126]
	(vii) Protects from **myocardial oxidative damage and cell death** in ischemia-reperfusion rat models	[106, 128]
	(viii) **Nephroprotective** effects	[129]
Curcumin	(i) Protective role on **endothelium** by inducing HO-1	[147]
	(ii) **Antiproliferative** and **antiapoptotic** effects on VSMCs, attenuating **neointima formation**	[149–151]
	(iii) **Hypolipidemic** effect and protection from **aortic fatty streak** development	[153, 154]
	(iv) Reduces collagen synthesis and **fibrosis and** improves **left ventricular end-diastolic volume, stroke volume, and ejection fraction**	[156]
	(v) Reduces **MI** size	[137]
	(vi) Protects from **adriamycin-induced cardiotoxicity**	[159]
	(vii) Protects from **cerebral ischemic insult**	[138, 139]
Berberine	(i) Improves the **proliferative ability of EPCs**	[180]
	(ii) Induces **endothelial-dependent vasorelaxation** and enhances **endothelium-independent VSMC dilatation**	[181]
	(iii) Inhibits VSMC proliferation and migration and reduces **neointima formation**	[182]
	(iv) **Lipid-lowering** properties	[185]
	(v) **Positive inotropic, antiarrhythmic, and vasodilator** properties	[188, 189]
	(vi) **Antihypertensive effects** in SHR	[195]
	(vii) Prevents **cardiac hypertrophy** and attenuates cardiomyocyte apoptosis	[193]
	(viii) Attenuates **adverse left ventricular remodeling and preserves left ventricular systolic function** in rat model of MI	[196]

ROS: reactive oxygen species; BP: blood pressure; eNOS: endothelial nitric oxide sinthase; ARE: Antioxidant Response Elements; ox-LDL: oxidized low-density lipoprotein; SHRSP: stroke prone spontaneously hypertensive rats; HO-1: heme oxygenase-1; ECs: endothelial cells; VSMC: vascular smooth muscular cells; MI: myocardial infarction; EPCs: endothelial progenitor cells.

Table 2: Clinical effects of vegetable compounds.

Vegetable	Clinical effects	Reference
Resveratrol	(i) Decreases **SBP** without affecting DBP	[37]
	(ii) Enhances **Ach-mediated vasorelaxation** in hypertensive and dyslipidemic pts	[38]
	(iii) Improves **FMD** in pts with either metabolic syndrome or previous MI	[39, 40]
	(iv) Decreases **total cholesteroland total** and **ox-LDL, triglycerides**, and **ApoB** levels in pts with T2DM, CAD, hyperlipidemia, and other CV risk factors	[53, 54]
	(v) Improves **insulin sensitivity** in both obese and metabolic syndrome pts	[66, 67]
	(vi) Improves significantly **diastolic function** and modestly **systolic function** in pts with previous MI	[40]
Brassica oleracea	(i) Reduces **total, LDL cholesterol** and **markers of oxidative stress** in smokers and hypercholesterolemic pts	[108, 119]
	(ii) Improves lipid profiles and **ox-LDL/LDL cholesterol ratio** in T2DM pts	[132]
	(iii) Lowers **arterial stiffness** and **central BP** in women	[133]
Curcumin	(i) Decreases both **total and LDL cholesterol** and increases **HDL cholesterol** in healthy subjects and in ACS pts	[164, 165]
	(ii) Increases **FMD** in postmenopausal women	[168]
Berberine	(i) Increases both **LVEF** and **exercise capacity** and decreases rates of **ventricular premature complexes** and **long-term mortality** in HF pts	[199]
	(ii) Reduces **total cholesterol, triglycerides, and LDL cholesterol** levels and modestly increases **HDL cholesterol** in hyperlipidemic and T2DM pts	[203, 205]
	(iii) Reduces **glycated hemoglobin, fasting plasma insulin, postprandial glucose, and fasting plasma glucose** in T2DM pts	[204]
	(iv) Lowers **SBP** and **DBP** in hypertensive, T2DM, and hyperlipidemic pts	[206]

SBP: systolic blood pressure; DBP: diastolic blood pressure; Ach: Acetylcholine; FMD: flow-mediated dilation; MI: myocardial infarction; ox-LDL: oxidized low-density lipoprotein; HDL: high-density lipoprotein; Apo: apolipoprotein; T2DM: type 2 diabetes mellitus; CAD: coronary artery disease; CV: cardiovascular; ACS: acute coronary syndrome; LVEF: left ventricular ejection fraction; HF: heart failure; pts: patients.

lifestyle intervention tended to lower the level of BP more than lifestyle intervention alone or than placebo [206].

6. Conclusions

Preclinical studies revealed several beneficial cardiovascular effects of resveratrol, *Brassica oleracea*, curcumin, and berberine. The benefits appeared to be mainly dependent on antioxidant, anti-inflammatory, and antithrombotic properties. In fact, the excellent results of both in vitro and in vivo studies induced researchers and clinicians to test the effects of phytochemicals in humans. However, evidences obtained from the few available clinical trials on the protective effects of these compounds in several CVDs are still controversial. A main limitation of current clinical studies relies on their heterogeneity and on small samples size. Furthermore, based on the literature discussed in the present paper, some confusion arises about the precise dose of each compound exerting more pronounced beneficial effects. In particular, whereas the use of a very high dose is associated with the most protective effects for few phytochemicals, the lowest dose turns out to be the most effective for other compounds. This phenomenon appears to be related to different animal models as well as to the specific disease under consideration. Therefore, there is a need for additional larger and well controlled human studies.

Altogether, the lack of a clear beneficial role in humans, the wide variety of in vitro, ex vivo, and in vivo experimental evidences that are summarized in Tables 1 and 2, suggests that resveratrol, *Brassica oleracea*, curcumin, and berberine may reveal very useful preventive and/or therapeutic tools for the treatment of CVDs, as a valid support to medical therapies.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Vascular Damage in Resistant Hypertension: TNF-Alpha Inhibition Effects on Endothelial Cells

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Inflammatory cytokines have been associated with the pathophysiology of hypertension and target organ damage (TOD). Resistant hypertensive patients (RHTN) are characterized by poor blood pressure control and higher prevalence of TOD. This study evaluated the relationship between plasma levels of TNF-α and arterial stiffness (pulse wave velocity—PWV) in 32 RHTN and 19 normotensive subjects. Moreover, we investigated the effect of TNF-α inhibition on human endothelial cells (HUVECs) incubated with serum from RHTN and normotensive subjects. HUVECs containing serum obtained from normotensive ($n = 8$) and hypertensive ($n = 8$) individuals were treated with TNF-α inhibitor (infliximab). Cell suspensions were used for measurement of DNA fragmentation and reactive oxygen species (ROS) content. RHTN patients showed higher levels of TNF-α compared to normotensive subjects, as well as higher PWV. Positive correlation was found between TNF-α levels and PWV measures in the whole group. HUVECs incubated with serum from RHTN showed increased cell apoptosis and higher ROS content compared to normotensive subjects. Infliximab attenuated the apoptosis of HUVECs incubated with serum from RHTN, but no effect in ROS production was observed. Our findings suggest that TNF-α might mediate, at least in part, vascular damage in resistant hypertension.

1. Introduction

Several studies have demonstrated the participation of inflammatory cytokines in the genesis of hypertension in humans [1, 2] and animal models [3, 4]. Mice lacking T cells (RAG-$1^{-/-}$ mice) showed attenuated hypertension after angiotensin II infusion and desoxycorticosterone acetate- (DOCA-) salt or norepinephrine administration [3, 5]. Moreover, increased secretion of cytokines such as IFN-γ, IL-17A, and TNF-α by circulatory spleen-derived T cells was observed in Ang II-induced hypertension. In these animals, the inhibition of TNF-α prevented increased vascular superoxide production and hypertension mediated by angiotensin II [3]. Although it has been suggested that several cytokines are involved in vascular damage induced by hypertension, TNF-α inhibition decreases blood pressure and prevents target organ damage in animal studies [3, 6]. Also, the inhibition of TNF-α in humans showed BP reduction [7]. On the other hand, the role of this cytokine in hypertensive subjects has been poorly studied.

Resistant hypertensive patients (RHTN) represent extreme phenotype of hypertension, characterized by poor blood pressure control and higher prevalence of target organ damage, which explain the unfavorable prognosis associated with this condition [8]. Vascular injury is a frequent characteristic induced by hypertensive disease. Our group showed that RHTN have higher arterial stiffness and impaired endothelial function compared to normotensive and mild to moderate subjects [9]. Conductance vessels gradually show reduction in distensibility and compliance, phenomena known as arterial stiffness. Arterial stiffness is an independent predictor of all-cause and cardiovascular mortality in hypertensive patients and is characterized by

structural changes in connective tissue proteins in the vascular wall [10]. In addition to extracellular matrix remodeling, oxidative stress and inflammatory markers are key players in vascular remodeling associated with hypertension. Angiotensin II stimulates NADPH oxidase activity, increases reactive oxygen species production (ROS), and reduces NO availability leading to endothelial dysfunction [11, 12]. In addition, ROS increases collagen secretion by vascular smooth cells [13], which may favor vascular stiffness. Both Ang II and ROS signaling activate cytokines production, including TNF-α, and the expression of cell adhesion molecules including VCAM-1 and MCP-1 [14, 15]. In turn, vascular inflammation stimulates vascular fibrosis and smooth muscle cells proliferation, subsequently increasing arterial stiffness [15]. Inflammatory markers such as TNF-α, C-reactive protein, and interleukin-6 are correlated with arterial stiffness in hypertensive subjects [16]. In fact, previous studies demonstrated that the infusion of angiotensin II in mice lacking T and B cells (RAG-1$^{-/-}$) or interleukin-17a (IL-17a$^{-/-}$) reduces collagen deposition in the aorta and superoxide production and preserved endothelium-dependent vasodilatation compared to wild type animals [17, 18]. Interestingly, human aortic smooth muscle cells treated concomitantly with IL-17 plus TNF-α showed increased expression of several genes related to vascular dysfunction and inflammation [18]. Taken together, these data demonstrated that T cells-derived cytokines may play a critical role in vascular stiffening.

We recently found that TNF-α inhibition with infliximab reduced systolic BP, left ventricular hypertrophy, and vascular inflammation in spontaneously hypertensive rats (SHR) [6]. Moreover, hypertensive subjects showed increased arterial stiffness and higher plasma levels of TNF-α compared with normotensive subjects [19]. TNF-α inhibition has been discussed as potential strategy to improve vascular function [20, 21]. On the other hand, the treatment with anti-TNF-α improves vascular endothelial function and decreases arterial stiffness in postmenopausal women [22] and rheumatoid arthritis patients [23].

Despite some studies suggesting TNF-α as a potential marker of vascular inflammation, the causal role of this cytokine in the pathogenesis of hypertension is underexplored. The association of TNF-α has been extensively reported in hypertension, but no previous study evaluated the effects of TNF-α on human endothelial cells. Thus, this study was designed to evaluate the relationship between plasma levels of TNF-α and arterial stiffness in RHTN and normotensive subjects. Moreover, we investigated the effect of TNF-α inhibition on human endothelial cells incubated with serum from RHTN and normotensive subjects.

2. Methods

2.1. Patient Population. This cross-sectional study was performed in the Outpatient Resistant Hypertension Clinic at the University of Campinas Hospital. Thirty-two patients classified as RHTN and 19 normotensive subjects were included in this study. Resistant hypertension (RHTN) was defined as blood pressure (BP) that remained above goals despite the concurrent use of 3 antihypertensive agents of different classes, including a diuretic, at optimal dose amounts. Also, patients whose blood pressure was controlled but required 4 or more medications were also considered resistant [24]. Hypertensive patients were followed up and treated for at least 6 months with regular scheduled appointments before being characterized as resistant to treatments. Exclusion criteria included secondary hypertension (identifiable and removable causes of hypertension, including Conn's or Cushing's syndrome, diabetes, renal artery stenosis, pheochromocytoma, and coarctation of the aorta), liver and renal disease, heart failure (ejection fraction < 50%), stroke, peripheral vascular disease, smokers, obesity (BMI $\geq 30\,kg/m^2$), pregnancy or oral contraceptive use, history or clinical evidence of recent infection, and use of anti-inflammatory drugs.

2.2. Laboratory Assessments. Blood samples were collected at 8:00 a.m. after overnight fasting. The plasma levels of TNF-α were determined by ELISA (R&D Systems, Inc., Minneapolis, USA) according to manufacturer's instructions. Biochemical assessments, including serum cholesterol, LDL, HDL, triglycerides, glucose, aldosterone, and creatinine, were performed by central laboratory at the University of Campinas Hospital.

2.3. PWV Measurement. Pulse wave velocity was measured using the SphygmoCor System (Atcor Medical, Sydney, Australia) with the patient in the supine position. Pulse wave of the carotid and femoral arteries was determined by estimating the delay with respect to the electrocardiogram wave. A measuring tape was used to measure the distance from the sternal notch to the carotid-femoral recording site. Carotid-femoral PWV was calculated by dividing traveled distance by transit time (PWV = distance/time). At least two measurements were performed in each patient. The PWV value was reported as the mean and the values were corrected for mean arterial pressure.

2.4. Collection and Preparation of Serum Samples. At the study visit, blood was collected by antecubital vein puncture using serum-separating tubes (BD Vacutainer System). After 30 minutes of resting at room temperature, whole blood samples were centrifuged for 10 minutes at 4,000 rpm. Serum was stored at −80°C in 1 mL aliquots until the cytokine measurements.

2.5. Human Umbilical Vein Endothelial Cells (HUVECs) Cell Culture and Plasma Incubation Conditions. HUVEC cell lines (CRL-2873, ATCC, Manassas, VA, USA) were cultured at 37°C in 5% CO_2 in Dulbecco's Modified Eagle's Medium (Vitrocell Embriolife, Brazil), supplemented with glucose 4,500 mg/L and 10% (v/v) fetal bovine serum (FBS). The cells were used at passage 3 for the experiments. HUVECs were plated in 6-well plates at a density of 2×10^5 cells/well (for flow cytometry assays) and in $25\,cm^2$ tissue culture flasks at a density of 4×10^5 cells/flask (for gene expression experiments). After 24 hours, the medium was replaced by FBS-free medium containing 10% of serum obtained from normotensive ($n = 8$) and hypertensive ($n = 8$) individuals.

Table 1: Primers sequences.

Name	Forward primer (sense) $5' \rightarrow 3'$	Reverse primer (antisense) $5' \rightarrow 3'$
Human *GAPDH*	GTTAGGAAAGCCTGCCGGT	AGTTAAAAGCAGCCCTGGTGA
Human *ENOS*	GTGGCTGTCTGCATGGACCT	CCACGATGGTGACTTTGGCT
Human *INOS*	GATCAAAAACTGGGGCAGCG	CTCATCTGGAGGGGTAGGCT
Human *ARGII*	TTAGCAGAGCTGTGTCAGATGGCT	GGGCATCAACCCAGACAACACAAA
Human *CAT*	ACAGCAAACCGCACGCTA	CACGGGGCCCTACTGTAATAA

GAPDH: glyceraldehyde-3-phosphate dehydrogenase, *ENOS*: endothelial nitric oxide (NO) synthase, *INOS*: inducible NO synthase, *ARGII*: arginase II, and *CAT*: catalase.

The cells were incubated for additional 24 hours with the patients' serum. The experiments were performed in duplicate. When used, infliximab (400 μg/mL) was added to the cells concomitantly with human serum.

2.6. Flow Cytometry Analysis of DNA Fragmentation and Reactive Oxygen Species (ROS) Content. The medium was discarded and adherent cells were washed with Krebs buffer. The cells were detached using a cell scraper. Cell suspensions were divided into two aliquots used for measurement of DNA fragmentation and ROS content.

For DNA fragmentation measurements, cells were centrifuged (2,000 ×g, 4°C for 10 min) and the pellets were resuspended in 250 μL of DNA fragmentation buffer (PBS containing 0.1% Triton X-100, 8 μg/mL propidium iodide, and 10 mg/mL sodium citrate) and incubated overnight at 4°C. After the incubation, samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Juan, PR, USA). Fluorescence of 10,000 events was acquired in the FL2 channel and analyzed using the CellQuest software. The cells with fragmented DNA emitted lower fluorescence signal, compared to cells with intact diploid and tetraploid DNA, which emitted characteristic two-peaked high-intensity fluorescence. The percentage of apoptotic cells was calculated using the number of low fluorescence events.

For ROS content measurements, cells were incubated with 1 mL of Krebs buffer containing 5 mM 2,7-dichlorodihydrofluorescein diacetate (DCFH) (Sigma, catalog number: D6883) for 30 min at room temperature. After incubation, samples were analyzed using a FACSCalibur flow cytometer. Fluorescence of 10,000 events was acquired in the FL1 channel and analyzed using the CellQuest Pro software. Mean fluorescence was obtained from a M1 population defined in the histograms. The limits for the M1 population were set based on the fluorescence of the unstained cells.

2.7. Real-Time Reverse Transcription Polymerase Chain Reaction. The total RNA was extracted from 25 cm^2 tissue culture flasks using QIAzolLysis Reagent (Qiagen, Germany) according to manufacturer's instructions. The concentration and purity of the isolated RNA were determined using UV spectrophotometry (NanoDrop, Thermo Scientific, Waltham, MA, USA). The integrity of the RNA was verified using agarose gel electrophoresis stained with ethidium bromide. Reverse transcription was performed using TaqMan Reverse Transcription Reagents (Life Technologies, Carlsbad, CA, USA) using 1 μg of total RNA. Real-time RT-PCR was performed using SYBR Green (Applied Biosystems, ONT, Canada, 367659). Specific sequences of the primers for endothelial NO synthase (*ENOS*), inducible NO synthase (*INOS*), catalase (*CAT*), arginase II (*ARGII*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Exxtend Biotecnologia Ltda., Sao Paulo, Brazil) as a housekeeping gene control are shown in Table 1. The primers were designed to comprise at least one intron to minimize the noise due to genomic contamination. The PCR parameters were an initial denaturation (one cycle at 95°C for 10 min), denaturation and annealing/amplification at 95°C for 10 s and 60°C for 30 s, respectively, for 40 cycles, and a melting curve, 72°C, with the temperature gradually increasing (0.5°C) to 95°C.

2.8. Statistical Analysis. Continuous variables were expressed as mean and standard deviation (SD). Normality of distribution was assessed by Shapiro Wilk test. Mann-Whitney test was used to compare clinical data in 2 groups, while 2-tailed unpaired *t*-test was used to compare apoptosis and ROS content between 2 groups. Comparisons among more than 2 groups were performed using 1-way ANOVA with Tukey's post hoc test. Categorical data were presented in percentages and compared by Fisher's exact test. Spearman correlation was performed between TNF-α and PWV. The level of significance (α) accepted was less than 0.05.

3. Results

3.1. Characteristics of Study Participants. General patients' characteristics are shown in Table 2. Normotensive and RHTN groups showed similar characteristics such as age, gender, body mass index, and systolic, diastolic, and pulse pressure. Resistant hypertensive patients showed higher levels of TNF-α compared to normotensive subjects (3.3 versus 2.1 pg/mL), as well as higher PWV (10.5 versus 7.2 m/s), represented in Figure 1. Statistically significant correlation was found between TNF-α levels and PWV measures in the whole group (Figure 1).

3.2. Effect of Infliximab on Endothelial Cell Apoptosis and Reactive Oxygen Species (ROS) Production. HUVECs incubated with serum from RHTN showed increased cell apoptosis (4.43 ± 1.9 versus 7.28 ± 1.5%; $p = 0.02$) and higher reactive oxygen species (ROS) content (987 ± 181 versus 1231 ± 127 mean fluorescence values; $p = 0.01$), compared to normotensive subjects (Figure 2). The treatment with infliximab attenuated the apoptosis of HUVECs incubated

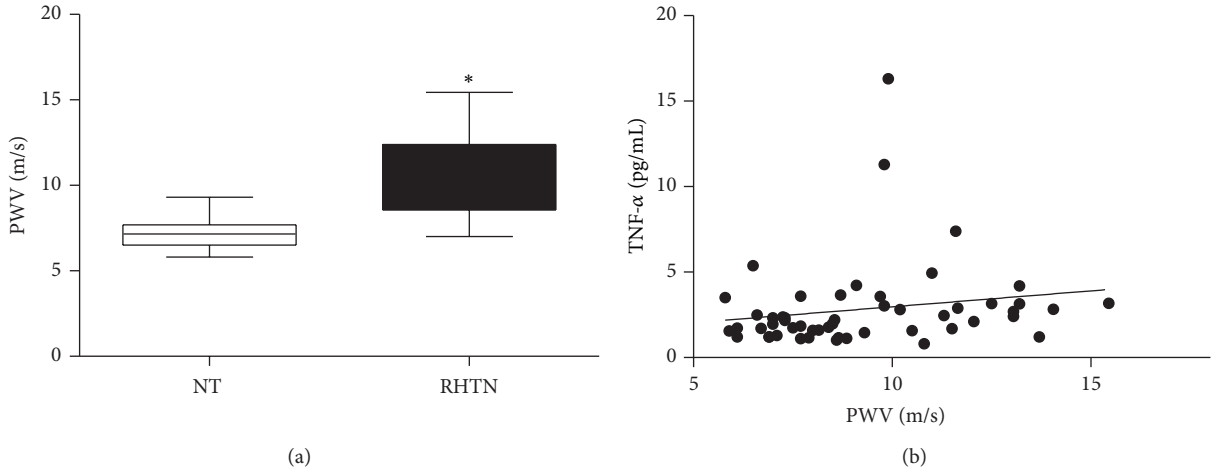

Figure 1: (a) Pulse wave velocity (PWV) in normotensive subjects (NT) compared to resistant hypertensive patients (RHTN) ($^*p < 0.05$ versus NT). (b) Correlation between TNF-α levels and PWV in the whole group ($n = 51$; $r = 0.31$; $p = 0.02$).

Table 2: General characteristics of normotensive and resistant hypertensive subjects.

	NT ($n = 19$)	RHTN ($n = 32$)	p value
Age (years)	52 ± 5.0	57 ± 13	0.06
Gender (F/M)	9/10	19/13	0.56
BMI (kg/m^2)	25.1 ± 2.3	26.3 ± 2.9	0.14
Office SBP (mmHg)	121 ± 14	146 ± 16*	<0.0001
Office DBP (mmHg)	78 ± 8	86 ± 14*	0.04
Office PP (mmHg)	43 ± 9	61 ± 13*	<0.0001
PWV (m/s)	7.2 ± 1.0	10.5 ± 2.2*	<0.001
TNF-α (pg/mL)	2.1 ± 1.2	3.3 ± 3.1*	0.04

Mean ± SD. NT: normotensive subjects; RHTN: resistant hypertensive patients; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; PP: pulse pressure; PWV: pulse wave velocity; TNF-α: tumoral necrosis factor-α. $^*p < 0.05$ versus NT.

with serum from RHTN (7.28 ± 1.5 versus 5.97 ± 1.39%; $p = 0.04$) but exerted no effect on ROS production. Although we observed a tendency pointing to an increase, infliximab did not change the apoptosis of HUVECs incubated with normotensive serum (4.43 ± 1.9 versus 9.66 ± 9.95; $p = 0.13$) (Figure 3).

3.3. Gene Expression Profile in HUVECs Incubated with Serum from Normotensive and Resistant Hypertensive Subjects. Normalized expression of the genes *ENOS*, *INOS*, *CAT*, and *ARGII* was shown in Figure 4. While no differences in *ENOS* expression were found between cells incubated with NT or RHTN serum, we found increased *ENOS* expression after treatment with infliximab only in the normotensive group (Figure 4(a)). No differences in *INOS* and *CAT* expression were observed among the groups. However, arginase II (*ARGII*) expression was lower in HUVECs incubated with serum of resistant hypertensive patients compared to normotensive serum, but no effect of infliximab was found in both groups.

4. Discussion

The present study demonstrated that resistant hypertensive subjects have higher arterial stiffness and increased TNF-α plasma levels compared to normotensive subjects. Moreover, TNF-α levels were positively correlated with carotid-femoral PWV. We also found that endothelial cells incubated with RHTN serum showed higher apoptosis rate than cells incubated with serum from normotensive subjects. The treatment with TNF-α inhibitor reduced apoptosis induced by RHTN serum. The inhibition of TNF-α increased the expression of *ENOS* in the cells incubated with normotensive serum, but no changes in *INOS*, *CAT*, and *ARGII* expression were observed. In addition, the expression of *ARGII* decreased in cells incubated with RHTN compared to NT serum.

The contributions of immune system to cardiovascular damage have been largely investigated. The lack of immune cells prevents vascular damage and the development of hypertension on several animal models of hypertension as angiotensin II infusion and DOCA-salt [3, 25]. Taken together, these findings suggest that the inhibition of inflammatory pathways may be beneficial for vascular damage prevention and treatment of hypertension. Recent study from our laboratory found that TNF-α inhibition reduced systolic BP and left ventricular hypertrophy and activated AKT/eNOS pathway, improving vascular function in hypertensive rats [6]. Indeed, higher levels of TNF-α and increased arterial stiffness were found in hypertensive subjects compared with normotensive subjects [19]. Also, arterial stiffness and TNF-α were positively correlated in hypertensive patients [16]. Previous studies reported that TNF-α plasma levels are associated with elevated blood pressure in apparently healthy subjects [1, 2].

Since RHTN patients have increased arterial stiffness and positive correlation with TNF-α levels, we investigated

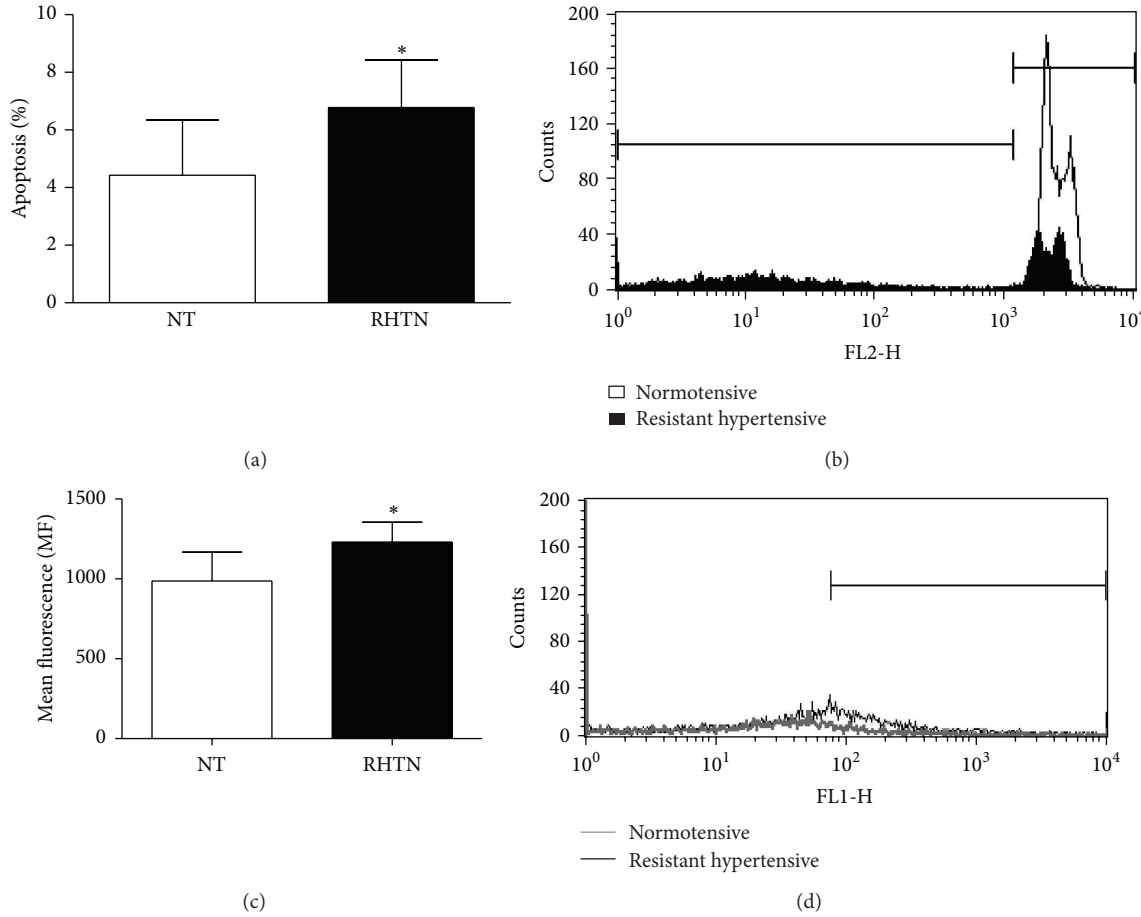

Figure 2: Percentage of apoptosis (a) and representative histograms overlay depicting fluorescence at FL2 (b) of HUVECs incubated with DNA fragmentation buffer and previously treated with serum from normotensive individuals (NT, $n = 8$) or resistant hypertensive patients (RHTN, $n = 8$). Mean ROS content (c) and representative histograms overlay depicting fluorescence at FL1 (d) from HUVECs incubated with DHCF and previously treated with serum from NT ($n = 8$) or RHTN ($n = 8$) ($^* p < 0.05$ versus NT). Mean ± SD. The experiments were performed in duplicate.

the effects of RHTN serum on endothelial cells in culture, by measuring apoptosis percentage and ROS content and whether those effects were mediated by TNF-α. We found that serum from RHTN promotes endothelial cells apoptosis and increases ROS content. We found reduction in cell apoptosis after the treatment with TNF-α inhibitor, but no changes in ROS content. Corroborating with these findings, we did not find changes in catalase gene expression after TNF-α inhibition. Our findings suggest that TNF-α may participate in apoptosis of endothelial cells but seems to not affect oxygen species.

TNF-α was also shown to stimulate endothelial microparticles (EMPs) releasing, which is a marker of endothelial dysfunction, and reactive oxygen species (ROS) production, which suggest that ROS are important mediators of TNF pathway [26]. Both higher ROS production and EMP releasing were associated with apoptosis. These findings are consistent with higher ROS content in HUVECs incubated with resistant hypertensive serum. Accordingly, we previously demonstrated that isoprostane levels, an oxidative stress marker, were associated with endothelial dysfunction in RHTN patients [27]. However, as we did not observe reduction in ROS content with TNF-α inhibition, other factors present in RHTN serum may be stimulating ROS formation.

The endothelium represents the main regulator of wall homeostasis through releasing of several molecules such as nitric oxide (NO) that is continuously produced by healthy endothelial cells. L-arginine is converted into NO by endothelial NO synthase. Also, NO can be produced in response to immunological stimuli through inducible NO synthase (iNOS). Interestingly, HUVECs incubated with RHTN serum had tendency of higher expression of eNOS compared to cells treated with NT serum, irrespective of the increased endothelial dysfunction found in those patients [9, 27]. Moreover, L-arginine is also a substrate for arginase, an enzyme expressed in the endothelium; TNF-α upregulates the expression of arginase in ECs, which decreases L-arginine availability to eNOS and leads to O^{-2} production. ROS production impairs vasodilatation mediated by NO [20, 28]. Surprisingly, we observed decreased arginase II (ARGII) expression in cells treated with RHTN serum compared to cells incubated with NT serum. Taken together, ECs treated with RHTN

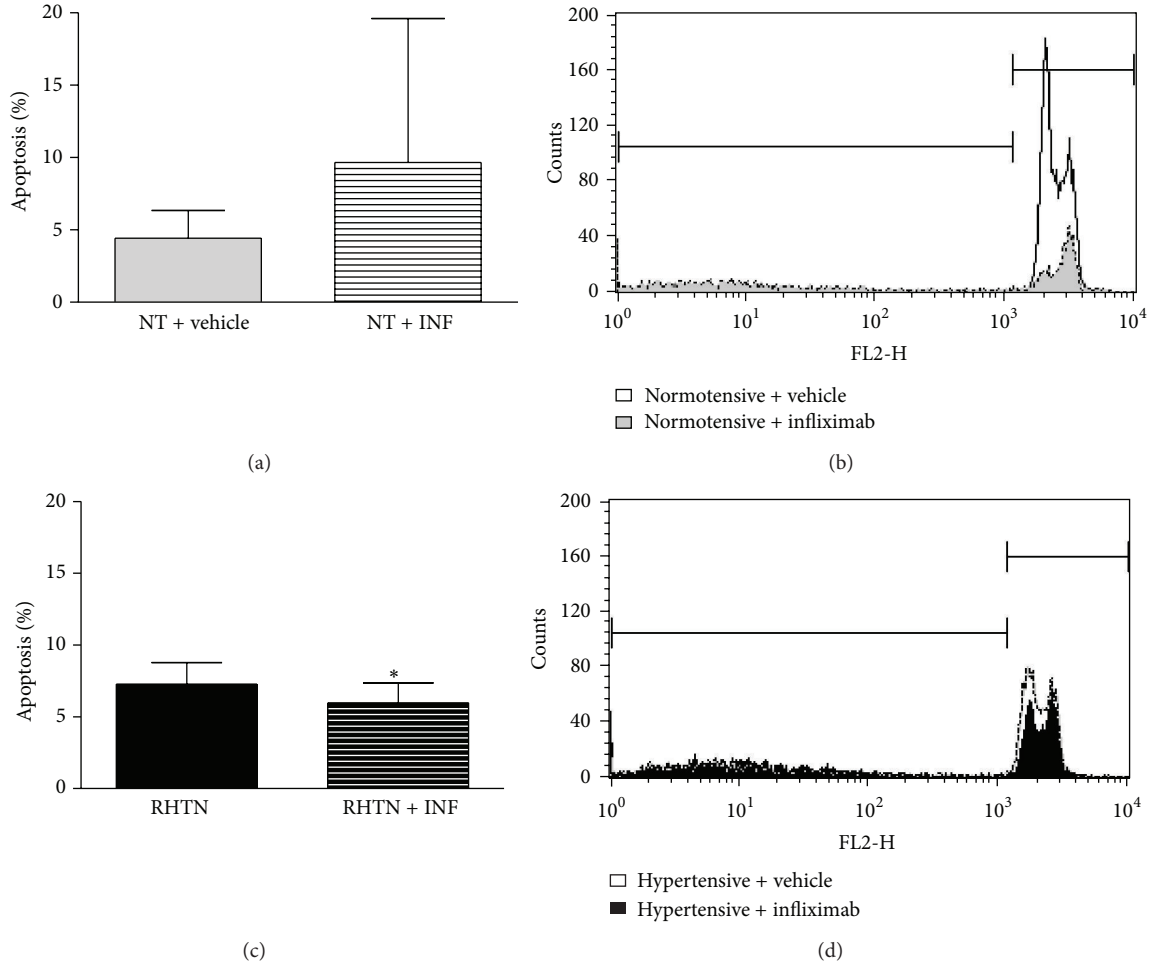

Figure 3: Percentage of apoptotic cells (cells with fragmented DNA) (a) and representative histograms overlay depicting fluorescence at FL2 (b) of HUVECs incubated with DNA fragmentation buffer and previously treated with serum from normotensive individuals (NT, $n = 8$) added with vehicle or infliximab. Percentage of apoptotic cells (cells with fragmented DNA) (c) and representative histograms overlay depicting fluorescence at FL2 (d) of HUVECs incubated with DNA fragmentation buffer and previously treated with serum from hypertensive patients (RHTN, $n = 8$) added with vehicle or infliximab ($^* p < 0.05$ versus vehicle). Mean ± SD. The experiments were performed in duplicate.

serum showed higher eNOS expression concomitantly with decreased ARGII expression. This enzymatic pattern would favor NO bioavailability and vasodilation in this *in vitro* system. Therefore, we may speculate that serum from RHTN has substances able to stimulate NO production in our *in vitro* system (functional endothelial cells) but endothelial cells of the patients may not respond to these stimuli *in vivo*, explaining the presence of endothelial dysfunction in these patients [9]. We also observed that the treatment with infliximab in cells incubated with serum from normotensive subjects displayed increased eNOS expression, suggesting that TNF-α might impair NO synthesis in ECs. Indeed, animal study with estrogen-deficient rats treated with anti-TNF-α had increased expression of tissue eNOS [29]. TNF-α has a crucial role in expression of eNOS suppressing eNOS mRNA and protein levels by decreasing mRNA stability [30].

On the other hand, we do not discard the possibility that TNF-α may modulate other NO synthases, as inducible NO synthase (iNOS), promoting nitrosative stress and endothelial dysfunction [31]; however, we found no statistical differences in iNOS expression between the groups or after infliximab treatment. We observed a large variability in HUVECs gene expression incubated with serum from different hypertensive subjects, which may reflect the differences between patients including severity and time of hypertension, presence of comorbidities, and differences in drug regimens.

The treatment of inflammatory disease (anti-neutrophil cytoplasmic antibody-associated systemic vasculitis) with infliximab improved endothelial function [32]; thus, TNF-α inhibitors have an emerging role in prevention of vascular dysfunction. Furthermore, arterial stiffness was improved in patients with rheumatoid arthritis treated with TNF-α inhibitors [21]. Further studies are needed to elucidate the mechanisms of the proinflammatory cytokine TNF-α on arterial stiffness. In conclusion, serum TNF-α in resistant hypertensive subjects induces apoptosis in human endothelial cells. These results suggest that TNF-α might mediate, at least in part, vascular injury in resistant hypertension. Finally,

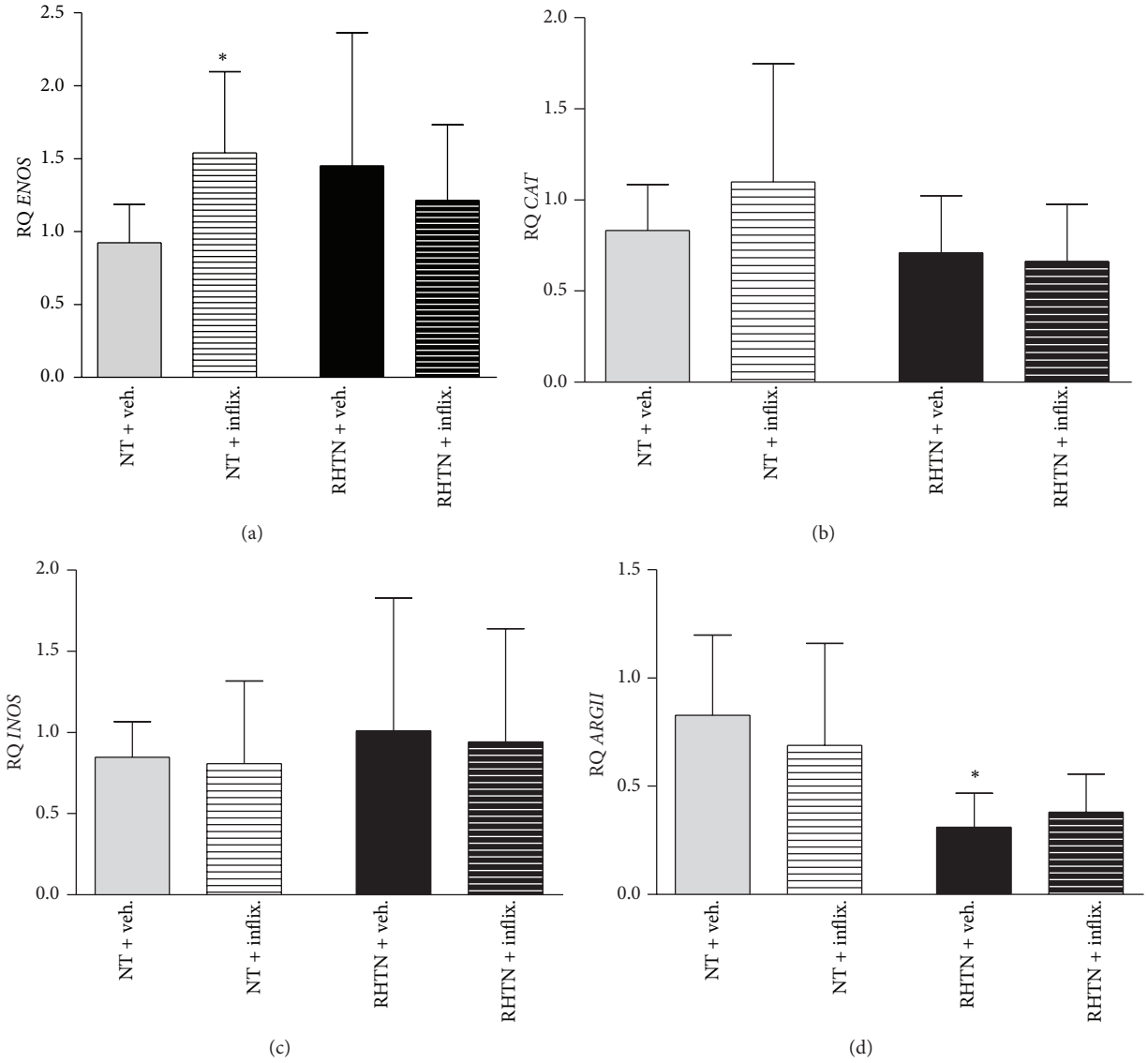

Figure 4: Gene expression of serum-treated HUVECs. Expression of genes normalized for GAPDH was investigated in human umbilical vein endothelial cells stimulated with plasma obtained from normotensive subjects (NT, $n = 8$) or resistant hypertensive patients (RHTN, $n = 8$) and treated with vehicle or infliximab. (a) Gene expression of endothelial nitric oxide synthase (*ENOS*). (b) Gene expression of catalase (*CAT*). (c) Gene expression of inducible nitric oxide synthase (*INOS*). (d) Gene expression of arginase II (*ARGII*) (mean ± SD, $^*p < 0.05$ versus NT vehicle). The experiments were performed in duplicate.

future clinical trials with TNF-α inhibitors in these patients may represent a field of interest.

Conflict of Interests

The authors have no conflict of interests to declare.

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Research Article

Presence of Periodontopathic Bacteria DNA in Atheromatous Plaques from Coronary and Carotid Arteries

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Objectives. Interest in periodontitis as a potential risk factor for atherosclerosis and its complications resulted from the fact that the global prevalence of periodontal diseases is significant and periodontitis may induce a chronic inflammatory response. Many studies have analyzed the potential impact of the *Porphyromonas gingivalis*, major pathogen of periodontitis, on general health. The purpose of this study was to find the presence of the *Porphyromonas gingivalis* DNA in the atherosclerotic plaques of coronary and carotid arteries and in the periodontal pockets in patients with chronic periodontitis, who underwent surgery because of vascular diseases. *Methods and Results*. The study population consisted of 91 patients with coronary artery disease or scheduled for carotid endarterectomy. The presence of *Porphyromonas gingivalis* DNA in atheromatous plaques and in subgingival samples was determined by PCR. Bacterial DNA was found in 21 of 91 (23%) samples taken from vessels and in 47 of 63 (74.6%) samples from periodontal pockets. *Conclusions*. *Porphyromonas gingivalis* DNA is frequently found in atheromatous plaques of patients with periodontitis. That is why more research should be conducted to prove if this periopathogen may have an impact on endothelium of patients at risk of atherosclerosis.

1. Introduction

Research over the past two decades has suggested that, beyond the conventional risk factors leading to the development and progression of atherosclerotic plaques, the condition may also result from microorganisms, increased levels of fibrinogen, the level of the white blood cell count, C-reactive proteins, and antibodies directed against heat shock proteins HSP 60.

On this basis, the immunoinflammatory theory of atherosclerosis was established, according to which atherosclerosis is a chronic immunofibroproliferative inflammatory response to factors that damage vascular endothelial cells [1]. Significant evidence proving the participation of chronic inflammation in the pathogenesis of atherosclerosis and the destabilization of existing atheromatous plaques in the arteries has led many researchers to focus their attention on searching for the cause of the inflammation. Many bacterial species have been suspected of playing an important (potential) role in atheroma development. The presence of the DNA of several microorganisms in atherosclerotic plaques, for example, *Chlamydia pneumoniae*, *Helicobacter pylori*, *Cytomegalovirus (CMV)*, and HSV, has been reported [2–5].

Interest in periodontitis as a potential risk factor for atherosclerosis and its complications resulted from the fact

Table 1: Characteristics of groups.

Characteristic	Group A ($N = 32$) Patients with coronary artery disease	Group B ($N = 31$) Patients scheduled for carotid endarterectomy	Group C ($N = 28$) Patients edentulous
Age (years)	59.3 (SD = 7.36)	63.9 (SD = 9.31)	65.1 (SD = ±7.2)
Gender (male/female)	31/1	24/7	23/5
Smoking (current and former smokers who stopped <5 years before entering the study)	15	19	17

that the global prevalence of periodontal diseases is extremely high and moreover, periodontitis may induce a chronic inflammatory response. Mild forms of periodontal diseases, including gingivitis, affect up to 70% of the general population, whereas approximately 10% to 15% of the population have a more severe process with destruction of the tooth-supporting tissues [6]. Many studies since 1989 have analyzed the potential impact of the *Porphyromonas gingivalis*, major pathogen of periodontitis, on general health and especially cardiovascular problems [7, 8]. Epidemiological studies have focused on proving this relationship. In patients suffering from periodontitis with damaged periodontal pocket epithelium, transient bacteremia not only occurs during and after medical procedures such as scaling and root planning, but also may be caused by toothbrushing and even simple mastication [9].

Some data show that periodontitis is significantly associated with biomarkers of endothelial dysfunction and dyslipidemia [10]. Other authors show that *Porphyromonas gingivalis* induces foam cell formation in murine macrophage cell cultures in the presence of LDL [11].

Experimental evidence has been provided in animal models. Li et al. [12] investigated the effect of repeated systemic inoculations with *Porphyromonas gingivalis* on the progression of atherosclerosis in heterozygous apolipoprotein E-deficient (ApoE(+/−)) mice. Lalla et al. [13] assessed the impact of oral inoculation with the *Porphyromonas gingivalis* on atherogenesis in hypercholesterolemic apolipoprotein E-null mice. Both authors concluded that long-term challenge with *Porphyromonas gingivalis* can accelerate atherogenic plaque progression.

Action of *P. gingivalis* is mediated through many virulence factors, such as gingipains, hemagglutinins, fimbriae, and lipopolysaccharides (Pg-LPS). Therefore it not only becomes destructive for periodontal tissues, but also can induce and enhance general inflammation [14].

The purpose of the present study was to assess the status of periodontal tissues in patients scheduled for surgery due to atherosclerosis and its complications. Also the presence of the DNA of the *Porphyromonas gingivalis*, the bacteria intimately related to periodontitis in the periodontal pockets as well as in the atherosclerotic plaques of coronary and carotid arteries in patients with chronic periodontitis, who were hospitalized and underwent surgery because of vascular diseases, was demonstrated.

2. Material and Methods

The study population consisted of 91 patients (78 men and 13 women) treated in the Clinic of Cardiac Surgery or in the Clinic of Vascular, General and Transplantation Surgery, Wroctaw Medical University.

Group A consisted of 32 patients from 44 to 74 years of age with coronary artery disease and group B consisted of 31 patients from 46 to 84 years of age scheduled for carotid endarterectomy. Chronic periodontitis had been diagnosed in all these patients.

Group C was made up of 28 patients from 50 to 77 years treated in both the abovementioned clinics, who had been edentulous for at least 2 years before examination (Table 1).

All participants were fully informed about the procedures and informed consent was obtained from all patients.

The study protocol was approved by the ethical committee of Wroctaw Medical University.

Periodontal examination was performed by one trained and calibrated periodontist [MS]. Measurements of the approximal plaque index (API), bleeding on probing index (%BOP), and periodontal pocket depth (PD) at six sites per tooth using a manual, UNC-15 periodontal probe were subsequently recorded. Moderate periodontitis was diagnosed if at least one pocket ≥5 mm was present and severe periodontitis when at least one lesion ≥7 mm was found [15].

Bacteriological samples were collected from the 3 deepest periodontal pockets of each dentate patient. After drying the site and isolation from saliva, a sterile paper point was inserted into the pocket for 10 s, then transferred to a sterile Eppendorf tube, and sent to the laboratory.

The surgical procedures for carotid endarterectomy and coronary artery bypass graft surgery (CABG) were performed 1 week after periodontal examination. Atheromatous plaques from patients with carotid arteries stenosis were harvested during the surgery and were placed in a sterile tube with 10 mL of saline solution and frozen at −20°C.

In patients operated on with CABG there was no possibility to harvest atheromatous plaques, so during the surgery sterile paper points were inserted into the coronary vessel for 10 s and then immediately placed in sterile tubes and frozen at −20°C [16].

The laboratory tests were carried out in the Department of Forensic Medicine, Molecular Techniques Unit, Wroctaw Medical University.

For DNA extraction, 100 mg of atherosclerotic plaque was homogenized in 680 μL 1x TEN buffer (0.1 M NaCl, 10 mM Tris-HCl pH 8.0, and 1 mM EDTA pH 8.0) using a bead beater at maximum speed for 20 s in three series. Then, the homogenized material was incubated for 16 hours at 55°C with 0.25 mg/mL proteinase K and 1.25% SDS. For purification of DNA, 1 mL of phenol : chloroform : isoamyl alcohol (25 : 24 : 1) was added and after centrifugation the aqueous phase was collected. DNA was precipitated with 99.8% ethanol and centrifuged. The DNA pellet was washed with 70% ethanol, dried, and dissolved in 100 μL water.

In the case of swabs from periodontal pockets (groups A and B) and coronary arteries, DNA extraction was replaced by alkaline lysis with 0.2 M NaOH. After incubation for 5 min at 75°C, the samples were neutralized with 0.04 M Tris-HCl, pH 7.5. Solutions thus prepared were used as templates in PCR.

For detection of *Porphyromonas gingivalis* DNA specific primers were used as described by Slots et al. [17]: forward primer 5′-aggcagcttgccatactgcg and reverse primer 5′actgttagcaactaccgatgt. Amplification reactions were carried out in 25 μL reactions with 0.2 μM forward and reverse primers, 0.2 mM of each dNTPs, 2U DFS-Taq DNA polymerase (BIORON), and ≈50 ng template DNA. The thermal profile consisted of initial denaturation at 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 10 s, annealing at 59°C for 20 s, and extension at 72°C for 1 min. The amplicons were visualized by electrophoresis using 1% agarose gel with 0.5 μg/mL ethidium bromide. A Gene Ruler 100 bp DNA Ladder (Fermentas) was used as a molecular size standard. As a negative control water and NAOH/TRIS-HCl buffer were used. In case of positive control, we based on the reference strain of *Porphyromonas gingivalis* W83 after lysis and neutralisation procedures or DNA isolated from it.

Results were expressed as mean ± standard deviation (SD) for quantitative variables. The gathered data was analyzed statistically with Fisher's exact test.

3. Results and Discussion

The mean number of teeth in patients from group A was 14.1 and from group B was 12.7. All patients had poor oral hygiene (API value within the range 46–100%), and the mean value of this index in A and B groups was 88.6% and 87.7%, respectively. Despite the low level of oral hygiene, the mean bleeding index (BOP) was 38.5% in group A and 30.4% in patients from group B. The mean pocket probing depth was 3.36 mm in group A patients and 3.28 mm in group B (Table 2).

The DNA of *Porphyromonas gingivalis* was detected in 28 samples from periodontal pockets in group A patients (87.5%) and in 19 patients in group B (61.3%). There was no statistically significant association between these findings. Comparison between these two test groups of patients showed a difference in the prevalence of *Porphyromonas gingivalis* DNA in samples taken from vessels. Only 3 samples from group A were positive (9.4%), whereas DNA of *Porphyromonas gingivalis* was detected in 15 atheromatous plaques from patients from group B (48.4%). In group C consisting of edentulous patients, *Porphyromonas gingivalis* DNA was isolated in three samples (2 from patients with carotid artery stenosis and 1 treated because of the reduced permeability of coronary arteries) (Table 3). There was no relationship between the presence of the DNA of the test bacteria in periodontal pockets and atheromatous plaques/swabs of the blood vessels.

Table 2: Mean values ± SD of periodontal indices in groups A and B.

Variable	Group A (N = 32) Patients with coronary artery disease	Group B (N = 31) Patients scheduled for carotid endarterectomy
Number of teeth	14.1 (SD = 5.8)	12.7 (SD = 6.1)
Mean API*	88.6% (SD = 13.9)	87.7% (SD = 14.1)
Mean BOP**	38.5% (SD = 14.4)	30.4% (SD = 19.8)
Mean probing depth	3.36 mm (SD = 0.77)	3.28 mm (SD = 0.93)

*API: approximal plaque index.
**BOP: bleeding on probing.

Currently, atherosclerosis is considered to be an inflammatory disease and not just a disease resulting from the accumulation of lipids in the vessel wall. An important element in prevention of atherosclerosis and its complications is an understanding of pathomechanisms leading to blood vessel wall damage. *Porphyromonas gingivalis*, the microorganism closely connected with chronic periodontitis, should be considered as one of the possible causes of eliciting general inflammation. With the use of PCR in several studies *Porphyromonas gingivalis* DNA was detected in atherosclerotic plaques of patients with chronic periodontitis. Toyofuku et al. [18] checked for the presence of bacterial DNA in samples obtained from 53 atherosclerosis patients. *Porphyromonas gingivalis* DNA was detected in 52% of arterial samples. Marcelino et al. [19] collected and analyzed DNA of periodontal pathogens in atheromatous plaques from 28 patients. Samples were positive for all bacteria except for *Fusobacterium nucleatum*. *Porphyromonas gingivalis* DNA was present in 50% of samples. Ishihara et al. [20] obtained samples of atheromatous plaques from the coronary arteries of 51 patients. As in previous studies, the PCR method was used. In 21.6% of the samples the DNA of *Porphyromonas gingivalis* was present. Similar results were published by Mahendra et al. [21], who examined 51 samples of atheromas from the coronary arteries of patients with chronic periodontitis. *Porphyromonas gingivalis* DNA was detected in 45.1% of samples. Equally high positive results (53.8%) for the presence of *Porphyromonas gingivalis* DNA have been obtained by Brazilian scientists. They evaluated atherosclerotic coronary arteries of 39 patients with chronic periodontitis [22].

The results of present study confirm the findings of the cited authors. *Porphyromonas gingivalis* DNA was detected in 21 samples from vessels. The majority of positive results (n = 15) originated from patients with carotid atherosclerosis from whom atherosclerotic plaques were taken to be tested.

Table 3: Positive results of polymerase chain reaction detection in samples from vessels and from periodontal pockets.

	Group A ($N = 32$) Patients with coronary artery disease	Group B ($N = 31$) Patients scheduled for carotid endarterectomy	Group C ($N = 28$) Patients edentulous
Periodontal pockets	28	19	NA
Atheromatous plaques/smear from coronary arteries	3	15	3

Since in patients operated on because of coronary atherosclerosis there was no possibility to harvest atheromatous plaques due to operation protocol, sterile paper points were tested after a 10-second contact with the vessel plaque. The percentage of positive results in this group was significantly lower and amounted to 9.4% (3 individuals). It seems that such a significant discrepancy in the detection of bacterial DNA may result from differences in the method of material collection, as *Porphyromonas gingivalis* has the ability to penetrate into the endothelial cells. Thus, there is a much higher probability of isolation of this pathogen from the complete atherosclerotic plaque. By contrast, despite the presence of these bacteria in endothelial cells, detection of their DNA in the filtrate obtained from the paper point contact with stable plaque covered with a layer of fiber may be much more difficult or not possible. However, it is interesting to note that in other published studies using a similar method a higher percentage of positive results was achieved. Zaremba et al. [16] checked samples from 20 patients with chronic periodontitis who were scheduled for coronary artery bypass grafting (CABG) because of coronary artery obstruction. They reported the presence of *Porphyromonas gingivalis* DNA in the filtrate of atherosclerotic plaques in 10 cases, which accounted for 50% of patients.

In contrast to the present study and the results of other authors cited above, Cairo et al. [23], when examining 40 samples of atherosclerotic plaques (obtained after carotid endarterectomy) by PCR, did not detect the presence of any periodontal pathogenic bacteria. Aimetti et al. [24] did not isolate any periopathogens in samples taken from atherosclerotic carotid arteries of patients with periodontitis. Padilla et al. [25] detected the DNA of *Aggregatibacter actinomycetemcomitans* (the authors checked the presence of four periopathogens) in only 2 out of 12 atherosclerotic plaques from patients who had periodontitis diagnosed and were operated on because of artery stenosis (carotid, popliteal, tibial, and femoral arteries). These authors suggest that such discrepancies in the results from different studies may be associated with the varying methods of laboratory analysis. In studies using a PCR differences may result from various methods of DNA extraction, use of unlike sequences of primers, and different reaction conditions. An example of such situation is a multicenter PCR comparison trial published by Apfalter et al. [26], who sent the same atheromatous plaques to nine different laboratories for the detection of *Chlamydia pneumoniae* DNA. Depending on the different test methods the positivity rate varied between 0 and 60%. These variations resulted from the different conditions and testing methods, since laboratories were free to choose the method.

Kozarov et al. [27] assumed that the presence of bacterial DNA in vessel walls does not prove the presence of live bacteria which are able to invade cells and to induce inflammation. Therefore, they conducted a study using quantitative PCR and then they stained the examined tissue. In samples of atheromatous plaques live bacteria *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* were present inside the endothelial cells. This study proved for the first time that detected bacterial DNA is derived from live bacterial cells, because only live microorganisms are able to penetrate into cells other than phagocytes [28].

The question also arises as to whether there is a correlation between the presence of *Porphyromonas gingivalis* in periodontal pockets and in the atherosclerotic plaques. In the present study there was no such relationship. In groups of dentate patients (A + B) the simultaneous presence of bacterial DNA in the samples from the periodontal pockets and in samples taken from blood vessels was confirmed in 19%, while in group B alone in 29%. Nevertheless, there was no statistically significant relationship. Similar results were published by Zhang et al. [29], who detected the simultaneous presence of *Porphyromonas gingivalis* DNA in the atheromatous plaques and periodontal pockets of 10 out of 51 patients (19.6%). In a group of 20 patients Zaremba et al. [16] marked the presence of *Porphyromonas gingivalis* DNA in both locations (periodontal pockets and the filtrate from atherosclerotic coronary arteries) in 5 patients (25%). However, in the studies conducted by Mahendra et al. [21] the rate was 39.22%. Ishihara et al. [20] reported the presence of *Porphyromonas gingivalis* 16S rRNA in samples of coronary atherosclerotic plaques obtained from 51 patients, and this was significantly positively correlated with the presence of this microorganism in periodontal pockets ($P < 0.01$). The fact that bacterial DNA is rarely isolated from atherosclerotic plaques compared to periodontal pockets and that usually there is no positive correlation between the simultaneous occurrences of periopathogens in both study locations is explained by the authors as being a result of the influence of the organism's defense system to the elimination of these bacteria, either through the cells of the first line of defense or infiltration of granulocytes, as well as a specific defense by running the humoral and cellular responses. This is not a convenient environment for their growth and reproduction. What is more in 3 cases *Porphyromonas gingivalis* DNA was present in samples from edentulous patients. It is worth emphasizing that edentulous patients had no periodontium, so there have been no periopathogens present in oral cavity for at least 24 preceding months.

In our study, in 6 patients no DNA of *Porphyromonas gingivalis* was detected in periodontal pockets but it was

determined in samples from blood vessels. Such a situation can be explained by the fact that in chronic periodontitis short periods of exacerbation of inflammation and prolonged periods of remission are observed. In addition, most patients in both study groups were elderly and they had lost some teeth (the "elderly" is defined as ≥65 years according to the contractual age limit adopted by most developed countries). As one of the main causes of tooth loss patients reported "mobility" of the teeth which can be linked to an advanced ongoing inflammatory-destructive state in periodontal tissues. It is likely that in deep periodontal pockets of teeth with increased mobility the bacteria of "red complex," including *Porphyromonas gingivalis*, were present. During this period there may have been an invasion of bacteria into the circulatory system; then the teeth were lost. Periodontal pockets of the teeth present in the oral cavity, from which samples for testing were taken, may be shallower and therefore not colonized by the most dangerous strains of strictly anaerobic bacteria.

4. Conclusions

In summary, we can conclude that it is possible that recognized periodontal pathogens present in subgingival oral biofilm may find a way to reach arteries, so ongoing periodontitis may be one of the factors affecting patients at risk of atherosclerosis and related diseases. For this reason, periodontal care should be treated as a necessity for all patients at risk of vascular and cardiac disorders.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Prevalence, Risk Factors, and Genetic Traits in Metabolically Healthy and Unhealthy Obese Individuals

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Objective. To assess prevalence of metabolically healthy individuals among patients with abdominal obesity (AO) and to determine phenotype and potential genetic traits associated with a benign metabolic status. *Methods*. 503 AO patients without cardiovascular diseases were examined. Waist circumference (WC), BMI, blood pressure, plasma glucose and serum insulin levels, HOMA-IR, lipid profile, and adiponectin (AN) and leptin (LEP) concentrations in serum were measured. Polymorphisms A19G and Q223R of the LEP and LEP receptor gene, and G276T and T45G of the AN gene were investigated. *Results*. 91.3% of patients were metabolically unhealthy obese (MUO), and 8.7% metabolically healthy obese (MHO). MHO patients were younger, and had lesser BMI and WC, while duration of obesity, frequency, and duration of physical training were greater than MUO patients ($p < 0.05$). In MHO and MUO patients distribution of the G19G, G19A, and A19A genotypes of the LEP gene and G276G, G276T, and T276T genotypes of AN gene did not differ. The T45T genotype was associated with increase of metabolic disorders' risk for patients with AO (OR = 2.331; 95% CI = 1.121 ÷ 5.132). *Conclusions*. Prevalence of MHO individuals among patients with AO is low. Benign metabolic status was associated with younger age, lower waist circumference, and higher physical activity, shorter duration of obesity, and G45G adiponectin genotype carriage.

1. Introduction

Abdominal obesity (AO) is one of the major risk factors for cardiovascular disease (CVD) and its complications [1]. On the one hand, this is thought to be the result of increasing prevalence of obesity in all age groups all over the world while, on the other hand, there is a distinct correlation between AO and early CVD and its complications, especially in patients with metabolic syndrome (MetS) [2, 3]. At the same time in a number of population studies, it has been shown that, despite AO, some subjects retained their insulin sensitivity and had normal lipid and glucose levels and blood pressure and cytokine profile and, accordingly, their risk of developing type 2 diabetes mellitus (T2DM) and CVD was rather low [4–7]. Such a subpopulation of obese individuals is usually called "metabolically healthy obese" (MHO). Data of different authors showed that the prevalence of this phenomenon in the population of obese individuals varies widely from 6.0% to 38.4%. Such inconsistencies are stipulated, first and foremost, by the absence of unified assessment criteria for this cohort. We could see that the "metabolic health" criteria in various studies were as follows: insulin sensitivity of the tissues measured by different methods and techniques, absence of the metabolic syndrome criteria based on diverse classifications, complete absence of any metabolic disorders, and, finally, at least 2 CVD risk factors [5–13].

Till now, the question of whether subjects with MHO have some specific phenotypic and/or genetic traits or they are at the intermediate stage of complicated obesity is still to be answered. A number of scientific papers showed that adipocytokine imbalance and genetic factors accounting for the expression of adipocytokines and/or polymorphism of the adiponectin gene as well as that of the leptin and its receptor gene might play a certain role in triggering or, vice versa,

preventing metabolic disorders. That is why in our study we tried to identify possible factors including genetic ones that determine these individuals' benign metabolic profile.

Objective. The objective of this study is to assess the prevalence of metabolically healthy individuals among the subgroup of patients with abdominal obesity and to determine the phenotype and potential genetic traits associated with a benign metabolic status.

2. Methods

A total of 503 residents of Saint Petersburg with AO aged 30 to 55 (average age 45.8 ± 0.3 years) have been enrolled in the study. The inclusion criteria were abdominal obesity (AO), that is, waist circumference (WC) ≥94.0 cm and ≥80.0 cm for men and women, respectively, and signed informed consent (IDF criteria of MetS, 2005). Patients with CVD were not included in the study. Among screened participants, there were 359 (71.4%) women and 144 (28.6%) men of comparable age (45.1 ± 0.6 and 46.1 ± 0.4, resp.; $p > 0.05$). Mean WC was 98.41 ± 0.62 cm in women and 108.39 ± 0.86 cm in men.

In the course of this study, the following anthropometric parameters were measured in accordance with the conventional rules: height, weight, WC, waist-to-hip ratio (WHR), and body mass index (BMI). The subjects were interviewed about their sociodemographic status (education level, income, and family status), familial predisposition to obesity, cardiovascular disease and diabetes, smoking and alcohol consumption, birth weight, duration of obesity, and physical activity (frequency and duration of training sessions per week).

Fasting plasma glucose (FPG) was measured with the automated biochemistry analyzer (COBAS INTEGRA 400/700/800) and standard kits (Roche, Germany). High-sensitivity C-reactive protein (hsCRP) quantitative determination was performed by immunoturbidimetric assay. Serum lipids profile (total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C)) was measured by enzymatic colorimetric assays with the analyzer COBAS INTEGRA 400/700/800 (Germany) and standard kits (Roche, Germany). Serum insulin was measured by solid-phase enzyme immunoassay (ELISA) with the DRG standard test kits (EIA-2935) (USA). Insulin resistance was assessed by calculating insulin resistance index, homeostasis model assessment (HOMA-IR) [14]. Leptin and adiponectin concentrations in venous blood serum were measured by ELISA with the testing kits manufactured by "Leptin ELISA" (DRG Diagnostics, Germany) and the "DRG Adiponectin (human) ELISA" kits (DRG Diagnostics, Germany).

Genomic DNA was extracted from the peripheral blood lymphocytes by the modified method of Blin and Stafford (1976) [15]. Polymerase chain reaction (PCR) was performed with the automated MJ Research (MJ Research Inc.) and Biometra (Biometra, Germany) cyclers with thermostable recombinant Taq polymerase manufactured by Sibenzyme (Russia). Identification of leptin gene A19G polymorphism was done by PCR followed by restriction analysis [16]. For identifying leptin receptor gene (LEPR) Q223R polymorphism PCR followed by restriction analysis (Gotoda et al., 1997) was performed [17]. For identifying adiponectin gene G276T polymorphism the method by Křížová et al. (2008) was applied [18]. Adiponectin gene T45G polymorphism was identified by PCR followed by restriction analysis [19].

Studying polymorphisms of these genes was prompted by the availability of data suggesting their possible involvement in both obesity and glucose and lipid metabolism disorders, which allows us to consider them candidate genes potentially contributing to physiological and/or pathological processes.

"Metabolically healthy obese" (MHO) were defined as individuals without metabolic syndrome (MetS) according to IDF criteria of MetS and without insulin resistance according to HOMA-IR, while patients with MetS or other metabolic disorders were identified as "metabolically unhealthy obese" (MUO).

2.1. Statistical Analysis. Biomedical research data was processed by SPSS software 17.0 for Windows. Sample characteristics were presented as an average ± mean error. Frequency characteristics of qualitative variables were analyzed by nonparametric techniques with the use of x^2, Fisher criterion. Odds ratio (OR) was calculated to define relative risk of the disease. OR = 1 was considered as indicating no association, OR > 1 indicated positive association, and OR < 1 indicated negative association of an allele or genotype with the disease (lower disease risk). Confidence intervals for frequency characteristics were estimated by precise Fisher's method, x^2 with Yates' correction (for small groups). Comparison of quantitative parameters in the study arms was done by ANOVA. Studied parameters were compared for various classification methods and over-time evaluation techniques (paired samples) by applying the sign test, Wilcoxon signed-rank test, and Friedman test.

3. Results

Among 503 subjects with AO, BMI ≥ 30.0 kg/m^2 was diagnosed in 57.0%. Subjects were divided into the following groups based on their BMI: 28 (5.6%) subjects had BMI ≤ 24.9 kg/m^2; 180 (35.9%) patients had BMI ≤ 29. 9 kg/m^2; 192 (38.2%) had BMI ≤ 34.9 kg/^{2}; 70 (13.9%) had BMI ≤ 39.9 kg/m^2; and 32 (6.4%) had BMI ≥ 40.0 kg/m^2. Various MetS components were found in 91.3% of the patients with AO, 66.5% out of those who were diagnosed with MetS. Only 8.7% of the patients with AO did not have insulin resistance or MetS components. Thus among patients with AO whose waist circumference met the IDF criteria of MetS diagnosis, prevalence of metabolically healthy subjects turned out to be rather low.

Comparative analysis between MHO and MUO subjects has shown that MHO individuals were younger and had lower BMI, WC, WHR, levels of total cholesterol, LDL-C, triglycerides, insulin, HOMA-IR, glucose, C-reactive protein, blood pressure, and intima-media thickness of the common carotid arteries but higher HDL-C as opposed to MUO patients (Table 1). Average leptin and adiponectin levels did not differ between these groups ($p > 0.05$). However, in

Table 1: Anthropometric parameters and laboratory parameters in metabolically healthy and unhealthy patients with abdominal obesity.

Parameters	Healthy AO ($n = 44$)	Unhealthy AO ($n = 459$)	p
Age (yrs)	42.2 ± 1.4	47.0 ± 0.4	0.001
BMI, kg/m^2	29.094 ± 0.511	31.561 ± 0.242	0.011
WC, cm			
M	104.65 ± 0.82	108.62 ± 0.95	0.013
F	93.29 ± 0.76	98.94 ± 0.65	0.014
WHR	0.857 ± 0.011	0.890 ± 0.004	0.002
SBP, mm Hg	113.28 ± 1.30	136.42 ± 0.88	0.0001
DBP, mm Hg	73.07 ± 1.08	86.75 ± 0.54	0.0001
Glucose, mmol/L	5.001 ± 0.072	5.624 ± 0.068	0.0001
Insulin, mIU/mL	15.189 ± 1.464	20.221 ± 0.776	0.024
HOMA-IR	3.464 ± 0.432	4.730 ± 0.245	0.043
TC, mmol/L	5.375 ± 0.123	5.866 ± 0.056	0.024
HDL-C, mmol/L	1.642 ± 0.061	1.212 ± 0.021	0.001
LDL-C, mmol/L	3.419 ± 0.130	3.955 ± 0.059	0.005
TG, mmol/L	1.025 ± 0.054	1.620 ± 0.042	0.0001
hsCRP, mg/L	3.009 ± 0.465	4.279 ± 0.180	0.012
Leptin, ng/mL			
M	29.304 ± 2.351	35.454 ± 3.410	NS
F	42.360 ± 3.101	57.504 ± 2.054	0.030
Adiponectin, mcg/mL			
M	17.330 ± 3.241	16.880 ± 0.851	NS
F	23.651 ± 0.864	19.014 ± 0.861	0.020

BMI, body mass index; WHR, waist-to-hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; HOMA-IR, homeostasis model assessment of insulin resistance; hsCRP, high-sensitivity C-reactive protein.

MHO women, adiponectin level was higher whereas that of leptin was lower than in MUO female.

We also determined that more than half of MHO individuals (58.1%) had had obesity for less than 6 years as opposed to 33.0% for MUO subjects ($p = 0.002$). Thus, MHO individuals had a shorter duration of obesity than MUO patients.

47.0% of MHO individuals and 33.4% MUO subjects practiced physical activity or had leisure-time exercise sessions ($p > 0.05$). In addition to this, the frequency of physical exercise sessions and their duration were significantly higher in MHO than in MUO patients (1.28 ± 0.31 times/week and 0.92±0.11 times/week, resp.; $p = 0.032$; 27.70±6.23 min/week and 15.81 ± 1.42 min/week, resp.; $p = 0.040$). This means that MHO individuals were more physically active than MUO.

There were no significant differences between the study arms in terms of social status, birth weight, number of meals, smoking and alcohol consumption status, familial predisposition to obesity, cardiovascular disease, and diabetes mellitus ($p > 0.05$).

Correlations between waist circumference, metabolic parameters, blood pressure, and adipocytokines were identified (Table 2).

Table 2: Associations between waist circumference and metabolic parameters, blood pressure, and adipocytokines in patients with abdominal obesity (correlation analysis).

Indicators	r	p
Systolic BP	0.348	0.0001
Diastolic BP	0.231	0.0001
Glucose	0.244	0.0001
Insulin	0.405	0.0001
HOMA-IR	0.402	0.0001
HDL-C	−0.327	0.0001
TG	0.219	0.0001
Leptin	0.326	0.0001
Adiponectin	−0.214	0.003
hsCRP	0.223	0.0001

Linear regression analysis has demonstrated that WC had made an impact on both systolic and diastolic blood pressure ($r^2 = 0.212$, $p = 0.0001$ and $r^2 = 0.101$, $p = 0.0001$, resp.), insulin ($r^2 = 0.233$, $p = 0.0001$), HDL-C ($r^2 = 0.116$, $p = 0.0001$), and leptin ($r^2 = 0.338$, $p = 0.0001$). Age affects BP ($r^2 = 0.107$, $p = 0.0001$), insulin ($r^2 = 0.221$, $p = 0.0001$), leptin ($r^2 = 0.312$, $p = 0.0001$), and adiponectin ($r^2 = 0.119$, $p = 0.0001$). Waist-to-hip ratio determines HDL-C ($r^2 = 0.120$, $p = 0.0001$) and adiponectin ($r^2 = 0.117$, $p = 0.0001$). Duration of physical activity influences blood pressure ($r^2 = 0.201$, $p = 0.0001$) and insulin ($r^2 = 0.224$, $p = 0.0001$).

At the same time, regression analysis has demonstrated that WC depended on sex, physical activity, duration of obesity, and number of meals.

Negative correlations were observed between adiponectin and TG ($r = -0.234$, $p = 0.003$), HOMA-IR ($r = -0.212$, $p = 0.006$), insulin ($r = -0.231$, $p = 0.0001$), WC ($r = -0.214$, $p = 0.003$), WHR ($r = -0.203$, $p = 0.002$), and BMI ($r = -0.128$, $p = 0.030$) whereas positive correlations were between adiponectin and HDL-C ($r = 0.209$, $p = 0.003$). In the course of regression analysis, it has been shown that the following indicators impacted adiponectin levels to a greater extent: insulin, WHR, and TG ($r^2 = 0.115$, $p = 0.0001$ for all parameters).

Conversely, positive correlations were detected between leptin and HOMA-IR ($r = 0.108$, $p = 0.011$), insulin ($r = 0.211$, $p = 0.0001$), WC ($r = 0.326$, $p = 0.0001$), and BMI ($r = 0.405$, $p = 0.031$). Regression analysis has shown dependence of the leptin level on BMI and TG ($r^2 = 0.211$, $p = 0.0001$).

Within the framework of this study, genotype distribution and alleles frequency of the leptin (019G polymorphism), leptin receptor (LEPR) (Q223R polymorphism), and adiponectin receptor (T45G and G276T polymorphisms) were analyzed in MUO and MHO as these genes potentially might be responsible for the development of obesity and metabolic disorders.

Distribution of genotypes and allele frequency of the leptin gene were analyzed in 459 metabolically healthy and unhealthy individuals with AO. In patients with and without

Table 3: Distribution of the G19G, G19A, and A19A genotypes and frequency of the 19A and 19G alleles of the leptin gene; distribution of the Q223Q, R223Q, and R223R genotypes and frequency of the 223Q and 223R alleles of the leptin receptor gene in patients with abdominal obesity.

Cohorts of AO patients	LEP genotypes			Allele frequency	
	G19G	G19A	A19A	19A	19G
MHO	13	22	5	0.400	0.600
MUO	169	186	64	0.375	0.625
p	NS	NS	NS	NS	NS
Cohorts of AO patients	LEPR genotypes			Allele frequency	
	Q223Q	R223Q	R223R	223Q	223R
MHO	14	18	7	0.590	0.410
MUO	108	245	57	0.562	0.438
p	NS	NS	NS	NS	NS

Table 4: Distribution of the G276G, G276T, and T276T genotypes and frequency of the 276T and 276G alleles; distribution of the G45G, G45T, and T45T genotypes and frequency of the 45G and 45T alleles of the adiponectin gene in patients with abdominal obesity.

Cohorts of AO patients	Adiponectin genotypes			Allele frequency	
	G276G	G276T	T276T	276T	276G
MHO	18	15	4	0.311	0.689
MUO	193	148	44	0.306	0.694
p	NS	NS	NS	NS	NS
Cohorts of AO patients	Adiponectin genotypes			Allele frequency	
	G45G	G45T	T45T	45T	45G
MHO	0	10	30	0.875	0.125
MUO	1	52	365	0.935	0.064
p	NS	0.034	0.042	0.005	0.005

metabolic disorders, distribution of the G19G, G19A, and A19A genotypes and frequency of the 19A and 19G alleles of the leptin gene did not differ ($p > 0.05$) (Table 3). Also, no differences between the groups of AO patients with and without metabolic disorders have been shown in analysis of the Q223Q, R223Q, and R223R genotypes and frequency of the 223Q and 223R LEPR alleles (Table 3).

Distribution of the G276G, G276T, and T276T genotypes and frequency of the 276T and 276G alleles of the adiponectin gene did not differ between MHO and MUO patients (Table 4).

In addition, there were more carriers of the 45T allele of the adiponectin gene among metabolically unhealthy patients with AO as opposed to metabolically healthy ones (Table 4). The T45T genotype was associated with over twofold increase of metabolic disorders' risk for patients with AO (OR = 2.331; 95% CI = 1.121 ÷ 5.132).

To make sure that both groups were comparable, we adjusted them to leave only overweight and class I obese patients based on the WHO classification, hence excluding all subjects with BMI $\geq 35.0\,kg/m^2$ regardless of the gender, a process that leads to removing 102 MetS subjects from the analysis groups. This approach was adopted since there were no class II and higher obese patients in MHO group. The comparative results confirmed that there were more carriers of the 45T allele of the adiponectin gene among MUO as opposed to MHO subjects (frequency 45T allele: 0.934 and 0.864, resp.; $p = 0.018$). Hence T45T genotype carriage was associated with an increased risk of metabolic disorders in subjects with AO (OR = 2.496; 95% CI = 1.127 ÷ 5.524).

Average values of leptin and adiponectin in carriers of various genotypes of the leptin and leptin receptor genes were not different. In MHO and MUO men and women with various genotypes of the studied genes, levels of leptin and adiponectin were the same. However, in women with AO with and without MetS/metabolic disorders (general cohort), carriers of the 223R allele of the LEPR gene, leptin levels were higher than those in individuals with the Q223Q genotype (57.124 ± 0.370 ng/mL and 46.301 ± 2.752 ng/mL, resp.; $p = 0.030$). Furthermore, in women with AO and metabolic disorders with the R223R genotype, leptin levels were higher than those in women, carriers of the 223Q allele of the LEPR gene (60.744 ± 5.581 ng/mL and 47.521 ± 3.243 ng/mL, resp.; $p = 0.045$). Leptin levels did not differ ($p > 0.05$) in the cohort of MHO women with various genotypes of the leptin receptor gene.

4. Discussion

According to the results of the study, prevalence of such a phenomenon as absence of the metabolic syndrome and/or metabolic disorders in persons with AO, residents of Saint Petersburg of employable age, turned out to be insignificant and amounted to 8.7%. These individuals did not have any signs of the metabolic syndrome except for their waist circumference value in accordance with the IDF criteria and insulin resistance according to HOMA-IR. For comparison, study conducted by Iacobellis et al. (2005) has proven that in an Italian cohort of obese patients, in which the MetS IDF criteria were also used as a discriminator of complicated and uncomplicated obesity in combination with assessment of such parameters as uric acid, fibrinogen, and insulin levels, prevalence of this phenomenon was higher and amounted to 27.5% despite the fact that selection criteria were stricter than those in our study [12]. These differences might be due to a number of factors like specific features of the study subjects (number of evaluated patients, their ethnicity, age, obesity duration, etc.), criteria used, and, perhaps, genetic peculiar traits, nutrition patterns, physical activity, and other environmental factors.

In our study, characteristic phenotypic features of obese individuals without MS or metabolic disorders were defined. These include younger age, less pronounced abdominal obesity with lesser duration, and a higher level of physical activity as opposed to persons with AO and MetS/metabolic disorders. According to the obesity classification by BMI, all metabolically healthy individuals fell into the category of overweight patients even though their waist circumference met the IDF criteria of abdominal obesity. Currently, it is proven that both waist circumference and waist-to-hip ratio strongly correlate with the amount of the visceral fat. It is also

known that the visceral adipose tissue generates large quantities of adipocytokines and anti-inflammatory substances that potentiate the development of insulin resistance and other metabolic disorders resulting in earlier debut of cardiovascular disease in obese patients. Presence of correlations between WC and metabolic parameters revealed in this study as well as other numerous studies confirms the relationship between AO and metabolic disorders. The obtained data is consistent with the results of other studies that also showed that individuals with uncomplicated obesity had less visceral fat than patients with obesity and metabolic disorders [20]. Brochu et al. have found that despite similar amount of total fat mass in postmenopausal obese women, MHO women had 49% less visceral adipose tissue than women with obesity and metabolic disorders.

Thus, lesser amount of the visceral adipose tissue may, to some extent, determine the favorable metabolic profile of this patient population [6].

In the vast majority of subjects with uncomplicated obesity that we evaluated, obesity duration was relatively short, less than 6 years. According to other studies' results, subjects suffering from obesity since childhood were encountered more frequently among MHO individuals. It is considered that children and adolescents develop hyperplastic obesity which is associated with preserved insulin sensitivity and with maintained adiponectin concentration, a combination determining the favorable profile of "metabolically" healthy obese individuals [6]. Another hypothesis was proposed by Muscelli et al. (1998), according to which early obesity was accompanied by some kind of metabolic adaptation enabling preservation of normal insulin sensitivity of the tissues [21]. It is also known that complicated obesity is often associated with ectopic fat depositions in the heart, liver, and muscles, which causes the development of insulin resistance and dysfunction of these organs. Conversely, uncomplicated obesity is characterized by a low degree of ectopic fat depositions especially in the muscles and liver [22].

Physical activity (PA) is a potential factor influencing the metabolic profile of obese patients. Thus, in our previous study, it was shown that in physically active AO patients with MS/metabolic disorders both insulin levels and insulin resistance index HOMA-IR were lower whereas the HDL-cholesterol level was higher compared with AO individuals with sedentary life styles while anthropometric indicators in these cohorts were comparable [23]. Therefore, PA can exert an independent positive influence on the metabolic profile of AO patients. Taking this into consideration, one may assume that MHO intense physical activity facilitates maintenance of the normal metabolic status, which is consistent with the results of the study carried out by Wildman et al. (2008) [9]. At the same time, other studies did not find any differences between the basal metabolic rate and energy expenditure on physical activity and level of physical performance in obese patients with the different metabolic profile [6, 22].

It is known that adipocytokine imbalance, in particular that of leptin and adiponectin, is associated with MetS. In a number of studies, it has been shown that an elevated leptin level potentiated insulin resistance and arterial hypertension and activated proinflammatory factors while adiponectin, on the contrary, possessed cardioprotective effects. This association is also supported by the results of correlation and regression analysis done in our study. However these adipokine levels are sex-dependent. For example, estimated levels of leptin and adiponectin in our study were lower in men than in women in both evaluated cohorts. Only metabolically healthy obese women had a lower leptin and higher adiponectin level as opposed to women with complicated obesity. That being said, it may be assumed that less significant adipokine imbalance in metabolically healthy women is one of their metabolic health "protective" mechanisms.

Undoubtedly, genetic factors also play a role in the development of obesity in subjects with the different metabolic profile. There are evidences of the association between the metabolic syndrome and obesity with polymorphic variants of some genes, products of expression which play an important role in adipogenesis and the regulation of carbohydrate and lipid metabolism. Potential candidate genes include the adiponectin gene, leptin, and LEPR genes studied in our trial.

According to our study results, distribution of the G276G, G276T, and T276T genotypes and frequency of the 276T and 276G alleles of the leptin gene were not different in AO patients with or without metabolic disorders. In MUO patients and in MHO individuals, frequency of the 19G allele of this gene was 0.625 and 0.600, respectively, which corresponds to the incidence of this gene in the European population. Thus, frequency of this gene's 19G allele varies by different data from 0.36 to 0.67 being higher in Finns than in Frenchmen and Italians as far as the European population is concerned [16, 24, 25].

The paper by Hager et al. (1998) states that homozygotes of the 19A allele have lower leptin levels than carriers of the 19A allele [25]. In AO patients we evaluated, leptin levels were not different in various genotypes of the leptin gene. In 1999, Li et al. have found that A19G polymorphism of the leptin gene was a predictor of obesity (heterozygotes had higher BMI than homozygotes of the 19A and 19G alleles) although this association was not confirmed in other studies [26].

However, in a number of studies no differences were identified in leptin levels or associations with BMI, waist-to-hip ratio, body mass, and body fat in carriers of different genotypes of the leptin gene [16, 24, 27]. The results obtained in this study also have not found any correlation between obesity and metabolic disorders in different genotypes of the leptin genes.

At present, three single-nucleotide polymorphisms of the LEPR gene are known to lead to amino acid substitution in the receptor protein: Lys109Arg (K109R) rs1137100; Gln223Arg Rs1137101 (Q223R); and Lys656Asn (K656N) rs8179187 [28–30].

A668G polymorphism of the leptin receptor gene is studied most frequently. A668G polymorphism is localized in exon 6 of the extracellular region of the leptin receptor's C domain that has a leptin-binding area, which leads to a single amino acid substitution, that of glutamine (Gln) with arginine (Arg) at position 223, and accounts for the change in functional activity of the leptin receptor [31–33]. In the literature, this polymorphism is most often referred to as Q223R whereas the allele types of the leptin receptor gene

are dubbed 223Q and 223R. Frequency of the 223Q and 223R alleles of this gene is highly variable across countries and ethnic groups. In particular, frequency of the 223R allele in Asian people is notably higher than that in other ethnic groups, up to 0.85 [33, 34]. Frequency of the 223R allele in healthy Europeans, by data of various authors, is in the range from 0.41 (England) to 0.44 (the Netherlands) [35–37]. For AO patients evaluated by us with and without metabolic disorders, frequency of the 223R allele of the LEPR gene was low and amounted to 0.438 and 0.410, respectively.

In our study we also found that in metabolically healthy and unhealthy women, carriers of the 223R allele of the leptin receptor gene, leptin level was higher than that in individuals with the Q223Q genotype. Furthermore, in MUO women with the R223R genotype, leptin level was higher than that in women who are carriers of the 223Q allele of the leptin receptor gene. In MHO women with different genotypes of the LEPR gene, leptin levels were not different ($p > 0.05$). These phenomena were not seen in men.

It has been noted in a number of studies that carriage of the 223R allele of the leptin receptor gene was associated with a high level of circulating leptin and reduced sensitivity of the leptin receptor [30, 32, 36] as well as increased BMI [36, 38, 39]. Other researchers received the data on the association of the R223 allele with glucose and insulin levels [40, 41]. Gottlieb et al. (2009) have found the correlation of this polymorphism with the metabolic syndrome: Q223Q and Q223R genotypes are more frequently encountered in MetS patients than in healthy individuals [42].

In the study carried out by us earlier, it was observed that in AO patients with MS/metabolic disorders carriership of the R223R genotype of the leptin receptor gene was associated with higher BMI and insulin levels; in men it was also associated with larger waist circumference [43]. In the study by van der Vleuten et al. (2006), it has been also shown that carriage of the 223R allele of the LEPR gene (homozygotes of the 223R alleles and heterozygotes) was associated with combined hyperlipidemia, reduced sensitivity to insulin and adiposity [44].

It is known that the adiponectin level is genetically controlled. Data of different authors suggest that heritability of the adiponectin level varies from 55% to 93% [45, 46].

Correlation between polymorphisms of the adiponectin gene and its production is identified, which may contribute to incidence of obesity, insulin resistance, type 2 diabetes mellitus, MS, and cardiovascular disease [47–51].

At present, single polymorphisms T45G and G276T are the most frequent ones among described polymorphisms of the adiponectin gene (Y111H, G-12823A, A-11426G, G-11391A, C11377G, A11426G, R112C, I164T, T45G, and G276T) [52]. Results of the studies devoted to these polymorphisms are controversial and largely dependent on studied cohorts.

In case of G276T (intron 2, rs1501299) polymorphism of the adiponectin gene, frequency of the 276G allele in the general population varies, by different data, from 0.701 to 0.830 [18, 48, 53–55]. According to our data, frequency of the 276G allele in AO patients with and without metabolic disorders did not differ (0.694 and 0.689). In a number of studies, association of the 276G allele with the low adiponectin level (France, Greece, Spain, and Japan), insulin resistance (Japan, Greece, Sweden, Spain, and Italy), obesity (Sweden, Japan), and T2DM (Japan) has been found [48, 53–57]. Yang et al. (2007) have found the association of the 276G allele with MS [55]. In our study, no differences in adiponectin levels in carriers of the 276G and 276T alleles have been observed.

With T45G (Gly45Cly) polymorphism (exon 2, rs2241766) of the adiponectin gene, frequency of the 45T allele in the general population, based on data of different authors, varies from 0.705 to 0.950 [18, 48, 53, 55, 58].

In a number of studies, relationship of T45G polymorphism and obesity, T2DM, and hypoadiponectinemia was noted. Thus, carriage of the 45T allele (wild type) was associated with low adiponectin level in healthy volunteers in the studies carried out in France and in Canada [56, 58]. Similar results were obtained in healthy individuals and patients with T2DM in Japan, Sweden, and Spain and Italy [48, 53, 57, 59]. Besides, in studies conducted in Sweden [57] and in Germany [60], carriage of the 45G allele was associated with adiposity. However, this association has not been found in studies carried out in Finland and in the multicenter trial done in Europe and Canada (STOP-NIDDM-study) [46, 61]. Carriage of the 45G allele has been also associated with increased risk of T2DM, impaired glucose tolerance, higher BMI and waist-to-hip ratio in the DESIR study (France) [62], increased T2DM risk (Japan) [48], and hyperinsulinemia and insulin resistance (Germany) [60].

Menzaghi et al. (2002) stated that the T45T genotype of the adiponectin gene was associated with insulin resistance and systolic blood pressure [53]. However, some researchers did not find any association between adiponectin gene polymorphism and insulin resistance and BMI [55, 63].

In the MetS patient population there were more carriers of the 45T allele of the adiponectin gene than among those metabolically healthy. Furthermore, T45T genotype of the adiponectin gene was associated with over twofold increased risk of metabolic disorders in AO patients even though adiponectin levels in AO patients with various genotypes of this gene were the same. Even when the groups were adjusted by excluding patients with class II and III obesity from MUO group the risk of metabolic disorders in AO patients had remained (OR = 2.496; 95% CI = 1.127 ÷ 5.524). Therefore, it can be assumed that carriers of T45T adiponectin gene genotype have a direct adverse effect on the metabolic profile in patients with AO. Also, as it was found in our previous trial, T45T genotype of the adiponectin gene and reduced adiponectin level in women were predictors of metabolic syndrome in AO patients [43]. Thus, lower frequency of the potentially unfavorable genotype of the adiponectin gene was found in metabolically healthy individuals, which, to some extent, might determine their metabolically favorable profile.

5. Conclusions

Prevalence of metabolically healthy obese individuals among patients with abdominal obesity is low, 8.7%. Benign metabolic status was associated with younger age, lower waist circumference, higher physical activity, and shorter duration of obesity. In women with uncomplicated abdominal obesity

the leptin level was lower and the adiponectin level was higher than in women with complicated obesity. In metabolically healthy individuals with abdominal obesity low frequency of T45T adiponectin gene polymorphism was found, which is associated with an increased risk of metabolic syndrome in patients with abdominal obesity.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

True Unipolar ECG Machine for Wilson Central Terminal Measurements

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Since its invention (more than 80 years ago), modern electrocardiography has employed a supposedly stable voltage reference (with little variation during the cardiac cycle) for half of the signals. This reference, known by the name of "Wilson Central Terminal" in honor of its inventor, is obtained by averaging the three active limb electrode voltages measured with respect to the return ground electrode. However, concerns have been raised by researchers about problems (biasing and misdiagnosis) associated with the ambiguous value and behavior of this reference voltage, which requires perfect and balanced contact of at least four electrodes to work properly. The Wilson Central Terminal has received scant research attention in the last few decades even though consideration of recent widespread medical practice (limb electrodes are repositioned closer to the torso for resting electrocardiography) has also sparkled concerns about the validity and diagnostic fitness of leads not referred to the Wilson Central Terminal. Using a true unipolar electrocardiography device capable of precisely measuring the Wilson Central Terminal, we show its unpredictable variability during the cardiac cycle and confirm that the integrity of cardinal leads is compromised as well as the Wilson Central Terminal when limb electrodes are placed close to the torso.

1. Introduction

Surface electrocardiography, by definition, is the time-domain representation of the electrical activity of the beating heart inside the chest, measured as voltage variation over time by surface electrodes placed in contact with the skin. Surface electrocardiography is represented by a vector quantity ($\overline{P}$) rotating around a fixed point (the electrical center of the heart) in the body frontal plane describing an angle (α) with a fixed direction identified by an imaginary line crossing the shoulders [1]. This definition was originally outlined in 1908 by E. Einthoven, later revised in 1931 by F. N. Wilson, who named the fixed point as the "central terminal," and further modified in 1942 by E. Goldberger, who invented the augmented leads [1]. From 1942, the mentioned definition and associated recording guidelines produced the so-called 12-lead ECG system, which is currently considered to be the best practice [1, 2].

The 12-lead ECG is so called because it produces twelve ECG signals. It uses a reference electrode placed on the right leg (RL) and nine exploring electrodes: three limb electrodes placed on the right arm (RA), left arm (LA), and left leg (LL) and six electrodes placed over the torso near the heart [1]. Electrode positioning and signals recordable from the six electrodes over the torso have been named precordial leads (precordials) and are also known simply as "chest leads" (see Figure 1(a)) or as V_1 to V_6 leads, while the signals recordable from the limbs have been named cardinal (or fundamental) Einthoven leads (see Figure 1(b)) and are referred to as Lead I, Lead II, and Lead III or simply as "limb leads":

$$\text{Lead I: } V_{\text{I}} = \Phi_L - \Phi_R;$$
$$\text{Lead II: } V_{\text{II}} = \Phi_F - \Phi_R;$$
$$\text{Lead III: } V_{\text{III}} = \Phi_F - \Phi_L;$$

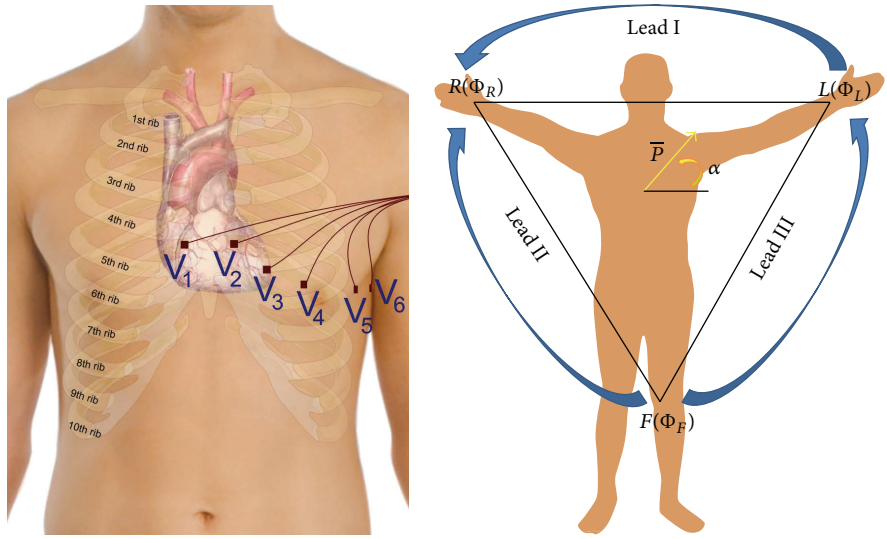
with

V_{I} being the voltage of Lead I;

V_{II} the voltage of Lead II;

V_{III} the voltage of Lead III;

Φ_L the potential at the left arm*;



(a) Precordial placement (b) Limb leads and electrodes

FIGURE 1: Twelve-lead ECG electrode placement and lead names [1] (a and b).

Φ_R the potential at the right arm*;

Φ_F the potential at the left leg*

*referred to the electrode on the right leg (Φ_{RL}).

The augmented leads are measured as the voltage difference between each of the limb potentials and the average of the other two limb potentials. For example, the augmented lead aV_F is measured as

$$aV_F = \Phi_F - \frac{(\Phi_L + \Phi_R)}{2}. \quad (1)$$

Because all of the limb potentials are implicitly referred to the potential of the right leg, it is possible to infer that cardinal leads are recorded as twice the voltage difference. For example, assuming the potential of the right leg Φ_{RL} being measured with respect to a point at a neutral potential (i.e., earth ground), Lead I can be rewritten as

$$V_I = (\Phi_L - \Phi_{RL}) - (\Phi_R - \Phi_{RL}). \quad (2)$$

Although at first glance it may seem that the potential Φ_{RL} will cancel out; due to the nonideal (not infinite) capacity to reject common signals that are present simultaneously at the inputs, known as the Common Mode Rejection Ratio (CMRR) [3, 4] of the employed amplifiers, any contact imbalance between the three electrodes may cause signal quality degradation and unpredictable drift of slow components. Intuitively, the effect of contact impedance imbalance gets worse when considering augmented leads as they require perfectly balanced contact from all of the four limb electrodes [1, 5, 6]. This is counterintuitive as the circuit that they form in the human body is an equilateral triangle that does not take into account the RL voltage at all (see Figure 1(b)).

Similarly, the voltage of a virtual point called the Wilson Central Terminal (WCT) is subtracted from each of the precordials' electrode potentials. The WCT is obtained by averaging the potential at the limbs referred to the reference electrode on the right leg using three identical resistors (5 kΩ or higher) connected to a single point [1]:

$$\Phi_{WCT} = \frac{(\Phi_L + \Phi_R + \Phi_F)}{3}. \quad (3)$$

Although Wilson himself used to refer to the precordials as "unipolar" [7], this has been repeatedly pointed out as a misnomer due to the repeated voltage difference required to obtain them [8–11]. It has also been demonstrated that the WCT cannot be considered a "null" potential [8, 9] nor should it be confused with the real center of the heart potential, because the ECG signals travel through different trunks of an inhomogeneous volume conductor and can be exposed to different sources of noise such as different expositions to RF fields and artifacts [9, 12]. In 1954, Frank [8] was the first to raise concerns about the potential fluctuations in the WCT during a cardiac cycle and how they could bias the ECG measurement [8, 13, 14]. He predicted that within a few years a new, refined cardiac conduction theory and ECG system able to work without the WCT would emerge. In the early days of modern electrocardiography, other researchers were also able to confirm that the WCT is not constant during the cardiac cycle. Confirmation of errors and variability of the WCT during the cardiac cycle have been measured employing an "integrator electrode." This procedure requires the entire human body to be encased in a metal structure and then immersed in water (neutral reference) during the measurement of ECG. Unfortunately, due to the cumbersomeness of the measurement process, this technique was used only for few experimental trials [15, 16]. In recent years, the significance of the WCT and even its physical location has also been debated [9, 10, 17]. However, aside from notable attempts in the 1940s and 1950s [14, 18, 19], until our

study, the WCT has never been correctly measured without a cumbersome procedure and in a repeatable way.

In this context, one must mention that not only has the WCT received scant research attention in the last few decades, but also there is a generalized lack of modern studies about the general placement of electrodes and the impact that electrode misplacement (particularly when intentional) may have upon diagnosis. Current common widespread medical practice is to move the limb electrodes to positions closer to the torso (shoulders and hips or sides of the navel). This is thought to reduce the obtrusiveness of the ECG recording as cables are not spread all over the body, which is particularly advantageous during stress recordings. However, there is evidence [20] that limb electrode positioning that affects the QRS influences the diagnosis of ischemic (including chronic) heart diseases [21, 22]. Although there is some evidence that in healthy subjects the variation in the ECGs imposed by alteration of the limb electrodes can be classified only as statistically relevant and not as clinically relevant [23], due to the significant shift in cardiac axis and waveform amplitude that can be observed in both ECG planes when the limb electrodes are in positions different from the standard ones [24], standardized recommendation for ECG clinical practice [25] confirms that misplacement of limb electrodes should be avoided [22] or used only where strictly necessary (i.e., stress test) and always noted on the recording [25].

Over the past two years, we have developed a new electrocardiographic device [3, 11, 12, 26–28] that allows real-time visualization and precise measurement of the WCT amplitude, shape, and variations; using this device we show that the WCT exhibits a clinically significant variation (>0.1 mV or >1 mm [2, 14]) across different recordings and during the course of the same recording. For the evaluation presented in this paper we have partially reused the unipolar ECG data that have been recorded from a small population of healthy subjects who volunteered during a previous study [11, 12, 26, 27] and agreed to have the data analyzed for publication purposes by expert cardiologists. The subject population comprises five males covering the age span of 29–36 years with an average age of 32.5 years. None of the subjects had a history of cardiac illness, and all the recordings presented normal sinus rhythms. We also recorded data from one volunteer subject again, performing two recordings consecutively to show the effect of placing the limb electrodes near the torso on cardinal leads.

2. Experimental Section

Our principal hypotheses for this study are as follows.

(1) The WCT is not a stable voltage reference exhibiting a clinically significant voltage variation.

(2) Moving the limb electrodes to a position near the torso can affect the shape and amplitude of cardinal leads as well as the WCT.

To demonstrate our hypotheses, we firstly introduce the true unipolar machine and a measurement technique that allows us to reliably measure and store the WCT; then, we present the data processing with a full example of WCT variability across the cardiac cycle and through a recording. Lastly, we show the effect that the placement of the limb electrodes near the torso (from ankles and wrists to hips, sides of the navel, and shoulders) has on limb leads and the WCT [25].

2.1. Hardware Development. Our hardware front-end and its pilot evaluation are properly described in [11, 12, 26–28]. However, for the sake of completeness, in this section we give a brief summary of the measurement hardware employed in this study. In Figure 2, we show a functional block diagram of the ECG amplifier (one single channel). In principle, we regard the unipolar ECG measurement as a combined observation of noise and useful signal. It is thus possible to measure the local signal of interest by subtracting the local noise (or what is regarded as such) from the measured signal. As it is possible to observe in Figure 2, the measured signal (measurement electrode) is fed to an instrumentation amplifier that subtracts from the signal a low-pass version of the same signal (the low-pass cut-off frequency is set at 0.1 Hz). With this technique, a pseudo-high-pass DC-coupled ECG front-end is achieved, preserving the ultrahigh input of the amplifier, which allows the use of dry electrodes. Experiments confirmed that the low-pass filter used to achieve the pseudo-high-pass filter can be implemented with passive components and its cut-off frequency can be positioned at very low frequency (i.e., 0.01 Hz), employing high value capacitors and resistors. This is possible because the ultrahigh-input impedance of the instrumentation amplifier employed can cope with several MΩ of impedance.

Amplifier referencing is achieved via the reference terminal of the instrumentation amplifier labeled as "Ref." The Ref terminal receives a damped version (low passed) of the summation of all the electrode signals and of the RL electrode. This technique, which is also known as "modified ground bootstrapping" [3, 12, 29–31], similar to the standard ground bootstrapping [3, 32], achieves power-line noise and electrodic noise suppression without the use of a driven right-leg technique [33, 34].

Signals recorded using this instrument can be regarded as being referred directly to the right leg. Therefore, a simple point-by-point subtraction between recorded signals allows real-time calculation of the 12-lead ECG. In Figure 3, an example of the calculation for Lead I is shown. In this example, prerecorded left-arm and right-arm signals have been simply subtracted to obtain Lead I. With this recording technique, the WCT is simply calculable from a point-by-point average of the recorded limb potentials. In order to allow reconstruction of traditional precordials (obtained by simple point-by-point subtraction of the WCT), our precordials are also directly referred to the potential of RL [11, 12, 27]. In our previous pilot study [12, 28], we demonstrated that correlation between the reconstructed signals and parallel recording of traditional signals exceeds 90% with minimal differences, which are due to components' tolerance [11, 12, 26].

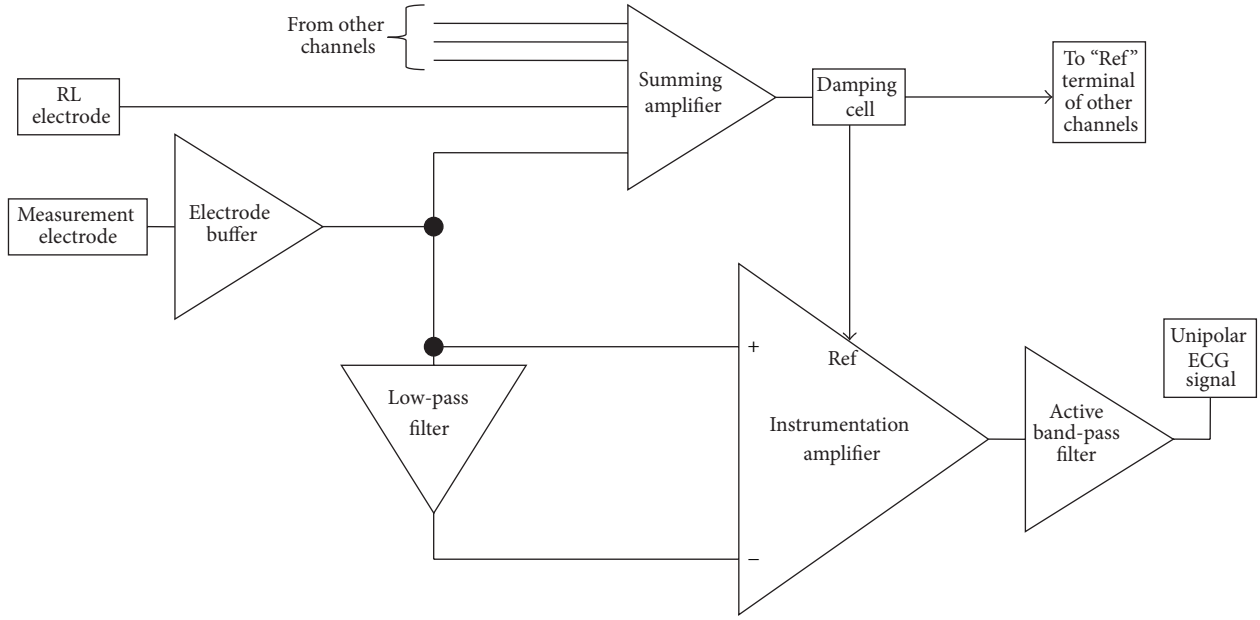


Figure 2: Block diagram of proposed ECG system.

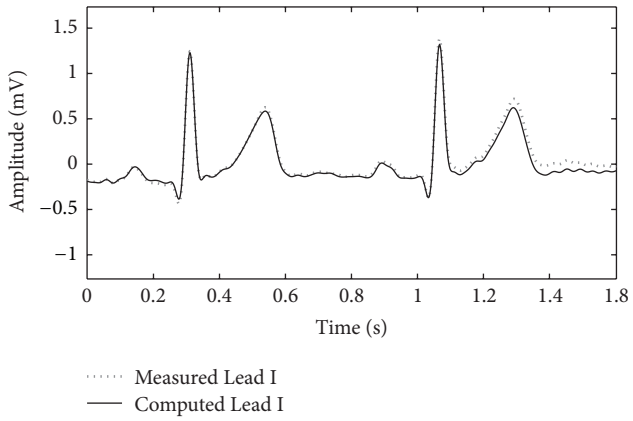


Figure 3: Example of a traditional ECG lead reconstruction from unipolar leads (point-to-point subtraction) (the data used to plot the image were recorded for the study [12]).

2.2. Measurement. For this study, we calculate the WCT by averaging the prerecorded limb potentials. As we have shown in our previous analysis, the WCT is profoundly different across subjects and may have the shape of ECG leads with sometimes very well marked characteristic waveforms such as a P wave, a QRS complex, and a T wave. For this reason, we measure the WCT's amplitude at its largest feature that is expected to normally coincide with the QRS-like complex. In other words, we measure this amplitude as the peak-to-peak amplitude. In this study, we show that the amplitude of the WCT varies during a recording and that, similar to what has been already demonstrated for standard ECG leads [20], its shape and amplitude are affected by the positions of limb electrodes. Using a case study we have also been able to justify the commonly observed shift of the cardiac axis towards the vertical direction [20, 23, 24].

3. Results and Discussion

(1) The WCT exhibits clinically relevant (>0.1 mV or >1 mm) amplitude variability during each cardiac cycle as well as clinically significant variation during the recording. In order to show this variability in a concise way, we selected a random starting point within the recording and measured the amplitude of the WCT for 10 consecutive beats after that point. As it is possible to observe from Figure 4, all of the 10 considered beats have an amplitude larger than 0.1 mV; moreover, between beat #3 and beat #6 there is the largest large extent of variability (0.12 mV) between cardiac cycles.

(2) Similar analyses performed for the other subjects of our database [11, 12, 26, 27] yield similar results.

(3) Our general WCT amplitudes are in accordance with values presented in the literature. We recall that amplitudes for the WCT of the order of 0.2 mV were already measured during a historical experiment that made use of a cumbersome procedure. During the experiment a volunteer was immersed in water whilst being encased in a metal structure called an "integrator electrode" [15, 16, 18, 35]. Our device instead allows continuous WCT precise measurement by recording straight from the limb electrodes.

(4) The WCT noise level is influenced directly by all three limb potentials; hence movement artifacts on any of the limbs or any contact impedance imbalance between the limb electrodes will directly affect the WCT signal quality and possibly degrade the precordials. Because the true unipolar device records limb components, noise affecting one of the limbs can be evaluated beforehand, and hence operators can decide

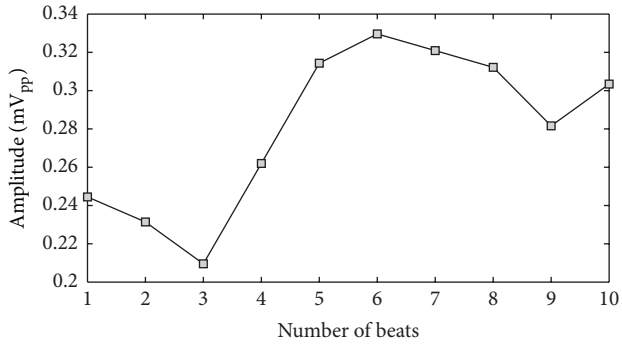


Figure 4: Variation in WCT amplitude measured across 10 consecutive beats selected starting from a random beat within the recording (see text).

not to use the WCT if it is compromised without experiencing loss of the entire set of precordials. To this extent, the amplitude of the WCT seems to be dominated by the right-arm (RA) component (which is the largest component observable from Figure 5(b)); similar observations were made for the other subjects enrolled in our pilot study and hence we can confirm the previous hypothesis that WCT may impair chest exploration due to biasing imposed by the right arm [14].

(5) The position of the limb electrodes directly affects the shape of the leads and WCT. A simple comparison of Figures 5 and 6 reveals that the QRS feature of the WCT is distorted. When electrodes are moved to the shoulders and hips (see Figure 6), the S-wave decreases in favor of a larger R-wave and this is particularly visible in Lead III, where the QRS is clearly larger.

(6) In unipolar components, there is a marked increase in the amplitude of the LL component and a reversion of the LA component polarity. For these reasons it is possible to say that the increase of information carried by the lower body (LL) and the simultaneous distortion of the information carried by the upper body (LA) justify the deviation of the cardiac axis in favor of more vertical directions, as observed in literature [24]. This finding is supported by an intuitive analysis of the correct formula for the calculation of the cardiac axis. Recalling that the cardiac axis is calculated by [36]

$$\text{Cardiac Axis} = \pm \tan^{-1} \frac{aV_F}{\text{I}} \tag{4}$$

which can be expressed in unipolar components as [28]

$$\text{Cardiac Axis} = \pm \tan^{-1} \frac{\text{LL} - ((\text{RA} + \text{LA})/2)}{\text{LA} - \text{RA}}, \tag{5}$$

it is easy to conclude that a marked increase in LL alone will increase the vertical component of the vector $\overline{P}$ representing the cardiac activity, shifting the value of its angle α towards a steeper value; one may note that a reversion of the LA polarity may also contribute to an increase of the numerator of the cardiac axis calculation formula, which, when limb electrodes are moved closer to the torso, is also always accompanied by a reduction of Lead I (the denominator), which may further increase the shift of α toward the vertical axis.

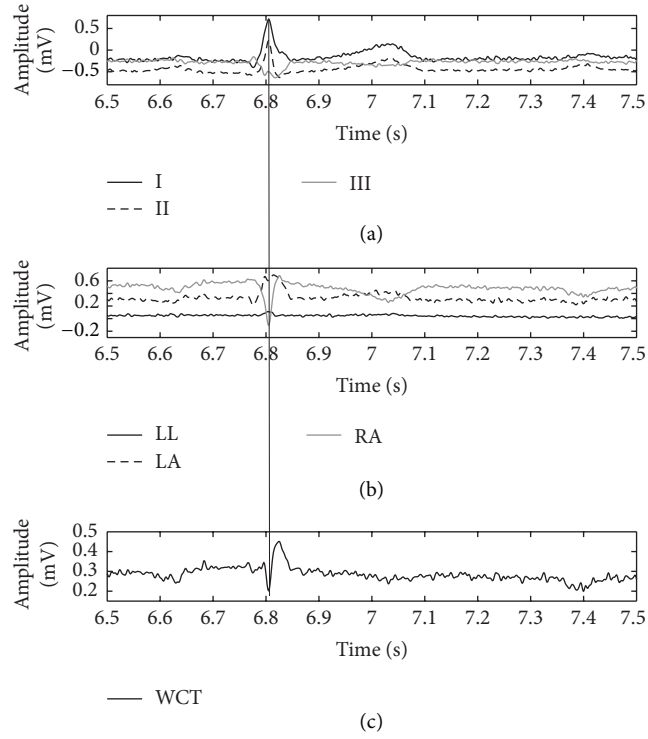


Figure 5: Direct comparison of WCT (c) with cardinal limb leads (a) and true unipolar components (b) when limb electrodes are placed on wrists and ankles. The QRS fiducial point is marked (thin vertical line) using Lead II as the reference.

Lastly, because the signals recorded with the true unipolar device are linearly independent, similar to what is done with EEG recordings, it is possible to increase the space of signals via rereferencing. Namely, the number of signal traces obtainable from the 10 placed electrodes will increase from twelve to at least thirty (nine independent unipolar ones, nine referred to the common average, and the twelve traditional signals), thereby increasing the redundancy of information present in the ECG, as has been sought since its invention more than 80 years ago [1]. In other words, a corollary of this new method is that the current practice is at the same time improved (more robustness to noise, larger redundancy of information, and visualization of WCT) and preserved (the traditional signal and diagnostic method are also useable). It is notable that reconstruction of 12-lead ECG based upon point-to-point subtraction of components can be more robust to noise. This is because signal analysts (medical practitioners annotating the ECG with or without the aid of automated procedures) will be able to estimate

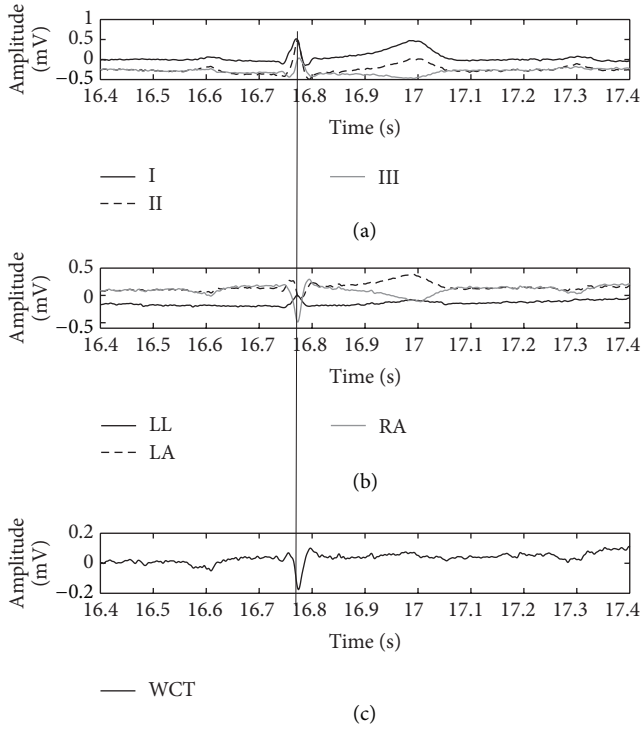


Figure 6: Direct comparison of WCT (c) with cardinal limb leads (a) and true unipolar components (b) when limb electrodes are placed on hips and shoulders. The QRS fiducial point is marked (thin vertical line) using Lead II as the reference.

the signal-to-noise ratio of each individual component (such as power-line noise and artifacts) and operate individual differentiated and customized software filters on the components before reconstructing the signal [11, 12, 26, 27].

4. Conclusions

We presented experimental evidence that the WCT is not a stable reference for ECG leads through the cardiac cycle, that its shape and amplitude (measured peak to peak) are comparable with the amplitude of other ECG leads, and most importantly that it shows clinically significant amplitude variability during the recording. With this study we also show that the WCT, like the limb leads, is directly affected by alteration of the electrode position and therefore it can pass this additional bias to precordials with unforeseen effects upon diagnosis.

Using our device, in this study, we have also been able to justify the shift of the cardiac axis toward the vertical direction that has been observed in several independent studies when limb electrodes are placed closer to the torso (i.e., stress ECG). Hence, since our analysis and experiment confirm concerns about the alteration of all standard leads when limbs electrodes are placed closer to the torso, we conclude that this practice should be avoided or used only where strictly necessary (i.e., when recording is not possible otherwise).

Lastly, our technique for measurement of ECG signals, allowing calculation of the WCT and standard 12-lead ECG, offers the construction of a larger space of signals, which adds redundancy to the ECG, as has been sought since its invention more than 80 years ago [1]. We are currently seeking ethical clearance for a large trial to confirm the extent and impact of our findings, particularly concerning the effect of the currently widespread practice of placing the limb electrodes closer to the torso.

Conflict of Interests

The author declares no conflict of interests.

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Review Article

An Update on Renal Artery Denervation and Its Clinical Impact on Hypertensive Disease

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Hypertension is a globally prevalent condition, with a heavy clinical and economic burden. It is the predominant risk factor for premature cardiovascular and cerebrovascular disease, and is associated with a variety of clinical disorders including stroke, congestive cardiac failure, ischaemic heart disease, chronic renal failure, and peripheral arterial disease. A significant subset of hypertensive patients have resistant hypertensive disease. In this group of patients, catheter-based renal artery denervation has emerged as a potential therapy, with favourable clinical efficacy and safety in early trials. Additional benefits of this therapy are also being identified and include effects on left ventricular remodeling, cardiac performance, and symptom status in congestive cardiac failure. Utility of renal denervation for the management of resistant hypertension, however, has become controversial since the release of the Symplicity HTN-3 trial, the first large-scale blinded randomised study investigating the efficacy and safety of renal artery denervation. The aim of this paper is to evaluate the history, utility, and clinical efficacy of renal artery denervation technology, including an in-depth appraisal of the current literature and principal trials.

1. Introduction

Hypertension is a clinical disorder that is defined as an aberrancy of blood pressure, with systolic blood pressure (SBP) equal to or greater than 140 mmHg and/or diastolic blood pressure (DBP) equal to or greater than 90 mmHg [1]. It is the most common chronic disease in developed societies and is associated with a variety of clinical disorders including stroke, congestive cardiac failure, chronic renal failure, ischaemic heart disease, and peripheral arterial disease [2, 3].

As blood pressure is a continuous variable, hypertension can be categorized into separate classes or stages, with current clinical classification systems encompassing Stages 1 (SBP 140–159 mmHg and/or DBP 90–99 mmHg), 2 (SBP 160–179 mmHg and/or DBP 100–109 mmHg), and 3 (SBP ≥ 180 mmHg and/or DBP ≥ 110 mmHg) [4]. These increments are divided as they provide prognostic value [5].

Poorly controlled hypertension is the predominant risk factor for premature cardiovascular disease, with an estimated contribution of 54% in all cerebrovascular accidents (CVAs) and 47% of all coronary events globally [6]. These risks, primarily attributable to accelerated atherogenesis and increased arterial impedance, have been found with elevations in SBP or pulse pressure in persons over the age of 60, and elevations in DBP in younger individuals [7].

Current therapeutic strategies in the management of hypertension are based on lifestyle interventions and pharmacological agents, with studies showing significant reductions in blood pressure (10–12 mmHg SBP, 5-6 mmHg DBP) to be associated with an overall decreased morbidity and mortality, including an estimated 38% risk reduction of CVA and 16% risk reduction of coronary artery disease [8]. In line with studies showing diminishing returns associated with aggressive blood pressure reduction below SBP of 140 mmHg, current treatment guidelines advocate for a uniform approach with suggested targeting of SBP to less than 140 mmHg for all hypertensive groups, regardless of cardiovascular risk profiles [9, 10].

Despite the obvious benefits as well as the wide range of available pharmaceutics today, the management of hypertension remains unsatisfactory even in industrialised nations. While this is primarily attributable to therapeutic inertia and poor adherence, an increasing proportion of patients are now recognized to have refractory or resistant hypertension, with

an almost threefold increase in cardiovascular risk in this group [11].

2. Aetiology, Classification, and Pathophysiology

Hypertension can be classified according to aetiology, with main subdivisions delineating primary and secondary forms. Primary hypertension, commonly referred to as essential hypertension, accounts for the majority of cases of hypertensive disease (>95%) [12]. It is a multifactorial disorder that has a strong heritable component with complex genetic and environmental interactions in which sympathetic overactivity and renal pathology form a large constituent of the pathogenesis [12–15]. Secondary hypertension, in contrast, embodies a small proportion of patients with diagnosed hypertension. It is caused by a variety of medical and medication-related conditions and disease states [12].

The pathophysiology of hypertension is complex. Under normal physiological processes, the renal system provides signals to the central nervous system to regulate whole body vascular resistance. These afferent and efferent pathways regulate blood pressure at multiple levels including the renin-angiotensin-aldosterone system, local nephron blood flow, and transporter regulation of sodium and water excretion [16]. A synergistic effect is further produced by the autonomic nervous system. Via actions on the heart, blood vasculature, and kidney, the sympathetic nervous system causes increases in cardiac output, vascular resistance, and sodium and fluid retention [13, 14].

In hypertensive individuals, high sympathetic drive accompanied by suppressed parasympathetic action contributes to hypertensive disease [17]. In addition, peripheral and central arterial baroreceptors are reset at higher thresholds in this group [18], leading to maintenance of higher mean arterial pressures, irrespective of intravascular volume. Compared to normotensive individuals, patients with hypertension also have an enhanced chemoreflex system, with heightened renal sympathetic stimulation and greater vasoconstrictor responses to noradrenaline [19]. With the increased activation of renal sympathetic function, increased spillover of noradrenaline ensues, resulting in augmentation of renin secretion from the kidney. The consequent increase in sodium and fluid retention, renal artery vasoconstriction, and decreased renal perfusion further perpetuates the hypertensive state, worsening renal perfusion and thus leading to a vicious cycle [20–22].

3. Resistant Hypertension

A subset of individuals with hypertensive disease are characterized as having resistant hypertension. As per The Joint National Committee 7 consensus, resistant hypertension is defined as a SBP at or exceeding 140 mmHg and/or a DBP at or exceeding 90 mmHg despite full compliance to the maximum tolerated dose of 3 or more antihypertensive medications, including a diuretic [1]. Resistant hypertension is not to be confused with poorly controlled hypertension or "pseudo-hypertension," a condition that is attributed to poor adherence, suboptimal medication regime, or secondary hypertension and which does not represent true treatment resistance [23].

To date, no large prospective studies are available to substantiate the prevalence of true resistant hypertension. Data extrapolated from small studies have established the prevalence ranging from 5 to 20% of cases [24]. It is acknowledged however that, for those diagnosed with resistant hypertension, therapeutic options are limited and patients are subject to an almost threefold increase in cardiovascular risk compared to those with controlled hypertension, conveying the need for alternative therapeutic options in blood pressure control beyond pharmacological strategies [25].

4. Renal Artery Denervation: Concept

Renal sympathetic nerves, afferent and efferent, are embodied within the wall of the renal artery and are required for maintenance of systemic hypertension [13, 14, 16]. The concept of sympathetic nerve modulation as a management tool for systemic hypertension is not new, with surgical intervention being used prior to the advent of pharmacotherapeutics.

Surgical resection of thoracic, abdominal, and pelvic sympathetic nerves has been utilised in the past for the management of Stage 3 hypertension. Despite sustained blood pressure control, these methods were associated with high perioperative mortality, and long-term deleterious effects including significant dysfunction of organs (bladder, bowel, and genitals) supplied by these nerves [26].

Given the efficacy of sympathetic modulation, the concept of selective disruption of sympathetic nerve supply was hypothesized. The idea was that, via percutaneous approach, a catheter can be inserted via the femoral artery and placed into the major renal artery on each side to deliver radio-frequency (RF) energy to the adventitia of the vessel, one of the layers of the arterial wall which houses the renal sympathetic and afferent nerves. The primary theoretical benefit would be sustained reduction of blood pressure, brought about by disruption of primary sympathetic output, without leading to the generalized adverse effects of broader sympathetic disruption.

5. Renal Artery Denervation: Clinical Trials

Percutaneous transcatheter renal artery denervation was first explored in the early 2000s. Regarded as a radical approach to hypertension, the idea was initially met with skepticism by most when first introduced. This perception persisted despite animal trials showing a significant reduction in blood pressure and renal noradrenaline content comparable to direct surgical renal denervation with minor procedural related complications [27–29].

5.1. Symplicity HTN-1 and HTN-2. The first human trial investigating the efficacy of catheter-based renal artery denervation was the Catheter-Based Renal Sympathetic Denervation for Resistant Hypertension (Symplicity HTN-1)

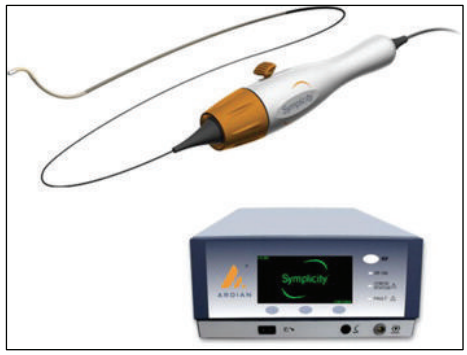


Figure 1: Symplicity catheter system.

Trial (2011). In this multicentre, nonrandomised study, the investigators looked at primary endpoints of safety and sustained blood pressure reduction from the procedure, with secondary endpoints of procedural effect on renal noradrenaline spillover and effect on renal function [30]. Inclusionary criteria were that of resistant hypertension (SBP ≥ 160 mmHg) despite three antihypertensive medications (including a diuretic) in the absence of haemodynamically significant valvular disease or renovascular abnormalities determined via angiographic assessment.

Patients undergoing the procedure were commenced on low dose Aspirin (100 mg daily) a week prior to the procedure with intravenous heparin cover during the procedure itself. RF ablation was performed with the Medtronic Simplicity Catheter (Figure 1) via a 6Fr or 8Fr guide. Four-to-eight ablations were delivered within each renal artery, each lasting approximately 2 minutes, and were separated both longitudinally and rotationally within the length of the artery (Figure 2). Median time of procedure from first to last RF ablation delivery was 38 minutes. After procedure, renal angiography was performed to identify any irregularities or stenosis. Patients were subsequently followed up at 1, 3, 6, 9, and 12 months with average office-based blood pressure measurements [30].

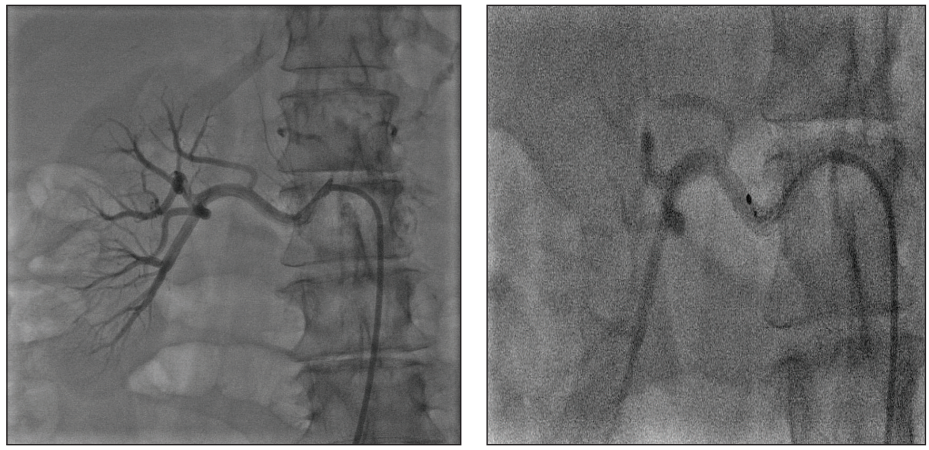
A total of 153 patients (60 females, 93 males), who were on average taking 5.1 antihypertensive medications, with a mean SBP and DBP of 177 mmHg and 98 mmHg (±17/14 mmHg), respectively, were included into this pilot study. Renal artery denervation was performed in 149 (97%) patients without complications. In the remaining 4 patients, renal artery dissection occurred in one patient prior to delivery of RF ablation that was treated with a renal artery stent without any further sequelae, whilst the other 3 patients developed a pseudo-aneurysm of the femoral access site that was managed conservatively with monitoring and analgesia [30]. Intraprocedural diffuse abdominal pains were reported in all cases that were managed with intravenous narcotics and anxiolytics. In terms of renovascular safety, repeat renal imaging that was performed in 81 patients at 6 months did not show any new abnormalities or stenosis in the treated arteries. One patient was noted to have progression of preexisting renal artery stenosis in the proximal portion of the renal artery, distant to the sites of RF ablation. This was treated successfully with a renal artery stent. There was no deterioration in renal function in the entire cohort [30].

Overall, the investigators found transcatheter renal artery denervation to be effective in the treatment of resistant hypertension. 92% of patients had significant (defined as a reduction in SBP of ≥ 10 mmHg) office-based blood pressure reductions at 1 month following procedure that was sustained to the 24-month follow-up period (average SBP reduction of 32 mmHg, average DBP reduction of 14 mmHg at 12 months following procedure) in the treated cohort. SBP and DBP were noted to be significantly lower than baseline readings at all time-points after procedure with the exception of DBP readings at 12 months [30]. Improvements in blood pressure dipping patterns were also observed with the procedure. Prior to treatment, 67% of the treated cohorts were either nondippers or reverse-dippers. This was noted to reduce to 33% after procedure. Importantly, there was no significant deterioration in renal function and a reduction in renal noradrenaline spillover was noted concurrently with the achieved blood pressure response [30].

A 3-year follow-up in a cohort of 88 (of the original 153 patients) showed that the reductions in blood pressure were noted to persist throughout 36 months, with an average blood pressure reduction of −32/−14 mmHg ($p < 0.01$). Of this group, approximately 50% achieved the goal of a SBP <140 mmHg. A drop in SBP of ≥10 mmHg was seen in 85% of the cohort at 12 months and 93% at 36 months. Furthermore, the proportion of patients with a SBP of 180 mmHg or higher had notably decreased from 30% at baseline to 5% at 36 months. One new renal artery stenosis was reported at 24 months, which was managed successfully with renal arterial stenting, and three deaths unrelated to the procedure were noted during this follow-up period [31]. The results were encouraging, highlighting the efficacy and safety of the procedure and alluding to the lack of functional reinnervation of the kidney over a longer time-frame [31].

Following the success of the Symplicity HTN-1 trial, a second trial was undertaken to evaluate the efficacy of transcatheter renal artery denervation within a randomised cohort. Titled the Renal Sympathetic Denervation in Patients with Treatment Resistant Hypertension or Symplicity HTN-2, the trial essentially mirrored its brother trial in terms of methodology but with the addition of a randomised (but nonblinded) control arm. The primary endpoint of the trial was the between-group change in average office-based measurements of SBP from baseline to 6 months following randomization. Secondary endpoints were of procedural safety, composite of cardiovascular endpoints, and additional measurements of blood pressure reduction at 6 months after randomization [27].

After anatomical screening of the renal artery to confirm eligibility, patients were randomly assigned to the interventional group to undergo catheter-based renal denervation or to a control group, which were isolated to medical therapy only. All patients assigned to the interventional cohort were administered heparin cover intraprocedurally and RF ablation was performed with the Medtronic Symplicity Catheter with the same technique. For both interventional and control groups, changes to baseline doses of antihypertensive therapy



(a) (b)

Figure 2: Catheter-based renal artery denervation procedure.

were advised against unless deemed medically necessary. All patients were followed up at 1, 3, and 6 months following procedure for office-based blood pressure assessment [32].

A total of 106 patients (45 females, 61 males) were included into the study, 52 of whom were allocated to the denervation group and 54 to the control group. Baseline characteristics of patients in both groups did not differ significantly in terms of age, sex, race, baseline blood pressure, and length of antihypertensive therapy [32]. With regard to the primary endpoint, catheter-based renal artery denervation was associated with a significant reduction in blood pressure compared to the control group. At 6 months after randomisation, office-based measurements of blood pressure in the renal denervation group were significantly reduced compared to baseline, a benefit that was reproducible by concordant measurements of home blood pressure and 24-hour ambulatory blood pressure monitoring. Of the treated cohort, 84% had >10 mmHg reduction in SBP, 80% had SBP values below 160 mmHg, and 40% had SBP values below 140 mmHg. Only 10% of the patients had no reduction in SBP. In line with the reduction of blood pressure, a greater reduction in urine albumin-to-creatinine ratio was also observed with the treated cohort [32].

No serious procedural complications were reported after procedure. Seven of 52 patients developed transient intraprocedural bradycardia that resolved with atropine. One femoral artery pseudo-aneurysm and one postprocedural hypotension were reported which was managed with manual compression and reduction in antihypertensive therapy, respectively. No postprocedural renal artery stenosis or aneurysms were noted in the 43 patients (37 renal duplex imaging procedures, 5 MRI procedures, and 5 CT angiographies) receiving renal imaging at 6 months. No deterioration in renal function was noted from baseline in both groups at 6 months [32].

A retrospective cost-benefit analysis of the Symplicity HTN-2 trial was performed by Geisler et al. (2012), which concluded that, given the robust sustained reductions in blood pressure, extrapolation to lifetime clinical probabilities (including CVA, myocardial infarction, all coronary artery diseases, heart failure, and end-stage renal failure) revealed an improvement in median survival for patients undergoing renal artery denervation compared to standard therapy (18.4 years versus 17.1 years). This benefit extrapolated to a cost-saving of 31,460 USD per quality-adjusted life-year, alluding to long-term savings in the face of high short-term costs [33].

All in all, the results of both Symplicity trials highlighted the clinical efficacy and safety of catheter-based renal artery denervation in resistant hypertension. Despite the promising results however, both trials were not without limitations that exceeded just the relatively small employed sample sizes.

Of note was the potential bias in trial design. The Symplicity HTN-1 trial was a "proof-of-principle" study that was unblinded allowing possible selection bias in recruitment of patients and observer bias in the measurements of office-based blood pressure. Though randomised, investigators in the Symplicity HTN-2 Trial were not blinded to the treatment modality and no sham procedures were performed in the control group, similarly raising the possibility of observer bias with the measurement of blood pressure. In both trials, patients with moderate-to-severe renal impairment were excluded, discounting any definitive statements regarding its efficacy in this population. Moreover, only patients with favourable renal artery anatomy to catheter-based renal artery denervation were included into both trials, emphasizing the prospect of dissimilar efficacy and procedural safety in patients with less favourable anatomy undergoing the procedure.

Though office blood pressure measurements were substantiated with ambulatory blood pressure measurements in both trials, they were limited to a small subset of participants and were used primarily to confirm the blood pressure lowering effect of the procedure, casting doubts as to the exact blood pressure response after procedure. Furthermore, the causes of response variability as seen in the 10% nonresponders in the Symplicity HTN-2 trial have not yet been well elucidated, with queries of incomplete denervation

Figure 3: EnligHTN catheter system.

or sympathetic reinnervation foremost in mind. From an operator perspective, the absence of an objective measure of procedural success was also seen as problematic.

5.2. The EnligHTN I Trial. The EnligHTN I trial was a prospective multicentre, nonrandomised, "first-in-human" trial of the multielectrode EnligHTN catheter. The primary efficacy endpoint of the trial looked at the reduction of office BP measurements following denervation procedure at 6 months compared with baseline. Primary safety endpoints were all adverse events that occurred during the study period. Unlike the Symplicity catheter, the EnligHTN catheter (St. Jude Medical) (Figure 3) consisted of an expandable electrode basket housing four Platinum-Iridium-based electrodes. Each electrode was capable of delivering low-level RF ablations to the renal arterial wall and the expandable feature of the basket allowed for establishment of better apposition of electrodes in reference to potential ablation sites on the vessel wall.

Recruitment in this study included patients in varying age brackets (18–80 years of age) who were selected by referral from primary healthcare providers or specialists across four participating centres. Enrolled subjects were required to have a clinic-based SBP measurement of ≥160 mmHg (≥150 mmHg for diabetics) despite prolonged use of at least three antihypertensives (including a diuretic) and suitable renal artery anatomy assessed via renal artery angiography. Participants with small (≤4 mm diameter, ≤20 mm length) [$n = 1$], multiple [$n = 2$], or highly tortuous renal arteries [$n = 0$] were excluded from the procedure as were those with significant (>30%) renal artery stenosis [$n = 4$] [34]. All eligible participants had 24-hour ambulatory blood pressure monitoring with strict compliance to regular antihypertensive regime for at least two weeks prior to enrolment. After this period, participants then had a complete baseline assessment which included collection of basic biochemistry (full blood count, serum creatinine, estimated glomerular filtration rate (eGFR), cystatin C) and urine analysis (urine albumin-to-creatinine ratio) [34].

Renal artery denervation procedure was performed under conscious sedation and local anaesthesia [34]. A minimum of four and maximum of eight ablation sites were performed in each main renal artery in a circumferential pattern, with each ablation lasting 90 seconds. Patients were monitored postprocedurally, with scheduled follow-up visits at 1, 3, and 6 months, and continuing to 24 months. Renal arterial imaging via computed tomography and duplex ultrasonography was repeated at 6 months [34].

A total of 46 (15 females, 31 males) patients were included into the study and underwent the renal artery denervation procedure. With regard to the primary efficacy outcome, investigators of the trial found significant blood pressure reductions with the renal artery denervation procedure performed with the EnligHTN catheters compared to baseline. At 1, 3, and 6 months, average blood pressure reductions of −28/10 mmHg, −27/10 mmHg, and −26/10 mmHg ($p < 0.0001$) were observed, respectively. The blood pressure reductions were sustained to the 18-month period, whereby an average SBP reduction of 24 mmHg was noted, with the majority (77%) of studied participants having a clinically significant response to therapy. In terms of safety, no serious adverse events were noted in the cohort. A nonclinically significant reduction in eGFR was reported at 6 months (baseline 87 ± 19 mL/min/1.73 m^2; at 6 months 82 ± 20 mL/min/1.73 m^2). At 18 months however, no clinically significant changes to renal function were noted [34, 35].

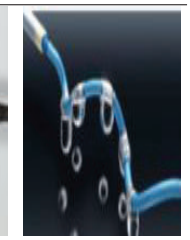
Like the Symplicity HTN-1 study, the EnligHTN trial was a "proof-of-principle" study that was nonrandomised and unblinded, allowing for possible selection bias in the recruitment of patients and observer bias in the measurements of office-based blood pressure. Despite its drawbacks, the results of the trial were promising, essentially mirroring the findings of both Symplicity HTN trials and further supporting the efficacy and safety of renal artery denervation as a highly effective therapeutic option in resistant hypertension. With the success of these early trials, demand for percutaneous devices rose, driving the development of a wide variety of alternative catheter-based systems for renal artery denervation (see Table 1).

6. Renal Artery Denervation: Potential Extended Efficacy

The OLOMOUC I Study (The Effect of Renal Denervation in Patients with Advanced Heart Failure) is an unpublished pilot study by Taborsky et al. investigating the efficacy of renal artery denervation in advanced cardiac failure. In this study, 51 patients with advanced cardiac failure (New York Heart Association (NYHA) Functional Class (FC) III/IV) were randomised to either catheter-based renal denervation plus standard medical therapy or to solitary standard therapy with follow-up over a 12-month period. Primary endpoints of the study looked at left ventricular systolic function calculated by 2D echocardiography and safety profile of the denervation procedure. Secondary endpoints were that of resting heart rate, renal function, NT-proBNP levels, and status of NYHA FC [36]. Inclusionary criteria comprised patients with NYHA FC III and/or IV heart failure who were stable on optimal medical therapy over a 6-month period prior to the intervention, suitable renal artery anatomy, resting heart rate > 70 beats per minute, and a eGFR > 50 mL/min/1.73 m^2 [36].

Overall, the intervention group saw a modest improvement in left ventricular ejection fraction (LVEF) [mean LVEF 25% at baseline to 31% at twelve months ($p < 0.01$)] relative to the control group [mean LVEF 26% at baseline to

Table 1: Renal denervation catheter systems.

Catheter type	BSC Vessix	MDT Symplicity	MDT Spyral	STJ EnligHTN	COV OneShot	ReCor Gen-2 Paradise	JNJ Thermo-Cool
Picture							
Catheter design	Balloon catheter 4–8 electrodes	Catheter with single electrode	Pigtail catheter 4 electrodes	Basket with four electrodes	Balloon catheter helical electrode and cooling	Balloon catheter, internal cooling	Pigtail catheter with 5 electrodes and cooling
Energy	Bipolar RF	Monopolar RF	Monopolar RF	Monopolar RF	Monopolar RF	Ultrasound	Monopolar RF
Power	~1 W	8 W	8 W	6 W	25 W	~12 W	15 W

28% at twelve months ($p = 0.36$)]. Other markers of left ventricular impairment (LVESVI, LVEDVI, and NT-proBNP) were similarly improved. In addition to the improvements in left ventricular function, there was a trend towards lower rehospitalisations for heart failure (8 versus 18) in the intervention arm ($p < 0.001$). Two complications, however, were registered in the intervention arm; one patient developed a femoral fistula formation and the second had formation of postoperative thrombus [36].

The findings of this yet-to-be-published trial indicate an additional benefit of denervation therapy, with proposed reduction in neurohormonal substrates for maintenance and progression of cardiac failure. These changes in sympathetic activity, with downstream changes in hormones related to left ventricular remodeling, may represent an additional tool in the management of advanced heart failure with reduced ejection fraction, independent of its effects on afterload reduction.

Another study, published in early 2012, investigated the effect of renal artery denervation therapy on left ventricular parameters, including echocardiographic indices of systolic and diastolic function. This study employed 64 participants, which were either placed into the treatment arm ($n = 46$) or control arm ($n = 18$). Patients over the age of 18 with a clinic-recorded SBP ≥160 mmHg despite management with three antihypertensives (including a diuretic) were included into the study. These subjects were followed over a 6-month period, with transthoracic echocardiography performed at baseline, 1 and 6 months [37].

Echocardiographic endpoints included LVEF, left ventricular mass index (a marker of left ventricular hypertrophy), mean interventricular septal thickness, and mitral inflow parameters (lateral E/E′, isovolumic relaxation time) measured via Doppler echocardiography. Besides a sustained reduction in office-based blood pressure (SBP/DBP −27.8/−8.8 mmHg at 6 months, $p < 0.001$), a significant reduction in markers of diastolic impairment (interventricular septal thickness, left ventricular mass index, mitral valve lateral E/E′, isovolumic relaxation time) was appreciated in the treatment group. Additionally, a statistically significant improvement in LVEF was noted (baseline LVEF: 63.1 ± 8.1% versus 70.1 ± 11.5% at 6 months, $p < 0.001$) [37].

Interestingly, although regression of parameters of left ventricular diastolic dysfunction was incremental with SBP reduction, "nonresponders" (patients which demonstrated <10 mmHg SBP reduction at 6 months following renal artery denervation) still demonstrated a marked reduction in these indices [37]. This finding would support the hypothesis that renal artery denervation causes regression of left ventricular remodeling independent of its effects on blood pressure.

This improvement in cardiac function has been shown to translate to a symptomatic benefit in a recent trial by Davies et al. (2013), which assessed seven patients with chronic heart failure with reduced LVEF receiving renal artery denervation therapy over a six-month period. Of interest, the mean blood pressure on referral was 112/65 mmHg (normotensive range), significantly lower than that employed in the majority of trials [38].

Following denervation, a nonsignificant trend towards blood pressure reduction was found (SBP −7.1 ± 6.9 mmHg, $p = 0.35$; DBP −0.6 ± 4.0 mmHg, $p = 0.88$) at 6 months, with no hypotensive events noted. Renal function was unaffected. All seven patients reported a significant symptomatic improvement, with significant quantitative improvement in six-minute walk distance at 6 months ($\Delta = 27.1 \pm 9.7$ m, $p = 0.03$). No procedural or postprocedural complications were noted [38]. The novel finding of this study, in addition to improvement of symptom status, was the lack of haemodynamic instability achieved after renal artery denervation in normotensive patients, which may indicate a secondary compensatory mechanism to maintain SBP within acceptable limits for organ homeostasis despite sympathetic disruption or may indicate nonefficacy of the procedure in blood pressure control.

In addition to the previously mentioned effects on left ventricular remodeling, cardiac performance, and symptom

status in congestive cardiac failure, studies have also demonstrated the efficacy of renal denervation in patients with impairment of the baroreflex sensitivity. In a study by Zuern et al. (2013), 50 patients with resistant hypertension and a mean ambulatory SBP of 157 ± 22 mmHg were enrolled in the prospective cohort study and underwent renal denervation therapy. At six-month follow-up subsequent to procedure, 26 patients (52%) achieved a drop in mean ambulatory SBP of ≥10 mmHg. Upon review, impaired baroreflex sensitivity was strongly associated with response to renal denervation ($p < 0.001$) [39].

7. The Symplicity HTN-3 Study

The Symplicity HTN-3 study was a multicentre, prospective, double-blinded, randomised study investigating the efficacy and safety of renal arterial denervation using the Symplicity catheter system in patients with medically refractory hypertension. Like the previous Symplicity HTN trials, the primary efficacy endpoint of the study looked at the change in office SBP measurements at 6 months. Secondary efficacy endpoints of the trial differed, looking at the change in mean 24-hour ambulatory SBP. The primary safety endpoint was a composite of major adverse events (defined as death from any cause, end-stage renal disease, an embolic event resulting in end-organ damage, renal artery or other vascular complications, or hypertensive crisis within 30 days or new renal artery stenosis of more than 70% within 6 months) [40].

Inclusionary criteria were that of resistant hypertension (SBP ≥ 160 mmHg) despite three or more maximally tolerated antihypertensive medications (including a diuretic) in the absence of haemodynamically significant valvular disease or renovascular abnormalities determined via angiographic assessment. All recruited patients underwent a confirmatory screening visit beforehand to confirm SBP of >160 mmHg and adherence to medications. Once included, patients were then randomised in 2 : 1 fashion to treatment arm and control arm, respectively. Those in the control group underwent renal angiography only (sham control). Patients in the interventional arm underwent the renal denervation procedure which was performed with the Medtronic Symplicity Catheter [40].

A total of 535 patients (325 males, 210 females) were included into the study, 364 of whom were allocated to the interventional cohort and 171 to the control cohort. Procedural technique and periprocedural pharmacotherapy of the interventional arm were unchanged from the previous Symplicity trials. Regardless of group, all blood pressure assessors were unaware of study group assignments and a blinding index was utilised to verify the effectiveness of blinding at hospital discharge and at 6 months. Patients were subsequently followed up at 6-month intervals after randomisation (with an aim follow-up up to 5 years). Changes to baseline doses of antihypertensive therapy were not encouraged unless deemed medically necessary [40].

In terms of safety, no significant difference was noted in terms of overall composite adverse events between denervation and control groups. The rate of major adverse events in the denervation group was 1.4% compared to 0.6% in the control group ($p = 0.67$). No significant changes in renal function were observed between both groups [40].

With regard to efficacy endpoints however, investigators surprisingly found no clinically significant changes in baseline SBP between both groups. At 6 months, an average office blood pressure reduction of −14.3 ± 23.93 mmHg was noted in the intervention arm compared to −11.74 ± 25.94 mmHg in the control group with a between-group difference of −2.39 mmHg (95% confidence interval [CI], −6.89 to 2.12; $p = 0.26$ with a superiority margin of 5 mmHg) [40]. Ambulatory blood pressure reductions were similarly non-significant, with an average reduction of −6.75 ± 15.11 mmHg in the denervation group and −4.79 ± 17.25 mmHg in the control group at 6 months, for a between-group difference of −1.96 mmHg (95% CI, −4.97 to 1.06; $p = 0.98$ with a superiority margin of 2 mmHg). There was also no significant between-group difference in terms of change in heart rate from baseline to 6 months (−3.8 ± 11.2 beats per minute in the denervation group and −2.7 ± 10.9 beats per minute in the sham-procedure group; $p = 0.30$) [40].

Though the trial essentially confirmed the safety of the procedure, the negative findings in terms of efficacy were sobering and essentially contradicted the findings of the other Symplicity HTN and denervation trials.

Investigators and stakeholders raise several possibilities to explain the discrepancy of the findings. Of note was the difference in population studied. Whilst there were no significant differences in terms of baseline characteristics between the two arms in the Symplicity HTN-3 trial, the trial included a significant number of African Americans (90 patients in the intervention arm, 50 patients in the control group), a demographic that was not present in the previous Symplicity trials. Subgroup analysis of this demographic showed a paradoxical effect of denervation therapy, with preferential (albeit not statistically significant) blood pressure lowering effects in the control arm as opposed to the Caucasian cohort [41, 42].

Patient characteristics and medication profiles between the Symplicity HTN-2 and Symplicity HTN-3 trials likewise differed, with a higher proportion of obese patients, patients with increased cardiovascular risk factors, and patients with greater use of diuretics and aldosterone antagonists as part of their antihypertensive regimen included into the Symplicity HTN-3 trial. In addition, participants included in the trial only received maximal antihypertensive therapy for two weeks prior to evaluation of efficacy, whilst a recommendation for at least two months is endorsed by current guidelines on hypertension, raising the possibility that patients with an incorrect diagnosis of resistant hypertension were included into the trial [43].

From a procedural and technical perspective, a large proportion of operators in the Symplicity HTN-3 trial had no previous experience with the procedure and as such may have been less experienced compared to the site-specific trained Symplicity HTN-1 and -2 operators. Moreover, whilst indirect electrical impedence was utilised to discern contact with the arterial wall and thus guide the positioning of the catheters, there were unfortunately no objective measures of procedural success. As such, inadequate thermoablation was possible, regardless of the experience of the operator [41, 44].

Finally, in the Symplicity HTN-3 trial, there were tighter entry control criteria with regard to ambulatory blood pressure. As part of the inclusionary criteria, ambulatory blood pressure monitoring was performed with the aim of excluding patients with pseudo- or white-coat hypertension. The same did not hold true for the Symplicity HTN-2 trial. This may have led to overestimation of initial blood pressure measurements in the earlier Symplicity trials and thus led to lower follow-up blood pressure results.

8. Conclusion

Given the heavy clinical and economic burdens of hypertensive disease, new treatment methodologies are currently being explored. Catheter-based renal artery denervation technology is one of the novel treatments in the management of resistant hypertension. Based on the concept of sympathetic nerve modulation, many were initially in favor of the technology given the promising results of the early trials. In view of the disheartening results of the Symplicity HTN-3 trial however, widespread utilisation of the procedure has now become controversial.

Skeptics allude to the lack of compelling evidence regarding efficacy in the trials and the significant profit driven goals with the technology. Advocates on the other hand argue that further vigorous randomised trials are required before complete disbanding of the technology and state that while the use of renal artery denervation in blood pressure reduction may be contentious, the efficacy of the technology in other areas is yet unproven and potentially beneficial.

As smaller studies have shown benefit in left ventricular remodeling, cardiac performance, and symptom status in patients with cardiac failure when utilized as an accessory tool to medical therapy, larger trials are recommended to assess the effect of this modality in this group. Additional double-blinded trials would also be recommended in patients with impaired baroreceptor sensitivity, as this represents a potentially vulnerable group of sympathetic hypertensive diseases which may be amenable to percutaneous catheter renal denervation therapy.

Disclaimer

All authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Hemodynamic and Biologic Determinates of Arteriovenous Fistula Outcomes in Renal Failure Patients

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The outcome of patients with end-stage renal disease on hemodialysis depends on a functioning vascular access. Although a variety of access options are available, the arteriovenous fistula remains the best vascular access. Unfortunately the success rate of mature fistula use remains poor. The creation of an arteriovenous fistula is followed by altered hemodynamic and biological changes that may result in neointimal hyperplasia and eventual venous stenosis. This review provides an overview of these changes and the needed research to provide a long lasting vascular access and hence improve outcomes for patients with end-stage renal disease.

1. Introduction

End-stage renal disease (ESRD) affects an increasing number of patients each year with a staggering estimate of almost 640,000 patients receiving dialysis at the end of 2012 in the United States [1]. The primary cause of ESRD is diabetes followed by hypertension [1]. Patients with ESRD have a high prevalence of concomitant cardiovascular disease, which is the primary cause of morbidity and mortality in this patient population [1]. The most common modality chosen for renal replacement therapy is hemodialysis, for which a vascular access is required. The vascular access choice influences and contributes to the overall morbidity and mortality of the patient [2, 3].

Hemodialysis vascular access type includes the preferred arteriovenous fistula (AVF), the arteriovenous graft followed by a central venous catheter [1, 2]. The best access to place with least complications is the AVF. When an AVF is placed, an artery is anastomosed to a vein and over a period of 2-3 months the vein becomes "arterialized," a process that is necessary prior to use of hemodialysis [4]. The first access recommended is the lower arm radiocephalic fistula (RCF) although these commonly fail especially in the elderly and those with diabetes [5, 6]. The second preferred site for an AVF is the brachiocephalic (BCF) which are being placed at an increased number. The third fistula configuration recommended is a brachiobasilic (BBF). The problem is that many of these fistulas fail for unknown reasons. One-year patency rates range from 60 to 65% [7, 8], with 60% of fistulas not suitable for dialysis between 4 and 5 months after surgery [9]. Medical management with antiplatelet agents such as ASA and Clopidogrel have failed to make a difference [10, 11]. This is likely due to the fact that these agents do not address the primary cause of access failure, neointimal hyperplasia (NH) leading to venous stenosis.

Once venous stenosis occurs with clinical symptoms such as painful swelling of an extremity, skin ulceration, venous hypertension, or subsequent poor function of the access, treatment options include angioplasty, stent, or surgical revision [12]. The treatment is dependent on the specific site, characteristics, and hemodynamics of the lesion [12]. For example, the most common location for stenosis in a RCF is near the anastomosis, while cephalic arch stenosis is frequently encountered in BCF [12, 13]. Cephalic arch stenosis is often treated with repeat angioplasty and stenting until fistula failure occurs [13].

Venous stenosis as a result of NH is poorly understood [14, 15]. There are multiple factors which influence the outcome of an AVF including demographics, adjuvant therapies, underlying histology, cytokines, oxidative stress, and hemodynamics [16, 17]. There are few trials which look at the biology of why a fistula fails or address treatment

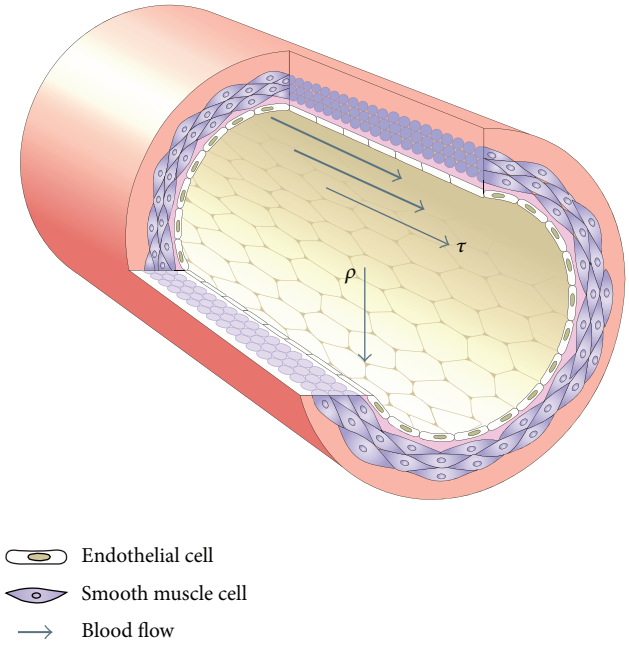

Figure 1: Schematic of a vessel. The white layer shows smooth endothelial cells; the purple layer smooth muscle cells; ρ shows direction of pressure; τ shows direction of wall shear stress. Figure reprinted by permission from Macmillian Publishers Ltd.: Nature Reviews Molecular Cell Biology, 10, 2009.

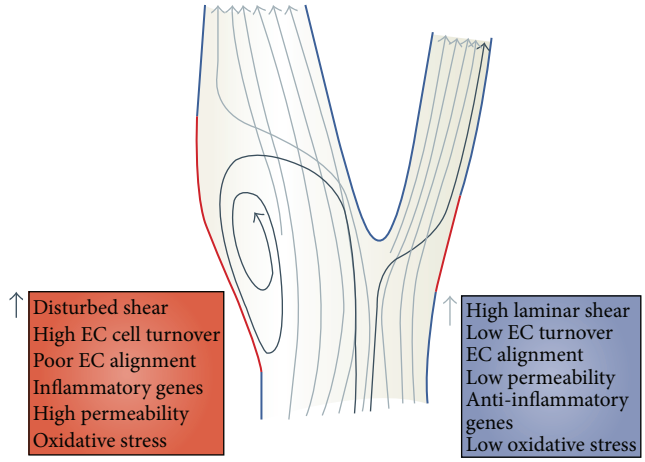

Figure 2: Schematic of a vessel with disturbed shear flow on the left and laminar flow on the right. Figure reprinted by permission from Macmillian Publishers Ltd.: Nature Reviews Molecular Cell Biology, 10, 2009.

options in prospective trials. This review highlights known hemodynamic and biologic determinates of fistula failure and suggest research areas which need to be explored.

2. Hemodynamics of an Arteriovenous Fistula (AVF)

Creation of an AVF requires a surgical anastomosis of a high pressure artery to a low pressure vein which causes an increase in wall shear stress (WSS) and tension. The pressure increase in the venous outflow tract will lead to medial thickening, the definition of venous arterialization. Pressure is defined as the perpendicular force (ρ) exerted in a vessel, whereas the WSS is the parallel force (τ) (Figure 1) [18]. Normally the luminal diameter will increase in an attempt to reduce the WSS back to pre-AVF levels. The next result is a dilated vein with a thickened media, the perfect vessel for a fistula suitable for use for hemodialysis. Corpataux et al. [19] summarized this phenomenon in a study where hemodynamic changes were demonstrated in six patients with a lower arm AVF using an ultrasound Doppler device. Within the first week after fistula creation, the blood flow and WSS increased substantially in the vein. The increased flow resulted in a venous luminal diameter increase, a process necessary for cannulation. The WSS gradually returned back to normal by 12 weeks. In this study, findings were also apparent at the arterial side, though to a lesser extent [19].

Problems arise in vasculature physics when a bend or curve happens which is frequently the case especially when an AVF is being constructed. Normal flow through a straight vessel is smooth and laminar as shown by the vessel on the right side of Figure 2 [18]. The endothelial cells in this instance are at steady state, with low cell turnover, low permeability, and low level of anti-inflammatory genes and oxidative stress. The area of abnormal WSS (red) is minimal. However, when a bend or curve arises laminar flow becomes turbulent as shown in the left side of Figure 2 [18]. With turbulent flow the endothelial cell turnover is high with poor alignment, inflammatory genes activation, and increase in oxidative stress. The area of abnormal WSS is much larger. Abnormal turbulent flow causes low WSS, denuding endothelial cells, excitation of pathways which eventually lead to NH [16]. Jia et al. have recently shown in study of AVF creation in canines that NH has a strong inverse correlation with WSS levels and also is related to flow patterns [20].

3. Underlying Histology and Progression to Neointimal Hyperplasia

The basic histology of an artery and a vein is very different (Figure 3) [21]. A normal artery has a smooth endothelial cell lining with the tunica intima defined as the boundary of the endothelial cell to the elastic lamina. The tunica media in an artery is normally much thicker than a vein with an increased amount of elastin [21]. When a fistula is created for hemodialysis, the patient often had chronic renal failure for several years, which causes underlying changes in the vein and artery including increased arterial and venous calcification [22] and NH [23]. When a fistula is then created, the changes in WSS and pressure sensed by the endothelial cells signal vasodilating agents, such as nitric oxide (NO), growth factors that control vascular smooth muscle cell (VSMC) migration and proliferation, and cellular adhesion molecules [17]. Upregulation of proteases such as matrix metalloproteinase and cathepsins result in matrix degradation and restructuring of the luminal expansion [17]. Little is known about the necessary outward remodeling of

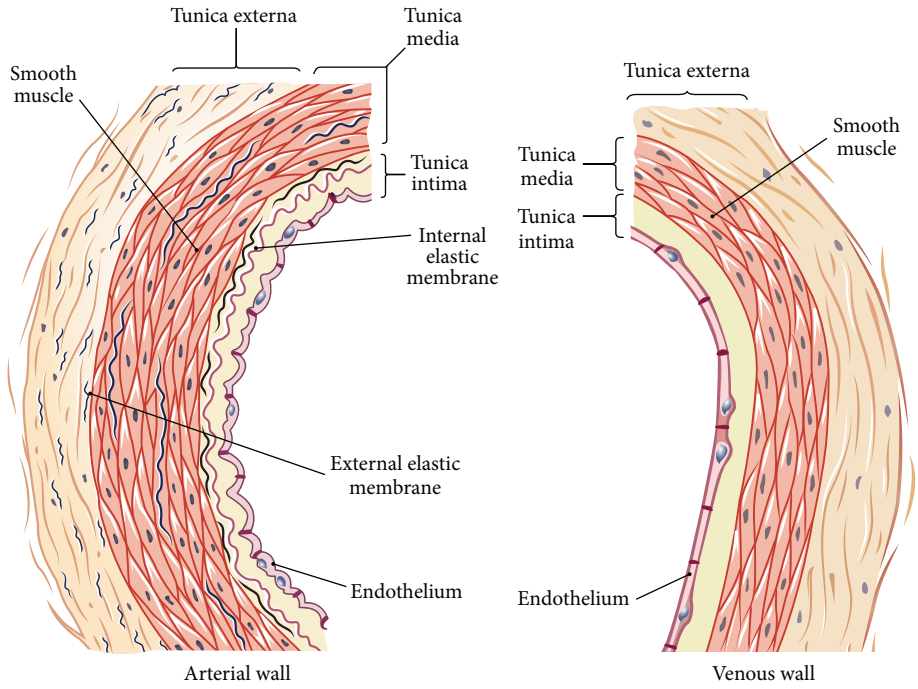

Figure 3: Schematic of an arterial wall on the left and venous wall on the right. Note increased tunica media in an arterial wall.

VSMC, although this is highly important [17]. In summary, the underlying histology of the vessels used to create an AVF, specifically calcification, elastin, and collagen deposition, predicts the ability of a vein to dilate after fistula creation.

The morphometric and histologic characteristics of the veins used for fistula creation have been studied. Lazich et al. have shown that the vein used to create BCF is larger in diabetics when compared to nondiabetics [24]. In particular the internal lumen and intimal and medial area were all found to be more dilated in diabetics [24]. This altered remodeling may be beneficial as previous reports have shown that cephalic arch venous stenosis is attenuated in diabetics with BCF [25–27]. Vascular wall remodeling differs between diabetics and nondiabetics with increased NH in the former [23, 24]. As NH progresses, this can dramatically decrease the lumen size and lead to negative vascular remodeling and vasoconstriction. All components of the vein including the adventitia are now recognized as contributing to the process of NH after fistula construction [8]. Most past research focuses on NH although outward remodeling is an equally important process that could preserve the vein lumen and predict the outcome of the AVF over time [8]. This critical balance between NH and outward remodeling in a vein when a fistula is created requires further exploration.

4. Nitric Oxide (NO), Asymmetric Dimethylarginine (ADMA), and Vasodilator Effects

Fistula creation causes a passive vascular distension and a dramatic release of NO from the endothelial cells [28]. The turbulent flow induced by creation of a fistula causes an increased global shear stress which increases nitric oxide synthetase converting L-arginine to NO (Figure 4) [28]. The NO activates soluble guanylyl cyclase (sGC) to cause GTP to be converted to cyclic guanosine monophosphate cyclic GMP causing smooth muscle cell relaxation. There is a delicate balance between endothelial cell activation which is needed for vein dilation and endothelial cell dysfunction [29]. Endothelial cell dysfunction could result from increased oxidative stress and lead to vasoconstriction and smooth muscle cell proliferation which could result in NH and contribute over time to access failure.

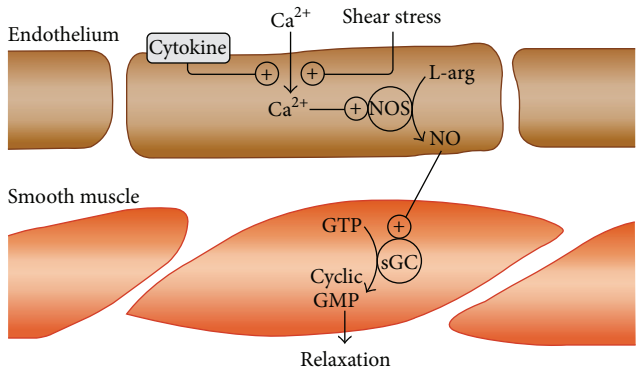

Figure 4: Schematic of vascular endothelium. Shear stress induces calcium dependent activation of nitric oxide synthetase resulting in smooth muscle relaxation. Figure reprinted by permission from Molecular Diversity Presevation International: International Journal Molecular Science, 13, 2012.

A contributing factor to endothelial cell dysfunction is a dramatic elevation of asymmetric dimethylarginine (ADMA) known to be increased in patients with renal failure [30]. ADMA, a metabolic by-product of protein metabolism inhibits the conversion of L-arginine to NO, reducing vascular compliance, increasing vascular resistance, and limiting blood flow [30]. Hammes et al. showed a 10-fold increase in ADMA in patients with ESRD when compared to patients without renal failure [31]. The procedure of hemodialysis itself is also associated with an even greater rise in ADMA [31]. Efforts to understand the complex pathway that leads to devastating ESRD and corresponding vascular disease have identified an accelerated calcific process, observed even in small vessels. Elevated ADMA is a step in the pathway of vascular disease in renal failure as it has been found to contribute to CKD progression with an associated increase in transforming growth factor TGF-β1 and subsequent vascular collagen deposition. [32]. The biologic effects of ADMA on AVF contribute to venous stenosis [33] and are the subject of future research investigation.

The vascular endothelium in an artery and vein respond differently to blood flow, especially if there is associated atherosclerosis [34]. The release of eNOS and resultant NO is necessary for adequate vein dilation, a part of arterialization. Endothelial cells in arteries and veins are structurally and functionally different including the ability to adjust to changes in shear stress and release of eNOS [35]. There are many inhibitors of NO, of most interest are Rho-kinases (ROCKs). ROCKs are small guanosine triphosphate binding proteins which mediate smooth muscle contraction, cell migration, and proliferation by inhibition of eNOS [36]. Inhibition of ROCKs has been shown to decrease NH [37]. Molecular events which cause NO release are under the control of ROCKs and warrant exploration in an AVF model.

5. Cytokines/Inflammation

Inflammation is a primary stimulus for NH. There is marked upregulation in proinflammatory genes and progressive neointimal formation in the venous vasculature in an AVF which contributes to the aggressive venous stenosis observed. Nath et al. [38] has shown an upregulation of genes including TGF-β1 which, in the venous vasculature in the AVF model in the rat, are accompanied by intimal hyperplasia. NH occurred in variable degrees by 5 weeks after establishing a fistula, and by 16 weeks, such NH was progressive and pronounced with abundant extracellular matrix. In human subjects, levels of inflammatory biomarkers have been harvested in surgically thrombosed fistulae. Pronounced intimal thickening in stenosed fistulas was associated with expression of TGF-β1 and insulin-like growth factor when compared to controls [39].

Research in coronary artery bypass grafts has given clues as to the mechanism of venous stenosis in arterialized veins used for hemodialysis. Graft failure is common following coronary artery bypass grafting [40, 41] puzzling vascular biologists and surgeons as to mechanisms. Yuan et al. [42] have shown that severe vascular wall degeneration and collagen deposition together with overexpressed TGF-β signaling cytokines were responsible for failure (early and late) of the saphenous vein and radial artery grafts. As TGF-β is responsible for NH in a number of vascular disease models that have similarities to the arterialized vein in an AVF, targets to impede this early gene signaling may be future directions to help retard the aggressive NH which occurs in AVF.

6. Oxidative Stress of Dialysis

Patients with renal failure have several risk factors which predispose them to oxidative stress, including age, and underlying disease including diabetes and hypertension. Hemodialysis is a treatment which contributes to this oxidative stress by evoking a dramatic change in the blood flow through a fistula. The continuous volume and pressure changes as a result of intradialytic volume gains can cause significant physiologic stress on the endothelium of a vein. Moreover the hemodialysis treatment itself has been shown to shear endothelial cells and reduce nitric oxide formation [43]. When an access is considered "mature" and cannulation begins, this could also worsen the oxidative stress contributing to NH [44, 45].

Oxidative stress has been linked to atherosclerosis by contributing to endothelial dysfunction and intima-media thickness. Weiss et al. [46] used markers of oxidative stress to study 11 AVF and 15 AVGs at the time of surgical resection or revision. Markers of cell growth and proliferation were endothelin-1 (ET-1), a potent mutagenic peptide implicated in the formation of intimal hyperplasia: TGF-β, a stimulus to vascular cell growth and matrix production and platelet-derived growth factor (PDGF), a mediator of intimal hyperplasia. All specimens studied showed significant NH. The neointima close to the vascular lumen of the AVF and the pseudointima close to the lumen of the ePTFE graft were positive for oxidative stress markers. At sites of injury, as evident by histological inflammation and healing, expression of oxidative markers was more intense. These findings support intimal injury and resultant oxidative stress as a direct result of fistula construction and cannulation contributing to NH.

7. Future Directions

Intensive research to determine the early events that trigger NH in an arterialized vein is needed as the process of NH starts when the fistula is created [17]. The optimal mismatch of shear stress and pressure in both the vein and artery are necessary to allow for some medial thickness without aggressive NH setting in. Anastomotic design and strategies and devices to define optimal WSS are in the process of being developed [47]. Computational models have been developed and are able to predict the clinical relevance of WSS in predicting AVF maturation and venous stenosis [48–50]. Randomized clinical trials are needed to determine the utility of CFD to improve AVF outcomes. NO production and VSMC reorganization in outward remodeling in AVF likely play a role in the ability of a vein to mature to support dialysis and are targets of future research. Early vascular biological events need to be unraveled.

The current approach to placement of a vascular access should be revisited. The goal of preoperative evaluation is to provide a well-functioning access for a patient that will last a life span of a patient with ESRD. Current work-up includes physical exam, preoperative color duplex Doppler ultrasound, and/or venogram to determine suitable arterial/vein diameters and adequate blood flow. Assessment of Doppler ultrasonographic assessment of flow-mediated dilatation has been used to assess preoperative vascular health but has not been found to predict fistula success [51]. The diameter of the vein has been shown to correlate with successful outcome in some but not all studies [52]. The intraluminal area and virtual histology available by such tools as intravascular ultrasound enable a more indebt assessment of endothelium [53]. This diagnostic procedure may provide intraluminal images, allowing for more precise assessment of veins, suitable and adequate for vein maturation, than external luminal diameters provided by traditional methods. A multifactorial approach evaluating arterial and venous function is necessary to predict AVF success.

The definition of a mature AFV is vague. Current guidelines define a mature access as one that has a blood flow of at least 600 mL/min and is 6 mm in diameter and less than 6 mm below the skin surface. In clinical practice, these parameters cannot be reliably measured in an outpatient setting. Recent investigation has defined the type of blood flow in a fistula as an important determinate in maturation [54]. Cannulation techniques and skills must improve. Continued education in the anatomy of the AVF and physical assessment is crucial. New techniques for cannulation of difficult or deep veins are being developed [55]. The rapid blood flow with hemodialysis causes excessive turbulence and may not be optimal to the endothelium of the arterialized vein, predisposing to NH. We need to decrease inflammation as much as possible with each dialysis treatment. Surveillance to detect access dysfunction including high flow states needs to be refined [56]. In summary, we must continue to investigate the hemodynamics and vascular biology of the AVF and develop better clinical parameters that confirm adequate AVF function if the outcomes are going to improve.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Review Article

Guided Tissue Regeneration in Heart Valve Replacement: From Preclinical Research to First-in-Human Trials

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Heart valve tissue-guided regeneration aims to offer a functional and viable alternative to current prosthetic replacements. Not requiring previous cell seeding and conditioning in bioreactors, such exceptional tissue engineering approach is a very fascinating translational regenerative strategy. After *in vivo* implantation, decellularized heart valve scaffolds drive their same repopulation by recipient's cells for a prospective autologous-like tissue reconstruction, remodeling, and adaptation to the somatic growth of the patient. With such a viability, tissue-guided regenerated conduits can be delivered as off-the-shelf biodevices and possess all the potentialities for a long-lasting resolution of the dramatic inconvenience of heart valve diseases, both in children and in the elderly. A review on preclinical and clinical investigations of this therapeutic concept is provided with evaluation of the issues still to be well deliberated for an effective and safe in-human application.

1. Introduction

Cardiac diseases (CVD), particularly heart valvulopathies, dramatically affect the world population representing in 2012 the primary cause of death for 17.5 millions of people. This number is expected to almost duplicate by 2030; thus, the World Health Organization is prompting a sustained programme for effective CVD prevention, management, and monitoring [1–3].

Characterized by rheumatic, degenerative, or genetic etiology, heart valve diseases interest both pediatric and adult patients and at end-stage, they require surgical intervention of reconstruction of the ventricular outflow tract, as well as correction of its components to restore the unidirectionality of blood flow among the heart chambers.

Standard healthcare procedures foresee the implantation of valve prostheses of either mechanical or biological composition, for which life-long anticoagulation, inability to repair/grow, or structural deterioration is considered the main hindrance for a long-lasting functional treatment in cardiopathic patients. Among the current valve prostheses, allogeneic valvulated conduits, commonly named homografts, represent the most akin replacement to a native tissue, even if their availability is seriously limited by organ donation shortage. Despite the high structural and functional resemblance, valve homografts have been reported to degenerate in nearly 15 years, like their animals-derived counterparts submitted to xenoimmunological shielding through glutaraldehyde treatment. At the basis of heart valve bioprosthetic degeneration are recipient's inflammatory and immune responses directed against allo-/xenogeneic epitopes, as, for example, the human leukocyte antigens (HLA) II or animal immunological triggers.

In the search for the effective replacement, a close similarity to the native valve is desirable. Adequate hemodynamic profile, facilitated diffusion of oxygen and nutrients, tissue adaptation to somatic growth, and full biocompatibility in terms of absent inflammatory/immune stimuli and thrombogenic power are pursued in the design and manufacturing processes of the ideal valve substitute [4] by application of tissue engineering principles. A multidisciplinary strategy is addressed to the development and creation of viable surrogate tissues for a prospective replacement of malfunctioning valves. Synthetic or natural scaffolds are engrafted with differentiated or stem cells and submitted to dynamic stimulation in bioreactors, conceived to simulate the complex mechanical and chemical prompts able to generate a physiological tissue/organ maturation [5].

Synthetic polymers, such as polylactic and polyglycolic acids (PLA and PGA resp.), have been the first scaffolds applied for tissue engineered heart valves (TEHVs). Thanks to the tunable porosity and bioadsorbability, these biopolymers facilitate cell infiltration and likely *de novo* deposition of extracellular matrix (ECM) by the same engrafting elements. Cell type selection for TEHVs has been heterogeneous. From differentiated cells, as saphenous vein-derived endothelial and fibroblast cells, to progenitors or less committed elements, as mesenchymal or endothelial progenitor cells, a strong propensity for adhesion and/or tissue integration has been demonstrated [6–8]. Specifically, accurate studies on heart valve embryonic developmental steps fostered the implementation of the manufacturing process with cues essential for a boosted cell differentiation [9–11]. A dynamic conditioning for at least 14 days was reported to be sufficient for effective maturation of cell-repopulated synthetic scaffolds prior to implantation.

While several technical advancements have been proposed for *in vitro* formulation and *in vivo* delivery of such TEHVs, specific flaws still hamper their clinical applicability. Inability to reach mature composition, distribution, and conformation of newly synthesized ECM, in terms of collagen, glycosaminoglycans, and furthermore elastin network, prevents the achievement of proper hemodynamic functionality in the right circulation system, where these valve prototypes are usually tested. Improper valve stratification and cusp thickening provoke incompetent leaflet coaptation and, hence, regurgitation events, which could be expected to worsen in conditions of arterial circulation.

Conversely, a natural scaffold has already reached the state of maturation of its extracellular matrix fibers. After effective removal of endogenous cellular elements by means of enzymatic and/or detergents-based decellularization, it can be implemented as a starter matrix for further colonization with selected cell types.

Apart from classical TEHVs, a novel valve substitute with *bona fide* self-regeneration ability has been advanced, namely, the tissue-guided regenerated replacement.

This review paper aims to evaluate the evolution steps of heart valve tissue-guided regeneration from the first bench experiments to the outcomes of the current bed applications.

2. Which Scaffold? and Which Decellularizing Manipulation?

The concept of tissue-guided regeneration has been first introduced with reference to the medical branches of dental and bone bioengineering, by implying the implantation of biomimetic scaffolds able to stimulate themselves the regeneration process without further factors or biological signals [12]. In sharp distinction to the classical tissue engineering conception, it does not contemplate the creation *in vitro* or *in situ* of a living tissue, but the induction of healing and adaptive remodeling through host's cell engraftment and natural physiological conditioning (body as a bioreactor). Conceived, instead, by many researchers as an exception or a peculiar modality of tissue engineering, it has been applied more recently to other medical fields, as in cardiovascular regenerative medicine.

The combination of cells and synthetic scaffolds has not be sufficient itself to reproduce the complexity of a vital and functional 3D tissue. In order to drive effective homing and instruct cells to proper differentiation and integration, scaffolds should ideally possess also matrikine signals and adhesion proteins typical of a healthy native tissue. Matrikines are classified as extracellular matrix domains able to interact with specific cell surface molecules belonging to the large family of cytokines, chemokines, and growth factors. In concert with adhesion receptors, for example, ligands to integrins, these proteins significantly influence cell proliferation, migration, and differentiation [13]. Composition and 3D spatial distribution of such domains in the starting matrices are thus fundamental for successful healing processes and discriminate a biomaterial with self-regenerating abilities from a scaffold leading to immature or pathological tissue formation [10, 14, 15].

Full reverse engineering of a native valve tissue ECM by artificial manufacturing is still a remote prospect. Outflow tract heart valves are, in fact, highly complex structures with intrinsic biological and biomechanical properties granting their opening and closure for virtually 3 billion times during the entire lifespan [10, 16].

Biological assets, as ECM anisotropy and its dependent cell distribution and specializations, as well as precise biomechanical characteristics, as specific coaptation height and minimum leaflet flexion, influence functionality and durability of native aortic valves.

Natural heart valves have been implanted in replacement surgery since the 1970s, by adopting animal tissues earlier submitted to glutaraldehyde treatment for immunologic shielding and ECM stabilization [17]. With the pioneering surgical procedure introduced by Ross and colleagues [18, 19], valve homografts entered the full clinical stage, being applied as replacement of the autologous pulmonary root, resected and heterotopically implanted in aortic position. Unfortunately, longevity of these valves in the patients is poor for mid-/long-term occurrence of structural deteriorations.

Many hypotheses have been advanced in relation to bioprosthetic degeneration, as, for instance, the higher mechanical stress introduced by the surgical implantation or the lack of viable cells induced during glutaraldehyde chemical treatment or after cryopreservation [20, 21]. In particular, the cytotoxicity of glutaraldehyde and its free aldehyde groups provokes in treated cells firstly a suffering state and then their death with extracellular space release of phosphate groups, as phospholipids, in the form of the so-called matrix vesicles, and nucleic acids that act as powerful nucleation sites for calcification [21]. To improve the stability of glutaraldehyde-treated tissues, several technical adjustments have been attempted. Organic solvents, for example, opportune mixtures of ethanol, octanediol, and/or octanol, have been applied during bioprosthetic valve manufacturing to extract cell membrane phospholipids, as well as to crosslink collagen-elastin network [22]. Moreover, detoxification processes have been introduced to neutralize free aldehyde groups of glutaraldehyde. These chemical agents are indeed particularly

toxic for their ability to interact with primary amino groups of proteins or nitrogen atoms in DNA, giving rise to Schiff bases. In addition, they are able to trap plasmatic calcium further contributing to the triggering or amplification of calcific events [23, 24]. Amino acids and amino compounds, that is, glycine, L-glutamic acid, and/or urazole, have been used as free aldehyde groups neutralizers [24, 25].

Current efforts are posed on the discovery of novel and less cytotoxic crosslinking agents: as an example, porcine heart valves treated with the natural gardenia fruit compound genipin were observed conserving a cell viability around 79–100% with effective induction of tissue stability [26, 27].

Through a different mechanism, cell viability is threatened in cryopreserved allografts by the formation of ice crystals during the cryocooling phase with induced mechanical stress, intercellular crystallization and thermal shock, and/or by the cytotoxic effect of osmotic phenomena during the thawing phase [28]. Controlled settings of temperature decrease (–1°C/min) and cryoprotector inclusion (DMSO or glycerol) are nowadays utilized in tissue banks for the effective storage of allografts before their clinical need.

Despite the optimizations operated, experimental evidences demonstrate that such modifications are not sufficient for the complete protection of allografts and xenografts from structural deterioration. A consistent set of data has been more and more demonstrating that immunological issues are the triggering factors in the onset of tissue degeneration. Biochemical and histological analyses on 7 commercially available animal-derived valve replacements revealed that only one bioprosthesis, that is, the Epic TM valve from St. Jude Medicals (St. Paul, MN), has a complete shielding of alpha-gal xenoantigens. Such xenogeneic epitopes are oligosaccharides of glycoproteins and glycolipids, displayed on the surface of vascular endothelial and stromal cells in all mammals except apes, Old World monkeys, and humans. An evolutionary gene silencing of the enzyme alpha 1,3-galactosyltransferase renders these species unable to metabolize alpha-gal [29, 30]. Retrieved at a concentration of at least 10^7 epitopes per porcine cell, alpha-gal is also expressed by the human gut bacterial flora, kept under control by the 1% serum circulating specific IgGs [8, 29–33].

Several studies correlated alpha-gal to the priming of heart valve calcification through an immune response mechanism [34]. The incomplete masking of the epitope could be exacerbated *in vivo* by the tissue fatigue, to which the valve is submitted in the patient's arterial circulation. The new crosslinker genipin is per se able to only attenuate the inflammatory and immune responses to xenogeneic tissues [26]. Moreover, non-gal xenoantigens have been identified and no clinical information is yet available regarding their impact on bioprosthetic graft survival.

The theoretically more biocompatible human valve allografts fail, however, in a similar time to the xenogeneic counterparts. Again, the main culprit of structural deterioration is the immune reaction against donor's antigens [35]. In particular, the endothelium has been considered to behave as antigen presenting cell element to the host's immune system [36]. The presence of HLA II (or HLA DQ/DR) has been related to humoral and cellular responses preventing any accommodation or tolerance of the graft and chronically inducing its rejection [37, 38].

A modality to render allo-/xenogeneic matrices immunoprivileged and possibly susceptible to further autologous-like regeneration has been offered by decellularization technology. This extraction process has as rational the removal of all endogenous cell elements, as, for instance, cell membranes, organelles, and nucleic acids, which can adversely prompt inflammatory, immune, and calcific events [39, 40].

Several methods for decellularization of organ and tissues have been formulated, mainly based on osmotic shock, detergents, and/or enzymes in possible combination with mechanical agitation and heat to facilitate tissue exposure to the chemicals.

A treatment with hypotonic or hypertonic solutions or their alternation, such as low and/or high ionic strength solutions, can easily induce cell lysis. Hypertonic stress is severely destructive for several proteins, including those of the contractile cell machinery, by causing their aggregation and misfolding in treated tissues and organs [41]. EDTA or other chelating agents are frequently added in the first decellularization steps to disrupt cell-cell binding and potentiate the action of the following treatments [42].

Ionic detergents, as sodium dodecyl sulfate (SDS), act very harshly on protein-protein interactions, while also solubilizing cell membranes of nuclear type [43]. Nonionic detergents, such as Triton X-100, show mild solubilization: their main targets are the lipids in interaction with themselves or with proteins [44].

Enzymes are usually applied to disrupt cell-cell binding and proteins. They are eventually used as a terminal step of the extraction process to remove any trace of nucleic acids released from lysed cells: such nucleases operate the hydrolysis of the interior or exterior bonds of ribo- and deoxyribonucleotide chains, otherwise closely sticking to the ECM fibers after release in the extracellular space [8].

Most commonly applied procedures relying on SDS and/or on the trypsin enzyme demonstrated efficacy in the decellularizing yield but, in turn, revealed inadequate preservation of the valvular ECM. Elastin fragmentation, collagen swelling, or, more generally, ECM denaturation and precipitation were frequently observed. Furthermore, these chemical treatments affect the glycosaminoglycan moiety of hyaluronan, cheratins and chondroitin sulfates [14]. As a result, the tertiary and quaternary structures of associated ECM macromolecular aggregates are altered and it is destabilized the ability to appease the shocks, provoked by the enormous pressure variations during the entire cardiac cycle [45–47].

Among ionic detergents, bile acids and their salt anions do not cause protein denaturation and, hence, are more conservative for treated ECMs. Sodium cholate and deoxycholate find large application in the decellularization procedures in combination with either Triton X-100 (TRICOL) [8, 47] or SDS [48], as well as alone [49]. They differ from SDS for the rigid steroidal groups in their backbone [43]. Less frequently, zwitterionic detergents, characterized by mixed properties of anionic and nonionic surfactants, are applied, but with several detrimental effects [50]. Biochemical and

ultrastructural evaluations of most scaffolds decellularized with bile acids-based treatments revealed good preservation of collagen and elastin network. Nevertheless, different results in terms of glycosaminoglycan and basal membrane integrity were evidenced. Collagen IV, proteoglycans, and glycosaminoglycans composing the basal *laminae* are minimally affected by sodium cholate [6, 8, 51], while they can be damaged and largely removed by sodium deoxycholate [52]. The conservation of the original basal membrane is essential for further endothelial cell adhesion and for the prevention of platelet attachment/activation once *in vivo*.

Zhou and colleagues investigated the effects of different decellularizing treatments on ECM preservation, as well as on thrombogenicity and immunogenicity after *in vitro* direct contact with human blood. No ECM disruption and complete decellularization were observed with sodium deoxycholate. Among all proposed methods, including also the use of SDS, trypsin/EDTA, or trypsin-detergent-nuclease, thrombogenic and immunological responses to sodium deoxycholate-treated ECMs were surely higher [53]. A confirmation to these observations was obtained through a quantitative methodology based on immunoblotting technique: Arai and Orton demonstrated that a conspicuous amount of soluble protein antigens were still detectable in bovine pericardium and porcine heart valves after decellularization with SDS and sodium deoxycholate [54]. On the contrary, the treatment with sodium deoxycholate alone for 48 hours revealed reducing smooth muscle actin to $0.96 \pm 0.71\%$, as well as the total soluble protein to $6.68 \pm 2.0\%$ [52].

3. Preclinical Proof-of-Concept of Heart Valve Tissue-Guided Regeneration

In the large panorama of *in vivo* preclinical TEHV testing, the researchers using decellularized scaffolds as starting matrices challenged the application of the sole ECM with respect to its standard arrangement with cells.

With a technology named SynerGraft (CryoLife, Atlanta, Georgia), O'Brien et al. were able to manufacture acellular porcine valve conduits by means of a decellularization treatment based on hypotonic solutions, enzymes for the removal of nucleic acids, and extensive washout. When implanted in sheep, the xenografts demonstrated competence for 6 months and, at *ex vivo* analysis, fibroblast repopulation interested leaflet stroma [55]. Similar results were obtained in the same ovine model with human cryopreserved decellularized heart valves in both aortic and pulmonary orthotopic positions [56].

The second heart valve tissue-guided regeneration trial was realized in pulmonary position by Konertz and colleagues. By comparison of the standard TEHV procedure to its exception, they implanted allogeneic sodium deoxycholate-decellularized valves, either nude or previously cell-seeded, to reconstruct the right ventricle outflow tract in juvenile sheep animals. The outcomes were surprisingly in favor to the sole scaffold implantation, as documented by regenerative processes initiated in the valve allografts at 6 months and by the increase in the annulus diameter in response to somatic growth, reported at nearly 1-year follow-up [57, 58].

As a further step towards the clinical stage, this group performed a comparative assessment of cryopreserved allografts versus decellularized heterografts (pig) in the sheep model for a time interval of 10 months. In a simulated Ross intervention approach, hemodynamic function of xenografts revealed to be satisfactory. In addition, sheep pulmonary homografts appeared partially devitalized in some valvular regions, while the acellular porcine valves (from now on referred with the proprietary name Matrix P) were free from calcifications and repopulated by endothelial cells and fibroblasts [59]. In recent times, aortic valve xenografts decellularized with the same technology were implanted orthotopically in juvenile sheep. After 4 months of evaluation in systemic circulation, no adverse events in terms of hemodynamic and biological performance were disclosed [60]. In another preclinical study in the systemic circulation, porcine aortic valves decellularized with sodium deoxycholate were implanted stented in an allogeneic model. Decellularized valves were well performing and prone to recellularization without calcified foci, which were revealed instead for the controls, that is, Carpentier-Edwards glutaraldehyde-treated commercial bioprostheses (Edwards Lifesciences, Irvine, CA) [61].

Allogeneic aortic valve conduits were evaluated by Baraki et al. in an ovine model after decellularization with sodium deoxycholate and SDS. Implanted in orthotopic position, such valves were trivially regurgitant and did not exhibit degeneration or dilatation after 9 months of *in vivo* follow up. While fresh native allografts used as control showed signs of mineralization, deterioration, and advanced insufficiency already at 3 months, decellularized aortic valves were interested by minimal calcification and incipient repopulation [62].

The ability to prevent calcification introduced in heart valves by decellularization was also described by Hopkins and colleagues in an allogeneic sheep model: calcium deposits were absent from acellular cryopreserved pulmonary valve allografts, whereas severely concerned their native analogues [63].

With the rational to test acellular aortic valves as substitutes for the reconstruction of the right ventricle outflow tract, we manufactured porcine valvulated conduits by applying TRICOL technology. Allogeneic decellularized valves were implanted in a porcine model for maximum 15 months, revealing a good hemodynamic performance over time. TRICOL decellularized substitutes exhibited *ex vivo* progressive regeneration without signs of degeneration (calcifications, fibrosis, and/or thrombosis) or immune rejection. Original ECM architecture was well preserved. Engrafted cell elements displayed native-like features, as the ability to synthesize novel ECM (collagen and elastin fibers). Regenerated valves appeared vascularized by newly formed, mature blood vessels, that is, capillaries and *vasa vasorum*, especially in *media* and *adventitia* of arterial walls. Interestingly, hallmarks of reinnervation were documented. Regenerative processes were also confirmed by the presence of reparative macrophages, namely, M2 populations, in higher amount with respect to proinflammatory M1. Stem

cell elements of embryonic, hematopoietic, neural, and mesenchymal lineages populated the regenerated aortic valves in specific topographic regional localization. Such regeneration signs further confirm the ability of the decellularized native ECM to behave as a stem cell niche providing cues for proper cell differentiation [64].

4. Heart Valve Tissue-Guided Regeneration in the Clinical Arena

Despite the initial difficulties in the acceptance of this experimental concept as therapeutic, this is the unique TEHV modality apart from solely endothelialized scaffolds to have reached to date translational applicability in heart valve diseased patients.

CryoLife designed, patented, and distributed two novel valve bioprostheses with the same SynerGraft decellularization technology based on osmotic shock: 500/700 and CryoValve biodevices.

After the positive outcomes observed in 6 months with 500/700 SynerGraft porcine valves in a weanling sheep right ventricular outflow tract reconstruction model [55], Simon et al. implanted these valvular substitutes in 4 pediatric patients. A catastrophic rapid failure of the xenografts led to 3 deaths in less than 1 year and a prophylactic valve substitution in the unique surviving child. At bioptic examination, a strong lymphocyte reaction was documented. In the disclosure study, the causes for such hyperacute and acute rejections were attributed to the xenogeneic extracellular matrix [65], even if possible culprits should have been researched in residual xenoantigens (see *Alpha-gal: the nightmare in xenogeneic valve implantation* in Section 5).

The other commercial option by CryoLife, that is, CryoValve, is a decellularized valve allograft. Following the data provided by the company, CryoValve has been already implanted in 5.700 patients since 2000 [66]. The biodevice was first evaluated in a small cohort of 36 individuals, where adequate functionality and no panel reactive antibody response were documented after 3 months from surgery [67].

In a comparison to historical controls, 14 pediatric patients, implanted with CryoValve for reconstruction of cardiac valve defects, displayed decreased titers of HLA I and II alloantibodies, when tested at 1, 3, and 12 months postoperatively [68]. Comparable outcomes were disclosed by Zehr et al. after aortic orthotopic implantation of the same cryopreserved decellularized allogeneic valve in 22 adult patients (range 31–80 years) [69].

A reduced titer of panel reactive antibodies is very advantageous especially for patients awaiting heart transplantation.

In 2005, Sayk et al. described histopathological findings regarding an explanted pulmonary Cryovalve from a 60-year-old patient who died for bronchopneumonia. After 5 weeks from implantation, no signs of degeneration could be appreciated. Indeed, coaptation was optimal without any leaflet thickening. At microscopic examination, polymorphonucleocytes and macrophages were found migrated in the proximal and distal sutures and involved in the elimination of nonviable donor myocardium. No progenitor, fibroblast, or myofibroblast infiltration was observed [70]. Such aspects conform to the outcomes described by Elkins and colleagues with reference to the two-phase progression of CryoValve regeneration in the ovine xenogeneic model [56].

After having received US FDA approval in 2008, CryoValve was adopted in many cardiosurgical centers rendering possible trials on noninferiority evaluation with respect to standard cryopreserved allografts. No postsurgery mortality, freedom from redo procedures, and lower incidence of severe regurgitation were valued in this comparison [71, 72].

Espousing both the classical and tissue-guided regeneration TEHV conceptions, Dr. Haverich and his research group started a clinical trialing with human pulmonary valve allografts, decellularized in 2002 with enzymatic (trypsin) treatment and from 2009 with sodium deoxycholate/SDS detergents. In contrast to conventional cryopreserved allografts and glutaraldehyde-fixed bovine jugular vein valves, decellularized fresh allogeneic valves (manufactured from 2006 within the company Corlife oHG, Hannover, Germany) were characterized by improved freedom from explantation, lower transvalvular gradients, and ability to adaptive growth. Moreover, immune responses mediated by T lymphocytes and natural killers were absent during the early-term evaluation of 3.5 years [73–75].

Analogously, Dohmen and colleagues evaluated the functional performance of acellular allogeneic valves, both alone or submitted to previous seeding with patient's vascular cells. From 2003 to 2007, 68 patients underwent Ross procedure with implantation of a pulmonary decellularized allograft. The patients' group was subdivided in equal number and submitted to implantation of either SDS- or sodium deoxycholate-decellularized pulmonary valves. Adequate valve performance was recorded in a 4-year-long follow-up with a low mortality rate. A discrete increase of transvalvular gradients was observed over time for control allografts and allogeneic valves decellularized with sodium deoxycholate, while no modification was reported for SDS-treated ones [76]. An immunological and echocardiographic evaluation was performed by the same group on sodium deoxycholate-decellularized allografts by comparison to the cryopreserved correspondents. A total of 20 patients were examined at specific frequency from surgery. In particular, HLA alloantibodies titers and eventually panel reactive antibody levels were determined at 5, 10, 30, 90, and 180 days postoperatively. In patients implanted with cryopreserved allografts, marked value elevations of HLA I and HLA II were documented already after 30 post-surgery days. Contrasting data were reported for decellularized allografts: nearly 60% of patients displayed no increased values, while for the rest of them either modest HLA I rise or abnormal panel reaction antibody levels were documented [77].

In the 2005–2010 lustrum, da Costa et al. implanted in aortic position 41 allogeneic heart valves decellularized with SDS. Submitted to arterial circulation, acellular valves demonstrated patency and low rates of calcification. One of treated patients underwent reoperation due to incoming mitral stenosis. During surgery, a small fragment of the implanted aortic conduit was excised. The histopathologic analysis of the aortic wall sample revealed intact ECM,

intimal hyperplasia, and trivial medial repopulation by fibroblast-like cells [78].

A clinical trial with allogeneic aortic heart valves treated with TRICOL technology decellularization is now ongoing.

Apart from valvular allografts, another xenogeneic acellular valve, that is, Matrix P, has been tested in clinical applications. As aforementioned, Matrix P is a porcine pulmonary heart valve decellularized through the sodium deoxycholate treatment. The technology has been developed and tested by Dohmen and colleagues and is commercially distributed by Autotissue Ltd. (Berlin, Germany). A variant of the Matrix P with equine pericardial patch extension, that is, Matrix P Plus, has been conceived for the surgical cases, where a distal or proximal patch extension is required. Both valves were granted with the European CE mark in the 2004-2005 biennium.

Matrix P substitutes were first applied clinically from 2002 in 103 patients undergoing to the Ross procedure and subsequent hemodynamic evaluation (transthoracic echocardiography and eventually multislice computed tomography) at discharge, 3, 6, and 12 months after intervention. Two patients died after surgery for multiorgan sepsis and one reoperation was performed for false aneurysm after 10 months from intervention. In the explanted xenograft, endothelial cell lining and stromal engraftment of recipient's cells were evidenced without signs of inflammation or immune rejection [79].

From 2006 to 2008 (30.5 months), 61 pediatric and adult patients were submitted to right ventricle outflow tract reconstruction with Matrix P or Matrix P Plus. Early mortality, settled at almost 8%, was due to non-xenograft related causes, while postinterventional valve failure occurred in 4 cases, retreated with the same Matrix P Plus. An additional valve with adequate hemodynamics was removed after 1.5 years during reoperation of an unsolved, complex cardiac defect reconstruction. No degeneration aspects were observed at macroscopic examination and histological evaluation revealed absence of inflammation but reendothelialization of leaflets and intimal pulmonary wall. Normal structure and lack of calcification were documented in the remaining xenografts for the entire intermediate follow-up by computed tomography and magnetic resonance [80].

In another study, serum samples were collected from 159 patients who underwent heart valve replacement with glutaraldehyde-treated bioprostheses (porcine or bovine) or Matrix P/Matrix P Plus. With reference to preimplantation values, immunological potential of decellularized xenogeneic valves was evaluated immediately after surgery and at 9–12 months postoperatively. IgG and IgM titers against porcine collagen I and alpha-gal were investigated to evaluate possible immune reactions and identify their triggers in ECM or cellular elements. No significant alteration was reported for anticollagen response in all implants. Anti-gal titers were indeed raised in commercial biological prostheses with respect to decellularized ones, for which a sole IgM response was documented [81].

Divergent results were although disclosed after implantation of the same Matrix P valves in other independent studies [82–86]. According to the outcomes of these clinical evaluations, freedom from graft dysfunction ranged from 50 to 60% with stenosis and pseudoaneurysm as main failure signs and inflammatory and fibrotic processes as histopathological evidences.

The controversial results with SynerGraft and Matrix P grafts should impose more caution in the application of xenogeneic tissues-derived biodevices in humans. A step back to the preclinical evaluation in human-like models (non-human primates) is mandatory to verify effective biocompatibility in xenointeractions.

5. Future Developments

Tissue-guided regeneration concept in heart valve surgery evolved quite rapidly considering that the first preclinical experiments were performed around the middle 1990s and the first-in-human trials were carried out in 2000.

Allogeneic decellularized heart valves can now be considered as a functional therapeutic replacement option; however, data about their long-term clinical evaluation are still incomplete.

Decellularized valve xenografts have entered the clinical stage too early after nonrobust preclinical investigation.

Many aspects remain hitherto to ponder for a prospective considerate clinical suitability of heart valve tissue-guided regeneration.

5.1. Uncomfortable Leavings of Decellularization Treatments: Cytotoxicity Issues. In the formulation of the decellularization cocktail, particular attention should be addressed to the biochemical properties of each agent used. More precisely, change of these features is strictly dependent on variations in the working microenvironment (temperature, pH, light, etc.).

Enzymes, applied for disruption of cell-cell interactions or for digestion of nucleic acids, work in specific regimens of temperature and ionic strength, and prolonged exposure can induce serious damages to treated cardiovascular scaffolds [44].

Detergents exert their activity depending on the critical micelle concentration (cmc), namely, the minimum concentration for individual molecules to spontaneously cluster and form micelles. The cmc is influenced by previously cited microenvironmental conditions, as well as by the presence of proteins, lipids, and other detergents. High cmc is a characteristic of bile acids, as sodium cholate and deoxycholate, but also of ionic detergents with low concentration of counterions.

Membrane proteins are solubilized by detergents through a mimic of the natural lipid bilayer environment, in which the same ones are found. An elevated cmc level is particularly crucial in decellularization technology, since the detergents can be more easily washed out from the scaffolds. Variations in the pH and temperature of the working solution can reduce detergent solubility and, for instance, cause gelation states, precipitation, or phase separations [43, 87].

Insoluble detergents remain entrapped among the fibers of the decellularized ECM. Such condition induces a cytotoxic microenvironment preventing further cell colonization [42, 88].

5.2. Age of the Donor. Several changes in valvular ECM architecture and composition are induced by ageing, as shown by some elegant works [10, 14]. Such varying ECM and cell properties should be taken into account for TEHV design, especially for the approaches relying only on the instructing abilities of the scaffolds.

It is still unknown whether a decellularization treatment may induce different effects on a young or adult ECM, but it is likely that the extraction power could be superior in a still immature scaffold.

Moreover, considering allogeneic valvular implantation, the donor's average age is rising to more than 50 years, the age in which heart valve pathophysiological alterations are frequently observed as consequence of hypertension and/or hypercholesterolemia [89].

5.3. Preclinical Heart Valve Evaluation: The Need for Standardization. Animal studies are undeniably essential to test the risk management associated with novel (bio)devices and prevent therapeutic design errors in patients. Following ISO 5840 standards, *in vivo* studies for the assessment of a valve device need to be performed site-specifically in at least 10 animals for a duration of 20 weeks. Two sham animals have to be included for controls, even if there is no specification, about which already approved bioprostheses should be used on this purpose. Hemodynamic, hematochemical and pathological tests must be carried out to evaluate performance and signs of structural damage, calcification, thromboembolism, inflammation, and degenerative processes.

ISO regulations do not specify the animal species of the testing model. So far, preclinical models for heart valve prosthetic evaluation have included sheep and pig animals. The ovine model is the most utilized [55–63]. Juvenile sheep (3–6 months) are excellent models to investigate calcification propensity. These animals well simulate the human valve physiology for similar anatomy, annulus size, heart rate, and relatively slow and limited somatic ingrowth. Conversely, platelet activity is reduced with respect to humans [90]. In addition, the juvenile ovine model failed to predict hyperacute immune rejection in xenointeraction with decellularized porcine valves [55, 65].

Pigs are adequate to assess valve-associated thrombotic complications: in fact, their platelets show *in vitro* an equal activity to human ones [61]. Porcine breeds with lower growth rate are to be preferred to standard animals in order to properly simulate a human pediatric being [64, 90].

Other animal models, such as the dog and the non-human primate, find fewer applications in studies of heart valve assessment due to socio-ethical limitations associated with their use for scientific aims. However, it will be pivotal to evaluate biocompatibility of xenogeneic decellularized valves in a model closer to the human, such as the non-human primate.

Apart from hemodynamic studies, most histopathological analyses are confined to a modest investigation with poor identification of engrafting cell elements and their effective viability and activity in the regenerated tissue. As an example, smooth muscle actin is frequently adopted as a marker of smooth muscle but can be expressed also by activated myofibroblasts: thus, alone, it is not indicative of *bona fide* cell differentiation and maturation. Additionally, extensive molecular and ultrastructural investigations should be performed to fully dissect the regeneration process and clearly distinguish inflammatory and immune responses in implanted decellularized valves.

5.4. Alpha-Gal: The Nightmare in Xenogeneic Valve Implantation. Most decellularization methods reach an effective yield in the removal of all endogenous cell components. However, the extractive power of allo- or xenoantigens from human or animal valve tissues may vary considerably. Kasimir et al. suggested in 2005 that the elimination of alpha-gal xenoantigens could be achieved by adding as a further step to their sodium deoxycholate/Triton X-100-based decellularization a treatment with the nonionic, nondenaturing IGEPAL CA-630 detergent. This variation to the standard protocol was reported to be efficient in the removal of alpha-gal as proven at the histological level [91]. Recently, we demonstrated through a proprietary ELISA test based on a commercially available antibody against alpha-gal that the epitope amount is only halved in porcine heart valves submitted to sodium cholate, Triton X-100, and IGEPAL treatment [92]. Despite the application of a similar decellularization protocol, such different experimental outcomes are to be ascribed to the methodology applied for alpha-gal detection, that is, the use of a high affinity isolectin versus an extremely specific antibody [8]. IGEPAL effects on extracellular matrix biocompatibility were also investigated in a rat subdermal model. In contrast with untreated acellular ECMs, IGEPAL counterparts revealed a high propensity for neoangiogenesis (unpublished data). For a future clinical application in humans, such an aspect has to be considered as pathological.

At the present time, TRICOL method is the sole decellularization technology proven to manufacture valve xenoscaffolds completely deprived of alpha-gal [8, 33, 92].

Other immunological modifications are currently tested to abolish the alpha-gal burden. Transgenic pigs knock-out for this xenoantigen have been generated: hyperacute rejection of obtained hearts was prevented in immunosuppressed baboons for at least 6 months [93]. Additional treatments with recombinant alpha-1,3 galactosidase obtained from *Bacteroides thetaiotaomicron* demonstrated being effective in the removal of the xenoantigen from decellularized cardiovascular scaffolds [94, 95].

While these modifications are likely efficacious, they are associated with high technical complexity and expensive costs of production.

As already mentioned, alpha-gal is not the sole xenoantigen in animal-derived tissues, though its role in hyperacute rejection manifestations is undisputed. Non-gal antibodies are described as further barrier to xenotransplantation. A captivating bioengineering hypothesis has been formulated in recent times by Uri Galili in order to favour an immediate regeneration and hence avoid rejection mediated by non-gal antibodies. Nanoparticles loaded with alpha-gal could

be co-injected in grafting procedures with the rational to bind anti-gal antibodies and force recruitment of pro-healing macrophages through the release of complement chemotactic factors, thus accelerating remodelling processes for a prompted autologous-like tissue formation. Positive preliminary results of this intriguing approach in fibrocartilage and ischemic myocardium settings have been lately disclosed [96–98]. Further confirmation and, especially, verification in other xenotransplantation challenges, such as the same heart valve xenointeraction, are attended. Potentially, such a tissue functionalization could be easily introduced in the manufacturing of decellularized heart valves for guided-tissue regeneration. A soak in a liquid medium containing these nanoparticles could be sufficient to allow diffusion through the collagenous network.

5.5. Increasing Biocompatibility, Bioactivity, and Calcification Protection: The Multifunctional Fillers. Biofunctionalization by means of hydrogel incorporation represents a smart modality to introduce regenerative signals or improve the biomechanical properties in the decellularized scaffold microenvironment. As suggested by Galili, the incorporation in hydrogel fillers of alpha-gal nanoparticles could allow for a homogenous distribution into treated scaffolds [98]. As space filler, Jeong et al. utilized polyethylene glycol, able to stabilize protein properties and protect them from degradation of proteolytic enzymes. This macromolecular substance demonstrated being superior in preventing calcification to polyacrylamide [99]. The same group developed a novel valve bioprosthesis based on a porcine tissue aortic root submitted to decellularization (SDS, Triton X-100, and sodium lauryl sarcosinate), α-galactosidase digestion, space filler functionalization, glutaraldehyde treatment, ethanol/octanol organic solvent, and glycine-mediated detoxification [100]. Although this extensively modified xenogeneic prosthesis demonstrated promising functionality and resistance to calcification both in an *in vitro* circulation experiment and in heterotopic (aortic-mitral) implantation in the sheep model, the utilization of glutaraldehyde excludes any valve remodeling and/or adaptation to the somatic growth of the recipient, that is, the key goals of tissue-guided regeneration.

5.6. Tissue Banking: The Need for Effective Procedures of Disinfection, Sterilization, and Preservation. In order to avoid the transmission of pathogenic contaminants and be distributed as sterile biodevices, decellularized valves need to be submitted to proper disinfection or sterilization methods.

A further advantage of decellularization procedures is given by the possibility to manipulate tissues in sterile settings without modifying the extraction power of the treatment.

Prior to allogeneic implantation, human decellularized scaffolds are currently submitted to similar antibiotic (/antimycotic) cocktails applied in tissue banks. Careful analyses should be accomplished to investigate the effects of residual antibiotics on scaffold repopulation. For instance, amphotericin B is known to modify cell differentiation in mononuclear cells, which are often participating in regenerative events *in vivo* [101].

Due to their animal origin, xenografts must be terminally sterilized before clinical application in order to eliminate any transmissible agent, such as bacteria, fungi, and species-specific viruses with human tropism (e.g., porcine endogenous retrovirus). While antibiotic solutions do not introduce any structural modification in treated valves, procedures of sterilization by γ irradiation, electron beam, or microwave alter significantly collagen, elastin, and other molecules of the ECM.

For valve bioprostheses, the guidelines contained in ISO 14160 standards indicate the application of terminal liquid sterilization (TLS) to ensure complete reset of the bioburden, that is, sterility assurance level of 10^{-6}. The worst-case scenario has to be simulated also by means of superinfections to verify the efficacy of the applied TLS. After the sterilization treatment, valve biodevices must be unmodified in their hemodynamic and biocompatibility assets. Among the TLS liquids, glutaraldehyde and formaldehyde find most application in the manufacturing of valve prostheses. However, TLS by means of glutaraldehyde or formaldehyde does not maintain the bioactivity of treated tissues and it is associated with cytotoxicity induction as previously reported.

For decellularized allografts and xenografts, novel sterilization methodologies should be designed.

In an attempt of sterilization of heart valves, Chen and Wika patented in 2003 a method for the induction of tissue drying with glycerol followed by ethylene oxide treatment or ionizing radiation (Patent file US 6534004 B2). The application of non-thermal microwave radiation was reported by Shamis and colleagues to be less aggressive on ECM scaffolding but not able to generate complete sterilization [102]. A sterilant treatment with 22.5 kGy gamma irradiation provoked, instead, a severe destruction of the fibers of the ECM [103].

The storage of decellularized heart valves in tissue banks could render immediate the distribution to the cardiac surgery units as off-the-shelf replacements. So far, cryopreservation has found large use for the preservation of heart valve allografts but also of their decellularized equivalents. The suitability of this technique for proper valve storage is much debated. Synergraft cryopreserved decellularized allogeneic valves did not show signs of structural damage upon observation with transmission electron microscopy and two photon-laser scanning confocal imaging [104]. Similarly, we evidenced that no collagen swelling/shrinkage or clearly distorted/disrupted elastin fibers could be observed in TRICOL-decellularized cryopreserved pulmonary valve leaflets [8]. Schenke-Layland et al. reported conversely the onset of detrimental effects on ECM fibers after cryopreservation [105] and proposed an alternative treatment, that is, vitrification, as more conservative for effective preservation. Vitrification can be achieved by adding formamide and 1,2-propanediol to the standard cryopreservation method, based on the cryoprotectant DMSO. Heart valves treated with this methodology demonstrated good hemodynamic profile *in vivo* with respect to standard allografts [106].

Residuals of cryoprotectants, such as DMSO, formamide, and 1,2-propanediol, may induce an ECM milieu toxic for engrafting cells.

5.7. Bioengineering Tools to Improve Recellularization. Different from other cardiovascular tissues, heart valves can exert their hemodynamic function even devitalised or acellular. In the mid-/long-term, however, remodeling abilities are required to sustain somatic ingrowth in children or, in general, to operate reparative processes. Such tissue competences may rely only on functional cells.

As also evidenced by Zilla et al. in the context of vascular graft repopulation [107], regenerative abilities could vary from species to species, with the human less inherently prone to a fast resolution in healing events.

Histopathological findings of resected decellularized valves prove the slower onset of repopulation in humans with respect to the previously observed animal models [78–80]. To improve recellularization, biofunctionalization methodologies are currently explored via nanotechnological modifications. In particular, coating with DNA aptamers or antibody-mediated enhanced homing revealed being optimal routes to drive the repopulation with selected circulating cell elements [108, 109].

5.8. Socio-Economic Concerns upon Costs and Beneficial Effects for the Cardiopathic Population. Even if decellularized allo-/xenogeneic valve replacements are relatively less labor-intensive with regard to standard tissue-engineered heart valves, the costs for their purchase are prohibitive for the healthcare system.

Moreover, the implementation of the manufacturing process with further technical innovations (i.e., transgenic alpha-gal KO pigs, recombinant enzymes, etc.) may drastically increase the Industrial production expenditures.

Tissue-guided regenerated heart valves risk therefore being a therapeutic option for few. As observed by Burch et al. [110], the basin of patients eligible for this valve surgical treatment depends on a close evaluation of risks/benefits. In this viewpoint, long-term follow-up outcomes will shed definitely light on the effective superiority ranking of decellularized valves with respect to common allografts.

5.9. Demand for Specific Guidelines in Decellularized Valve Risk Assessments for Clinical Applicability Granting. The rapid evolution of heart valve tissue engineering has not been accompanied by a similarly fast legislative response. As previously highlighted in several points of this review, the regulation for a considerate clinical application of novel decellularized biodevices is to date incomplete. In the lack for specific guidelines, such novel bioreplacements are rendered equivalent to standard bioprostheses, for which, however, characteristics of viability or support to living cell elements are not contemplated.

As we already pointed out elsewhere, no regulations are still available for the clinical application of xeno-derived biomaterials. Already granted with US FDA approval or CE marking, many commercial biodevices distributed as decellularized are actually devitalized and with a strong xenoantigenic load harmful for treated patients [65, 111].

6. Conclusions

The timesaving and the relative simplicity in the manufacturing of acellular valve scaffolds render this therapeutic concept easily up scalable in Industrial productions and possibly available for a population of both diseased infants and adults. With all the prospects for a life-long durability and abilities of remodelling and adaptation, tissue-guided regenerated valvular conduits could drastically reduce the number of reinterventions and hence the health system costs for heart valve disease management.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Dissemination of Health-Related Research among Scientists in Three Countries: Access to Resources and Current Practices

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Objectives. In public health and clinical settings insufficient dissemination of evidence-based practices limits the reach of new discoveries to broad populations. This study aimed to describe characteristics of the dissemination process by researchers across three countries (Brazil, United Kingdom, and United States), explore how designing for dissemination practices has been used, and analyze factors associated with dissemination. *Methods*. A similar online survey was used to query researchers across the three countries; data were pooled to draw cross-country conclusions. *Findings*. This study identified similarities and differences between countries. Importance of dissemination to nonresearcher audiences was widely recognized as important; however, traditional academic venues were the main dissemination method. Several factors were associated with self-rated dissemination effort in the pooled sample, but these predictive factors (e.g., support and resources for dissemination) had low prevalence. Less than one-third of researchers rated their level of effort for dissemination as excellent. Respondents reported limited support and resources to make it easier for researchers who might want to disseminate their findings. *Conclusion*. Though intentions show the importance of dissemination, researchers across countries lack supports to increase dissemination efforts. Additional resources and training in designing for dissemination along with improved partnerships could help bridge the research-practice gap.

1. Introduction

Though the United States (US), the United Kingdom (UK), and Brazil differ in many ways, across all three, there is significant spending on research to prevent and treat important health issues, such as cardiovascular disease [1–4]. While evidence is being produced, the pipeline to move this research into practice is far too long. It has been widely cited that it takes 17 years for research to make it into practice and that along the way most discoveries are lost, leaving only 14% of the evidence discovered to benefit health [5]. In both public health and clinical settings, there remains insufficient dissemination of evidence-based practices, resulting in new discoveries not reaching broad populations and populations most in need [6–8]. This is particularly true for cardiovascular disease prevention and treatment, for which many evidence-based strategies are available but have not had maximum population impact [9]. Dissemination has been defined as "an active approach of spreading evidence-based interventions to the target audience via determined channels using planned strategies" [10].

There is a need to speed up the pipeline from discovery to application (e.g., discovery of a new smoking cessation technique to widespread use across clinical and public health settings) [7]. Potential solutions to bring research to practice include involving stakeholders [11–16] in the research process (e.g., design, data gathering, and analysis) and/or evaluation process (also referred to as practice-based research [17]) and

using theories and frameworks to guide dissemination efforts [18, 19]. Designing for dissemination (D4D) has been recommended as a strategy to help bridge the research-practice gap [20]. Researchers are encouraged to involve dissemination partners early on in the research process. Encouraging a role for potential adopters in research discovery creates a more collaborative approach [21–25]. Involving this future target audience in early, developmental phases of the research process, rather than at the end of a study [22, 26], can help researchers better incorporate issues related to external validity and translation for use in practice settings into intervention development [27–29].

The objectives of the current study are threefold. (1) Describe characteristics of the dissemination process by researchers across three countries; (2) explore how D4D practices have been used; (3) analyze factors associated with dissemination.

2. Methods

2.1. Sampling. Methods for sampling for each country have been presented in detail elsewhere [25, 30]. Briefly, in the US, sampling was conducted using the 12 journals with the highest impact factors in the category "public, environmental, and occupational health" using the lead author's affiliation, the NIH RePORTER database (an electronic tool for searching NIH-funded research projects), and researchers affiliated with the Prevention Research Centers (PRCs) Program of the Center for Disease Control and Prevention (CDC) from each PRCs website. These sources resulted in an initial pool of 488 valid investigators. Researchers were surveyed in 2012. In the UK, investigators from eight funding agencies conducting applied health services and public health research were identified. From these sources, the sample included 536 potential participants. Surveys took place in 2008. In Brazil a sample of 536 potential participants (all researchers classified as part of one of the health sciences areas as defined by Brazilian research agencies) was drawn from the database available at the Brazilian Council for Scientific and Technological Development or CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico). Participants were surveyed from October to November 2012.

2.2. Questionnaire Development and Administration. Development of the UK survey [30] was informed by a systematic review of dissemination planning frameworks and strategies [18], which suggested key elements influencing the effectiveness of dissemination: planning activities, targeting audiences, selecting communication channels, and evaluating impact. Thus, the questionnaire had several parts. These were designed to elicit general researcher views and attitudes on the dissemination of research, think about a particular grant and capture any research impacts on health policy, clinical guideline development, or the organization and/or delivery of healthcare and services [31, 32], and obtain self-reported descriptions of research impacts (using four open-ended questions). This survey included 36 open and closed questions and took approximately 30 minutes to complete. Additional details about the UK survey can be found elsewhere [30]. This survey served as the basis for the surveys in the US and Brazil. The US and Brazil surveys included 35 items and 51 items and took respondents a median of 11 minutes and 15 minutes to complete, respectively. Only closed-ended items that were present on all three surveys were included in the current analysis. All surveys were conducted online; participants provided informed consent, and institutional review board approval was obtained from each of the three universities involved; additional details about the surveys and their administration can be found elsewhere [25, 30]. So that pooled analyses and those comparing across countries could be conducted, only survey items common across all three countries were included in the current analysis.

2.3. Data Analysis. Data were analyzed in SPSS version 20 (SPSS Inc, Chicago, IL). Distributions for each variable were explored across countries, and differences were explored using chi-squared tests. Associations between several predictor variables and self-rated effort to disseminate research findings to nonresearch audiences (i.e., "overall, how do you rate your efforts to disseminate your research findings to nonresearch audiences?" with response options as follows: excellent, good, adequate, poor, and not sure) were explored. The predictor variables included reasons why the researchers disseminate (including to influence policy/practice or to satisfy grant/contractual obligations), importance of dissemination, access to resources/structures (including a formal dissemination strategy or dedicated person for dissemination), adherence to designing for dissemination practices such as the stage at which dissemination-related activities occur, and dissemination processes and actions such as the frequency of producing research summaries. These predictors were selected as those hypothesized by the research team as most likely to be related to the outcome, and the set was limited to these variables to minimize the potential for extra comparisons and the likelihood of type 1 error. Multivariable logistic regression was used to determine odds ratios for excellent/good compared to poor self-rated dissemination effort for each country and in the pooled sample (with and without adjustment for country). The extreme categories were created to maximize differences between groups.

3. Results

Across countries, the samples included similar number of researchers with 277 respondents in Brazil, 232 in the UK, and 266 in the US. Response rates were 42% in Brazil and around half for the UK (50%) and the US (54%).

3.1. Reasons to Disseminate. Across countries, researchers cited similar reasons to disseminate their findings (Table 1). For the pooled data, roughly eight out of ten respondents considered "to raise awareness of the findings," "to influence policy or practice," and "to influence practice" as the main reasons to disseminate their findings. Some differences existed

Table 1: Reasons, resources, and methods used to disseminate the results of research in public health in Brazil, US, and UK %[a] (*n*).

Dissemination characteristics	Brazil	United Kingdom	United States	Total	*P* value (χ^2)
Reasons to disseminate[b]					
Raise awareness of the findings	92.1 (209)	93.1 (216)	94.4 (251)	93.2 (676)	.598
Influence practice or policy	91.2 (207)	93.1 (216)	90.2 (240)	91.4 (663)	.512
Influence practice	81.1 (184)	83.6 (194)	80.1 (213)	81.5 (591)	.583
Influence policy	78.0 (177)	85.3 (198)	75.6 (201)	79.4 (576)	.021
Stimulate discussion or debate	86.3 (196)	74.6 (173)	70.7 (188)	76.8 (557)	<.01
Attract future funding	52.4 (119)	62.5 (145)	52.3 (139)	55.6 (403)	.037
Raise the organizational profile	52.4 (119)	64.7 (150)	42.1 (112)	52.6 (381)	<.01
Promote public understanding of science	47.1 (107)	40.1 (93)	57.9 (154)	48.8 (354)	<.01
Justify public funding	60.4 (137)	48.3 (112)	39.1 (104)	48.7 (353)	<.01
Satisfy grant/contractual obligations	15.0 (34)	36.6 (85)	41.4 (110)	31.6 (229)	<.01
Improve your own communication	29.1 (66)	24.1 (56)	21.1 (56)	24.6 (178)	.117
Importance of dissemination to nonresearch audiences					
Importance to your own work					
Very important	77.7 (171)	69.6 (160)	54.2 (143)	66.4 (474)	
Important	19.5 (43)	24.3 (56)	24.2 (64)	22.8 (163)	
Somewhat/not important/NS	2.7 (6)	6.1 (14)	21.6 (57)	10.8 (77)	<.01
Importance to the work of your unit/department					
Very important	75.9 (167)	65.7 (151)	37.7 (100)	58.5 (418)	
Important	22.7 (50)	26.5 (61)	24.5 (65)	24.6 (176)	
Somewhat/not important/IDK[c]	1.4 (3)	7.8 (18)	37.7 (100)	16.9 (121)	<.01
Resources & structures for dissemination in unit/department					
Formal communication/dissemination strategy or plan	27.8 (62)	20.2 (46)	31.8 (84)	26.9 (192)	.014
Dedicated person or team responsible for dissemination-related activities	32.0 (71)	20.6 (47)	52.6 (140)	36.0 (258)	<.01
Method of dissemination[b]					
Academic journals	98.7 (224)	97.8 (227)	100.0 (266)	98.9 (717)	.067
Academic conferences	91.6 (208)	96.1 (223)	92.5 (246)	93.4 (677)	.117
Report to funders	79.7 (181)	91.4 (212)	68.0 (181)	79.2 (574)	<.01
Seminars and/or workshops	69.2 (157)	71.1 (165)	60.9 (162)	66.8 (484)	.035
Press releases	32.6 (74)	48.3 (112)	62.0 (165)	48.4 (351)	<.01
Face to face meetings	49.8 (113)	40.1 (93)	53.4 (142)	48.0 (348)	.01
Other conferences	21.6 (49)	55.2 (128)	42.5 (113)	40.0 (290)	<.01
Media interviews	32.6 (74)	31.9 (74)	50.8 (135)	39.0 (283)	<.01
Newsletters	13.7 (31)	39.2 (91)	45.1 (120)	33.4 (242)	<.01
Email alerts	6.2 (14)	7.8 (18)	22.2 (59)	12.6 (91)	<.01
Targeted mailings	3.1 (7)	16.4 (38)	16.2 (43)	12.1 (88)	<.01
Designing for dissemination/processes/actions					
Stage in the research process that dissemination-related activities are planned					
Early	60.0 (132)	35.2 (80)	45.1 (120)	46.6 (332)	
Late	36.8 (81)	64.8 (147)	39.1 (104)	46.6 (332)	
Never	3.2 (7)	0.0 (0)	15.8 (42)	6.9 (49)	<.01
Frequency that research summaries/key messages are written for specific nonresearch audiences					
Always/usually	34.7 (76)	31.3 (71)	32.0 (85)	32.6 (232)	
Sometimes	22.8 (50)	48.5 (110)	37.6 (100)	36.5 (260)	
Rarely/never/NS	42.5 (93)	20.3 (46)	30.5 (81)	30.9 (220)	<.01

Table 1: Continued.

Dissemination characteristics	Brazil	United Kingdom	United States	Total	P value (χ^2)
Frequency the impact of your research is evaluated?					
Always/usually	22.4 (49)	13.3 (30)	15.5 (41)	16.9 (120)	
Sometimes/rarely	55.7 (122)	69.5 (157)	56.8 (150)	60.5 (429)	
Never	19.2 (42)	17.3 (39)	26.5 (70)	21.3 (151)	
Not sure	2.7 (6)	0.0 (0)	1.1 (3)	1.3 (9)	<.01
Frequency that a framework/theory is used to plan dissemination-related activities					
Always/usually	10.8 (24)	8.8 (20)	16.7 (44)	12.3 (88)	
Sometimes/rarely	22.4 (50)	48.5 (110)	38.4 (101)	36.6 (261)	
Never	60.1 (134)	39.6 (90)	25.9 (68)	41.0 (292)	
Not sure	6.7 (15)	3.1 (7)	8.7 (23)	6.3 (45)	
Do not plan dissemination activities	0.0 (0)	0.0 (0)	10.3 (27)	3.8 (27)	<.01
Time dedicated to dissemination-related activities to nonresearch audiences.					
<5%	36.5 (80)	31.4 (71)	54.4 (143)	41.5 (294)	
5–20%	35.6 (78)	52.7 (119)	34.6 (91)	40.7 (288)	
>20%	27.9 (61)	15.9 (36)	11.0 (29)	17.8 (126)	<.01

[a]% within local; [b]those responding "yes"; [c]IDK = I do not know.

between countries. Stimulating discussion or debate (86%) and justifying public funding (60%) were reported most frequently by Brazilian researchers compared to researchers in the other countries, while satisfying grant/contractual obligations (15%) was lower among these researchers. UK respondents ranked influencing policy, attracting future funding (62%), and raising the organizational profile (65%) as higher than the other two countries, while they reported promoting public understanding of science (40%) as lower. Compared to researchers in the other countries, US researchers selected promoting public understanding of science (58%) and to satisfy grant/contractual obligations (41%) frequently. However, US researchers selected the reasons of justifying public funding (39%) and raising the organizational profile (42%) less often than researchers in other countries.

3.2. Importance. In the pooled sample, almost two-thirds of respondents reported that dissemination to nonresearch audiences was very important to their own research and roughly half of the participants reported that dissemination was very important for their unit/department. The importance of dissemination differed by country (Table 1). Seventy-eight percent of Brazilian researchers and 70% of UK researchers reported that dissemination of their own research was very important, but this was the case for 54% of US researchers. Though the number reporting importance of dissemination of their unit/department's work was slightly lower overall (59% versus 66% for their own work), the pattern by country remained, with 76% of Brazilian and 66% of UK, but only 38% of US, researchers reporting this was very important.

3.3. Resources/Structures. Access to a formal dissemination strategy (27%) and a dedicated person or team (36%) was low in the pooled sample (Table 1). However, compared to Brazil and UK researchers, US researchers reported the highest access to resources (53%). In Brazil and UK roughly a 30% and 20% reported access to a formal communication strategy/plan or a dedicated person, respectively, while one-third and one-half of US participants reported access to these resources.

3.4. Dissemination Processes and Actions. Table 1 also presents the methods researchers use for this dissemination by country and for the total sample. Across countries, the most frequently reported methods were academic journals (99% overall) then academic conferences (81% overall). Methods differed significantly by country. Brazil did not rank any of the methods significantly more frequently than any other countries but ranked press releases (33%), other conferences (22%), newsletters (14%), and targeted mailings (3%) less frequently. Researchers from the UK reported to funders (91%) and other conferences (55%) more frequently than other countries; they reported face-to-face meetings (40%) less often. The only method US researchers reported least frequently was seminars and/or workshops (61%). US researchers reported several methods more frequently than other countries; these included press releases (62%), media interviews (51%), newsletters (45%), and email alerts (22%).

3.5. Designing for Dissemination. Table 1 reveals that, in the overall sample, less than one-half of researchers reported planning dissemination-related activities early and roughly one-third reported they always or usually produce summaries for nonresearch audiences. Additionally, several important actions related to designing for dissemination and other dissemination-related activities showed differences across countries (Table 1). Brazilian researches most frequently reported planning dissemination-related activities early (60%, compared to 45% in the US and 35% in

Table 2: Regression of predictor variables on self-rated dissemination[a] effort separately for each country and pooled (unadjusted and adjusted for country) OR (95% CI).

Dissemination characteristics	Brazil	United Kingdom	United States	Pooled (crude)	Pooled (adj[b])
Reasons					
Influence policy OR to influence practice	2.1 (0.5–8.3)	5.5 (0.9–35.0)	**4.5 (1.2–16.5)**	**3.8 (1.7–8.7)**	**3.8 (1.7–8.8)**
To satisfy grant/contractual obligations	0.5 (0.2–1.3)	1.1 (0.4–3.0)	0.9 (0.5–1.7)	0.7 (0.5–1.2)	0.8 (0.5–1.3)
Resources					
Unit/department/school has formal communication/dissemination strategy	**3.0 (1.4–6.5)**	—[c]	1.8 (0.9–3.6)	**2.4 (1.5–3.9)**	**2.9 (1.8–4.7)**
Dedicated person/team for dissemination in unit/organization	1.7 (0.8–3.5)	2.6 (0.7–9.6)	1.3 (0.7–2.4)	1.2 (0.8–1.9)	**1.6 (1.0–2.5)**
Methods/actions					
Frequency that research summaries/key messages are written for specific nonresearch audiences	**5.9 (3.5–9.9)**	**4.8 (2.2–10.1)**	**21.9 (10.7–44.9)**	**8.5 (6.0–12.0)**	**8.2 (5.8–11.7)**
Stage in the research process when planning dissemination-related activities	**3.2 (1.5–6.7)**	3.2 (0.98–10.2)	**3.9 (2.4–6.6)**	**2.8 (2.0–4.0)**	**3.3 (2.3–4.7)**

[a]The reference category is poor (versus excellent/good); [b]adjusted for country w/dummy variables; [c]could not be estimated due to small cell sizes.

the UK). However, Brazilian researches most frequently reported rarely or never producing summaries for nonresearch audiences (42% compared to 30% in the US and 20% in the UK). Though the frequency across all countries was low (17%), 22% of Brazilian researchers reported that they always or usually evaluate the impact of their research on changing public health practice or policy; 26% of US researches reported they never do this. Use of frameworks was also quite low across all countries (12% reporting always or usually), though it was the lowest in the UK (9%) and the highest in the US (17%).

Overall, 18% of researchers reported spending greater than 20% of their time on dissemination-related activities (Table 1), with the highest in Brazil (28%) and the lowest in the US (11%). Finally, roughly one-third of the participants reported their own effort to disseminate their findings as excellent or good, though the UK had the highest percentage (33%) compared to Brazil (25%) and the US (28%) (Figure 1).

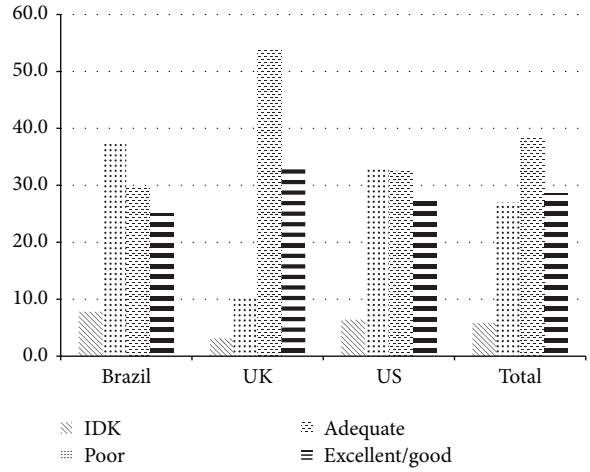

Figure 1: Self-rated dissemination effort to disseminate public health findings to nonresearch audiences in Brazil, US, and UK % (IDK = I do not know).

3.6. Factors Associated with Dissemination Efforts. Several factors were associated with self-reported dissemination effort, with important differences across countries (Table 2). Researchers reporting that they disseminate their findings to influence policy or practice were more likely to self-rate themselves as putting more effort toward dissemination (OR = 3.8; 95% CI = 1.7–8.8); this was not significant for the UK or Brazil. Interestingly, there was no relationship between disseminating to satisfy grant/contractual obligations and effort to disseminate; indicating intention to disseminate may not translate to behavior. Those reporting a unit/department/school with formal communication/dissemination strategy had higher odds of better self-rated effort in the pooled data (OR = 2.9; 95% CI = 1.8–4.7) as well as all individual countries except the US. However, the positive association between reporting a dedicated person/team and self-reported effort to disseminate was only significant in the pooled analysis (adjusted for country) (OR = 1.6; 95% CI = 1.0–2.5). In the pooled sample (OR = 8.2; 95% CI = 5.8–11.7) as well as all the individual countries, reporting producing research summaries for nonresearch audiences was also associated with higher self-rated effort to disseminate. Finally, researchers who planned dissemination at an earlier stage of their research process (pooled sample) were more likely to report excellent/good dissemination efforts (OR = 3.3; 95% CI = 2.3–4.7).

4. Discussion

This exploration of the characteristics of the dissemination process by researchers across three countries found a number of similarities as well as important differences between countries. Researchers seem to recognize that dissemination of their findings to nonresearch audiences is a main reason

for their research and rate it as important; however, the main method they use to reach this audience is through traditional academic venues. Overall, most of the variables explored were associated with self-rated dissemination effort in the pooled sample. However many of these factors had low prevalence in the countries explored. It seems there are limited support and resources to make it easier for researchers who might want to disseminate their findings. The study also revealed that less than one-third of researchers felt the level of effort they put toward dissemination efforts was excellent. This may in part explain the speed at which research is being translated into practice [7, 33]; many researchers may lack access or may be unaware of the effective methods of knowledge translation.

Our modeling indicated factors related to increased success in dissemination by researchers, and these included access to the right resources and practicing designing for dissemination activities. Researchers reporting a unit/department/school with a formal communication dissemination strategy had higher odds of better self-rated effort. Unfortunately, even though access was somewhat higher in the US, it was low across the three countries (with only about one-third of participants in the pooled sample reporting some access), indicating a lack of resources for dissemination in developed and developing countries. Thus our findings point to the potential for provision of such resources to enhance the ability of researchers to improve their efforts to disseminate. Alternately, formation of a transdisciplinary team may be a more feasible approach. It may not be feasible or even advisable for a public health researcher to develop all of the necessary dissemination skills. Teaming with a communications or marketing expert might allow for optimal dissemination. Whether the dissemination efforts are by the researchers themselves, team members with dissemination expertise, or outside experts, they will likely involve some type of technology [34–36]. Many of the methods of dissemination mentioned by researchers in the current study are conducted at least in part online. In particular, the growth of online publishing and potential of social media now offers a multitude of low cost, high reach channels for the dissemination of information about prevention and treatment of cardiovascular disease. Future efforts should explore how to maximize the multitude of technological platforms and utilize target audience preferences for effective dissemination [37].

Better connections between researchers and practice are another avenue which has been suggested as a way to improve translation of research to practice [38, 39]. This may be particularly true in low and middle income countries [40]. This analysis found significant associations between whether the researcher had practice-based experience and self-rated dissemination effort in two of the countries explored (data not shown) [25]. Training programs offering researchers practice-based experience and also valuing practice experience in faculty recruitment and promotion requirements may serve as a way to build these connections. For example, the American Heart Association conducts a course: Seminar on the Epidemiology and Prevention of Cardiovascular Disease that brings together researchers and practitioners working on cardiovascular health promotion [41]. Such programs could be expanded to include management and treatment of cardiovascular disease, furthering the impact of research into practice.

Designing for dissemination is an active process that helps to ensure that public health interventions, often evaluated by researchers, are developed in ways that match well with adopters' needs, assets, and time frames [25]. Additional training in designing for dissemination may be needed across countries for researchers developing cardiovascular disease prevention and treatment interventions [42]. Structural factors in the architecture of research institutions may also be important barriers to engage in designing for dissemination [43]. There was a difference in both the importance of dissemination and the use of designing for dissemination practices among Brazilian researchers; further, the US lagged the other countries. Though it was not universal across activities, Brazilian researches most frequently reported planning dissemination-related activities early, always or usually evaluating the impact of their research on changing public health practice or policy, and spending greater than 20% of their time on dissemination-related activities. These factors may be related to the fact that researchers embracing the importance of dissemination may also be the researchers who are more likely to engage in designing for dissemination activities, such as involving stakeholders earlier in the research process. Some cardiovascular disease prevention programs, which were designed with dissemination in mind, have been scaled up, allowing them to have broad, population health impacts [9]. For example, the Child and Adolescent Trial for Cardiovascular Health (CATCH), an evidence-based program for youth in United States schools, has engaged diverse stakeholders and has been widely disseminated and adopted [44–46]. In Brazil, Guide for Useful Interventions for Activity in Brazil and Latin America (GUIA), a cross-national academic-government partnership, used evidence of effectiveness to trigger political action to scale up Academia da Saúde (a community-based physical activity intervention) at the national level [47].

While the top reasons to disseminate were similar across countries, there were apparent differences observed, which indicate that messaging to enhance researcher dissemination may need to be different across countries, as the reasons behind such efforts differ. This may reflect local culture, political environment, and/or other factors. For example, in the UK applied health research has a strong emphasis (from the major funding source National Institute for Health Research) on producing research to address National Health Service priorities and to support decision making by health professionals and policy makers, which may have led to more frequent reporting of influencing policy and attracting future funding among this population. However, relative to the top reasons for dissemination, these were reported less frequently. The role of funders may therefore be important not only because many dissemination activities are often unfunded [48] but also because funders can set the priority of the project to include dissemination [49–51]. Funders of research in the prevention, management, and treatment of cardiovascular disease may consider making this an important priority in the development of calls for proposals

and review criteria. Repeating this survey in other countries might identify additional points for country-specific actions. There are additional benefits to repeating the survey in these three countries and replicating this project in other countries so they might identify areas for improvement. This might allow countries to see how they can improve dissemination to nonresearch audiences, as many countries are spending large amounts of money on research. Further, a coordinated approach to administration of surveys similar to the ones used in the current work in a large number of countries, with a variety of education and funding structures, would allow for additional pooled analyses. This might shed light on whether there are systematic differences in the reasons researchers disseminate their findings as well as what structures might be most supportive.

This study had limitations worth noting. First, each country conducted the study in a different context; thus common demographic measures are not available across countries, and there was variation in time of delivery. Considering the speed at which the field of dissemination research is moving, it is possible that substantive changes may have occurred in the use of research dissemination practices. The differences in demographics collected across countries prevent presentation of field of research and publication records for the survey respondents. However, this study did not aim to look at individual-level predictors of dissemination. Further, questions may have been interpreted differently across countries, perhaps leading to some of the cross-country differences observed. Additionally, the researchers in the study were not a systematic nationwide sample, but they were meant to represent sectors of research. The administration of the survey in only three countries limits the generalizability of the findings, though the countries included were diverse in context. As this data was collected by self-report, there is always the potential for social desirability bias. Further, information on who did and did not participate is not available, leaving open the potential for bias, as the sample likely includes those most interested. Finally, the cross-sectional nature of the study limits our ability to determine causality. Despite these limitations, this study provides a first of its kind look at dissemination and dissemination-related factors among researchers in health-related fields across three countries.

5. Conclusions

This study identified that researchers are interested in disseminating the results of their research to raise awareness and to influence practice and/or policy. Though intentions seem to show the importance of dissemination, researchers across countries appear to lack supports to enhance their dissemination efforts. Additional resources as well as training in designing for dissemination along with improved partnerships could help bridge the research-practice gap.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Does Defensive Medicine Change the Behaviors of Vascular Surgeons? A Qualitative Review

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Although in literature few successful claims have been shown in comparison with other medical specialties such as gynaecology and orthopaedics, vascular surgery is included among high-risk specialties. The high-risk of receiving medical claims may lead vascular surgeons to practice defensive medicine, as is normal in several other areas of clinical practice. No studies are available to our knowledge of the incidence of defensive medicine in the field of vascular surgery. Taking into consideration the scarce amount of information, the authors provide a critical discussion regarding the application of defensive medicine behaviour among vascular surgeons.

1. Introduction

Defensive medicine has been defined as the practice of ordering tests, procedures, and visits or the practice of avoiding treatments for patients considered at high-risk, in order to prevent medical malpractice claims [1, 2]. In the last decades, the culture of defensive medicine has grown worldwide because of the ever increasing number of medical claims, usually associated with high-risk medical areas [3]. As defensive medicine is used among physicians in order to lessen their exposure to medical malpractice litigation, this practice is constantly growing in all medical specialties on the presumption that every patient may be a potential litigant. In the course of time, defensive medicine has become a more and more transversal practice, encompassing all medical areas. A reduction of defensive medicine could help to improve the quality of medical care delivered by hospitals and in all medical specialties the diffusion of this phenomenon should be better controlled. Vascular surgery is a specialty of surgery in which diseases of the vascular system, or arteries and veins, are managed by medical therapy, minimally invasive catheter procedures, and surgical reconstruction. The specialty evolved from general and cardiac surgery as well as minimally invasive techniques pioneered by interventional radiology [1–3].

Although in literature a low incidence of successful claims has been shown in comparison with other medical specialties such as gynaecology and orthopaedics, vascular surgery is included among the high-risk specialties. A high-risk of receiving medical claims may lead vascular surgeons to practice defensive medicine, as in every other area of clinical practice. Nevertheless, to our knowledge, no studies are available in literature about the practice of defensive medicine in vascular surgery. The aim of this paper is to examine the defensive approach, not only in its current application, but also in the field of vascular surgery, discussing how being sued

can affect medical treatment therefore highlighting the need to assess the real incidence of certain defensive behaviours among vascular surgeons.

2. Materials and Methods

The following databases (from 1978 to January 2015) Medline, Cochrane Central, Scopus, Web of Science, and Science Direct were used, searching with the following key words: vascular surgery, defensive medicine, medical negligence, and misdiagnosis. The main key word "vascular surgery" was searched for singularly and then associated individually with each of the other keywords.

Of the 446 sources found, only 22 were considered appropriate for the purpose of this paper. All sources have been screened independently by three physicians and in order to be included they had to be selected by at least two of them.

3. Some Data about Defensive Medicine

The phenomenon of defensive medicine adds 5% to 9% to the cost of US medical care and the annual medical liability system costs along with defensive practice have been estimated in 2008 as $55.6 billion of dollars equal to 2.4% of total health care spending [4, 5]. Recently, the Italian parliamentary board of inquiry estimated the cost of defensive medicine in the public sector at 10.5% while in the private sector it amounts to 14% of total health care spending. Therefore, according to the board, defensive medicine costs 10 billion euros, equal to 0.75% of the gross national product. Although several studies have been published about various approaches to defensive medicine, the consequences deriving from it are still controversial and debatable [6]. Tancredi and Barondess [2], for example, reported a comprehensive study supporting how defensive medicine does not determine the increase of health care costs but rather increases the exposure of patients toward unnecessary risks. In the same way, defensive medicine has been further defined as a widespread practice with little impact on medical care costs [7]. Defensive medicine, as reported by several clinical studies, has a negative impact on the quality of medical care, even without taking into consideration the price of this practice [8]. Several studies have highlighted how lawsuits have a negative impact on physicians causing them stress thereby jeopardizing their future performance [9]. The increase of public attention towards adverse events together with the patients' expectation in obtaining higher compensation for damage from medical malpractice has created a significant pressure on health professionals, making it more difficult for them to obtain adequate insurance coverage, particularly in some specialized branches (such as gynecology and orthopedics) more exposed to this risk. Medical claims are clearly based on the law of medical malpractice which still represents the main guarantee for patients and a useful tool for protecting the patients' health. Although medical liability discourages substandard care and allows patients to obtain reasonable damage compensation, there is no evidence in the literature that a fear of being sued is useful for reducing the rate of medical error [10]. In this perspective, the increase of exposure to medical malpractice has been making physicians more careful and conscious of their own actions. Considering a defensive approach we can identify two different forms: positive and negative. While the positive form can be defined as the practice of performing unnecessary therapeutic and diagnostic treatments, the negative form can be identified as the practice of declining to provide medical care. Both in positive and in negative form, the primary aim is to prevent medical claims, rather than to promote the patient's best interest [11].

4. Vascular Surgery between Defensive Medicine and Medical Malpractice

The exposure to medical malpractice litigation has also increased in the field of vascular surgery, where physicians are called to cure disorders affecting arteries, veins, and lymph vessels. As for every other area of clinical practice, vascular surgeons tend to be sued for failing to deliver safe and appropriate care to patients [12]. Physicians involved in vascular surgery may be sued when the execution of surgical treatments or preoperative activities (diagnostic procedures) are the result of medical malpractice. Because vascular surgeons are also frequently involved in other surgical treatments (elected for different reasons), due to intraoperative complications such as bleeding disorders, in examining the phenomenon of defensive medicine the general division between general and vascular surgery could be misleading. A study conducted by Campbell et al. [13] in England involving 424 claims revealed that varicose vein is the most common pathology involved in medical claims and nerve damage was the most frequent subject of complaints, followed by incorrect surgery and damage to the femoral vein and artery. Markides et al. in 2008 found that 50% of all successful claims were based on intraoperative problems, while 14% and 11% were assigned, respectively, to failure/delay of treatment and diagnosis. Varicose vein surgery was identified as the most common area of litigation, followed by peripheral vascular disease and abdominal aortic aneurysm. Both the studies above mentioned highlight the prevalence of varicose vein surgery in the management of medical claims regarding vascular surgery, taking into consideration that other diseases such as intraoperative nerve and vessel damage may cause worse permanent damage to the patient [14].

5. Discussion

The probability of defensive performances, among physicians, is directly proportional to the specific risk level. Among surgical specialties, vascular surgery is considered to be a high-risk of litigation as confirmed by Jena et al. [15] in a study of 2011, where the proportion of physicians dealing with malpractice claims has been evaluated taking into consideration each specialty. The results obtained show an almost 19% per year probability of facing a claim for vascular surgeons together with thoracic and cardiac surgeons [15]. Nevertheless, no study has yet been conducted in the literature about the incidence of defensive medicine in the

specific field of vascular surgery. The exposure of vascular surgeons to medical malpractice litigation may be relevant both in emergency and in elective surgery, where the control of unexpected intraoperative bleeding may differently affect the physicians' performance. "Type of treatment" and "time factor" may also represent two important key points in the evaluation of defensive approaches among vascular surgeons. Reporting the main types of disease and procedures involved in successful medical claims recorded by the NHS Litigation Authority, Markides et al. highlighted the importance of a correct and appropriate treatment in the management of patients with peripheral vascular disease (PVD), abdominal aortic aneurysm (AAA), and carotid artery disease (CAD) [14]. The main issue involving vascular surgeons is due to the management of CAD and the probability of causing serious neurological damage (e.g., ictus) or, in the worst case, the death of the patient, posing an interesting question whether or not vascular surgeons should operate on patients with asymptomatic carotid stenosis. The proper treatment of carotid stenosis has always been of great interest for the vascular surgeons. In order to standardize the approach to this pathology, the Society for Vascular Surgery (SVS) published in 2008 specific guidelines for the treatment of carotid stenosis, providing recommendations on the basis of an evidence-based medicine approach [16, 17]. According to these guidelines, recommendations for medical therapy rather than surgical treatment depend on the grade of carotid stenosis, distinguishing among patients with a low, moderate, and severe grade of carotid stenosis. In this perspective, practical examples of positive defensive approaches with the aim of preventing medical claims in case of ictus or death should only be not following specific guidelines and performing surgical treatment without specific symptoms, together with an overestimation of diagnostic test results (e.g., Eco-Doppler), thereby exposing the patients to unnecessary surgery.

Further aspects should be considered in the occurrence of mistakes in surgical procedures. An interesting approach is the evaluation of "time factor" in vascular surgery, carried out by Sirignano et al, who highlighted, between 2009 and 2011, 63 claims involving vascular procedures and how the erroneous timing in surgical, medical, or diagnostic intervention may generate errors and how even more frequently errors occur in cases treated electively rather than in urgent or emergency cases [18].

An "emblematic" question may arise in the evaluation of erroneous timing in vascular surgery: which factors can affect the timing of elective procedures? In this perspective a useful support in the attempt to provide a valid answer is to underline the role of "defensive medicine," which still remains controversial. A "positive" defensive medicine approach can occur in the assessment of "cardiac risk stratification" for vascular surgery, which is considered a very high-risk category, associated with cardiac morbidity rates greater than 5% in many reports. Examples include aortic and other major vascular surgeries, as well as peripheral vascular surgery [19].

For this reason, in order to avoid underestimation of the cardiac risk, vascular surgeons may assume an "assurance behavior," which increases the time of preoperative investigation with economic repercussions.

With the aim of reducing this phenomenon, the guidelines of the American Association College of Cardiology and the American Heart Association recommend preoperative cardiac testing only when the results may influence patients' management. Moreover, they individuated four high-risk conditions, showing how to assess and treat them preoperatively; they include unstable coronary syndromes, decompensated heart failure, significant cardiac arrhythmias, and severe valvular disease. Applying these guidelines in an appropriate manner could help in reducing the risks of incurring in medical defensive procedures.

If, on the one hand, the application of positive defense medicine in vascular surgery can impact on the consequences above reported, on the other hand, the effects of negative defense medicine can be even more dangerous, as it is based on "*avoidance behaviors*" only with the aim of reducing the risk of litigation in certain medical activities. However, according to data reported below, this attitude may sometimes increase the number of claims. The authors of the same study [18] have also tried to evaluate the "time factor" and how it can affect the final outcome in patient treatment.

In a paper of 2012 [18] five cases of claims in vascular surgery, in which the "time factor" played a key role in delaying or in not performing a surgical procedure, were reported. In one case, the delay in performing the ligation of an arteriovenous fistula in the left arm of a 75-year-old woman undergoing haemodialysis, which was diagnosed in September and not operated on until the next December due to numerous follow-up visits, caused a severe bleeding in the arm and the death of the patient shortly after. Another case involved a 63-year-old male, who after an intervention of saphenectomy complained of a severe pain in his foot, which was not treated causing, on the 4th postoperative day, the amputation of the leg, for a severe ischemia of the foot due to a thrombosis of popliteal and tibial arteries. Because amputation of a limb is a drastic solution, intolerable for a patient to take into consideration even if suffering from critic ischemia, a therapeutic approach based on an excessive use of revascularization therapies aiming only to obtain a defense in a potential lawsuit is another pragmatic example of positive defensive medicine.

Other important issues worthy of attention, which should be evaluated, are the different medico-legal implications of endovascular versus open surgery and the peculiar features of vascular surgery from the perspective of defensive medicine and the consequent medico-legal problems that may result. Regarding the first aspect, over the past 30 years, vascular surgery has undergone a significant evolution process: all has changed with the advent of endovascular procedures and, thanks to the rapid evolution of available grafts, everything is still changing. The approach to patients has varied as well as indications. Critically impaired patients, in poor general condition, can be subjected to effective treatments that were not first to be proposed for the high-risks associated with open surgery [20]. The availability of those new "less invasive" endovascular procedures could have influenced some "inexperienced" surgeons of the existence of an easier way to treat patients. However, this assumption has to be considered wrong. The new endovascular procedures represent, together,

Table 1: Vascular surgery pathologies: carotid stenosis, aortic aneurysms, aortic dissections, and peripheral arterial disease, evaluated in the light of the indication, timing, and technique.

	Indication	Timing	Techniques
Carotid stenosis	To treat symptomatic stenosis or only haemodynamic asymptomatic lesions	Within 24 hours from symptoms onset or within 14 days	Carotid endarterectomy or carotid artery stenting
Aortic aneurysms	To treat on the basis of the diameter or on the basis of accompanying symptoms and/or aortic morphology	In case of symptoms: as soon as possible or after careful patient evaluation	Open repair or endovascular repair
Aortic dissections	To treat all the dissection or selectively on the basis of visceral malperfusion, aneurismatic dilatation, and uncontrolled pain and hypertension	Within 15 days from symptoms onset or after 15 days	Open repair or endovascular repair
Peripheral arterial disease	To treat patients presenting with claudication or only patients with critical limb ischemia To recognize patients presenting with acute limb ischemia	Critical limb ischemia presenting patient should always be treated in urgent/emergent setting	Open repair or endovascular repair. In case of endovascular repair, use or do not use stent, covered stent, and drug eluting devices

a very efficient weapon for the clinician but for an experienced clinician, at the end of an adequate learning curve. A modern vascular surgeon must be a surgeon, capable of working with the same skills in both open and endovascular surgical procedures [21].

Finally, in considering the fields of application of vascular surgery from the perspective of defensive medicine and the consequent medico-legal problems that may result, three key aspects have to be carefully evaluated: the indication, the timing, and the technique used; in Table 1 are reported four pathologies of vascular surgery: carotid stenosis, aortic aneurysms, aortic dissections, and peripheral arterial disease, which are evaluated in the light of the three indicators above mentioned [20, 21].

6. Conclusions

In conclusion, the little literature available about defensive medicine related to vascular surgery leads us to take seriously into consideration the need to assess the real incidence of defensive medicine in this area. In order to assess the current trend of defensive medicine (positive and negative) in vascular surgery, we suggest focusing not only on the personal perceptions of vascular surgeons practicing it, which however could be underestimated taking into account "*avoidance behaviors*," but also on reporting and reevaluating any suspicious case with a team of specialist surgeons, who can evaluate all diagnostic and surgical procedures undertaken together with the compliance to the guidelines and runtimes. Only in this way will it be possible to have a true picture of this phenomenon and therefore be able to correct the negative impact it has in terms of cost and quality on the health service.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Full GMP-Compliant Validation of Bone Marrow-Derived Human CD133$^+$ Cells as Advanced Therapy Medicinal Product for Refractory Ischemic Cardiomyopathy

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According to the European Medicine Agency (EMA) regulatory frameworks, Advanced Therapy Medicinal Products (ATMP) represent a new category of drugs in which the active ingredient consists of cells, genes, or tissues. ATMP-CD133 has been widely investigated in controlled clinical trials for cardiovascular diseases, making CD133$^+$ cells one of the most well characterized cell-derived drugs in this field. To ensure high quality and safety standards for clinical use, the manufacturing process must be accomplished in certified facilities following standard operative procedures (SOPs). In the present work, we report the fully compliant GMP-grade production of ATMP-CD133 which aims to address the treatment of chronic refractory ischemic heart failure. Starting from bone marrow (BM), ATMP-CD133 manufacturing output yielded a median of 6.66×10^6 of CD133$^+$ cells (range 2.85×10^6–30.84×10^6), with a viability ranged between 96,03% and 99,97% (median 99,87%) and a median purity of CD133$^+$ cells of 90,60% (range 81,40%–96,20%). Based on these results we defined our final release criteria for ATMP-CD133: purity ≥ 70%, viability ≥ 80%, cellularity between 1 and 12×10^6 cells, sterile, and endotoxin-free. The abovementioned criteria are currently applied in our Phase I clinical trial (RECARDIO Trial).

1. Introduction

In the last decade, bone marrow (BM) and peripheral blood- (PB-) derived CD133$^+$ endothelial progenitor cells have been tested in controlled clinical trials as therapeutic agent for heart failure both in acute [1, 2] and chronic [3, 4] setting, with the aim to achieve neoangiogenesis in ischemic myocardial territories. Published Phase I and Phase II studies, although heterogeneous in terms of revascularization strategies and cell delivery routes, reported a partial or complete restoration of global left ventricular function (LV) and improvements in regional myocardial perfusion [1, 5–13]. A number of adequately powered controlled Phase II and Phase III trials are currently ongoing to confirm these preliminary clinical evidences [14].

Mechanistically, a large body of preclinical evidence has shown that CD133$^+$ cells, a subset of CD34$^+$ progenitors [15, 16], exert their mode of action in ischemic tissues by directly

differentiating into newly forming vessels [17] and, predominantly, by indirectly activating proangiogenic signaling through indirect paracrine mechanisms [7, 18]. Due to their nonhomologous use, notwithstanding immunomagnetically clinical-grade purified [19], $CD133^+$ progenitors [20] have to be considered in the cardiovascular setting as an Advanced Therapy Medicinal Products (ATMP), in compliance with the European Medicine Agency (EMA) guidelines [21] and the Committee of Advance Therapies (CAT) Reflection paper on human stem cell-based medicinal product [22]. As ATMP, $CD133^+$ cells require manipulation in certified facilities operating with pharmaceutical standards in order to ensure high-quality and safety manufacturing processes in compliance with Good Manufacturing Practice (GMP) criteria [23]. Specifically, the final $CD133^+$ cell product must be released upon a strict manufacturing characterization, as well as definition of release criteria and quality controls. This validation process is in fact the prerequisite for the release of batches intended for clinical use.

Importantly, in the cardiac cell therapy field the ATMP validation process relies upon intrinsic features of the starting material. It is in fact well known how the number of BM progenitors, their viability, and functionality may be severely affected by multiple cardiovascular risk factors of self-donor patients with ischemic heart failure [24, 25]. In a previous proof-of-concept paper, we have reported that a GMP-compliant implementation of cord-blood- (CB-) derived $CD133^+$ cells for cardiovascular repair does not alter the angiogenic potency *in vitro* and *in vivo* [26]. Using BM of patients with ischemic cardiomyopathy as starting material, we have here developed a standardized final GMP-compliant clinical-grade manufacturing protocol for human $CD133^+$ cells fulfilling clinical-grade ATMP standards (ATMP-CD133). Data generated in the present work have been included in the Quality Section of the Investigational Medicinal Product Dossier (IMPD) "ATMP-CD133" [21], recently cleared by the competent Italian Authority (Istituto Superiore di Sanità, Rome, Italy) as therapeutic agent of the actively enrolling Phase I clinical trial RECARDIO trial [27].

2. Materials and Methods

2.1. Quality Documentation Concerning ATMP-CD133. IMPD quality documentation structure has been set up following EMA guidelines (CHMP/QWP/185401/2004). Specifically, our active ingredient consists of human BM-derived $CD133^+$ endothelial progenitor cells (drug substance). The drug substance resuspended in physiological saline plus 5% of human serum albumin (HSA) represents the ready-to-use ATMP medicinal product (ATMP-CD133).

2.2. Equipment and Facility Characteristics. Manufacturing and quality control test have been performed in a GMP cell production facility (Laboratory of Cell Therapy "Stefano Verri", Monza, Italy) authorized by the Italian Competent Authority (Agenzia Italiana del Farmaco, AIFA) with the licence aAMM-70/2013, according to European and Italian regulatory rules [23]. In particular, all cell manipulations were assessed in B/A GMP classes.

2.3. Assessment of Risk Analysis. An *ad hoc* risk analysis was assessed according to international guidelines [28], with particular focus on the Investigational Medicinal Products (IMP) [29]. Unsuitable starting material, unmet quality standards of starting raw materials, manufacturing process contamination and cross-contamination, positive selection failure, overnight storage and transportation of ATMP-CD133, and miss-labelling were identified as in-process critical steps. Strategies to specifically avoid and manage those steps have been developed and documented in IMPD Standard Operative Procedures (SOPs). As an example, a worst case scenario of production process failure has been considered. This procedure allows to recover a discrete number of $CD133^+$ cells found in the negative fraction, as the negative fraction bag is stored at 4°C until batch release, and a supplemental back-up CliniMACS kit is available at the Cell Factory for emergencies. An evaluation of adventitious risk contamination was also performed [30].

2.4. Aseptic Validation Media Fill. The aseptic process has been validated according to the European GMP legislation [29]. Media fill validation is required in order to ensure robustness, reproducibility, and safety of manufacturing process. In the simulation (i.e., media fill), all aseptic operations were performed using Tryptic Soy Broth (TSB) according to SOPs. At the end of the simulation all collected samples were tested for sterility according to Eu.Ph.2.6.1. To verify the potential microbial growth, samples collected were incubated at +20–25°C for 7 days followed by 7 days at +30–35°C. During all this time (14 days) culture medium turbidity was observed. Subsequently, growth promotion was performed on media pool to verify the fertility of the medium and the absence of growth inhibition factors, according to Eu.Ph.2.6.1.

2.5. Premises and Environment Condition during Manufacturing Process. Premises were fully compliant with GMP rules in order to protect the manufacturer and to minimize risk of cross-contamination [23]. Working conditions of premises such as temperature, humidity, and pressure were maintained and controlled by DESIGO system (Siemens, USA). Adjacent rooms of different grades have been maintained with a controlled differential pressure of 10–15 Pascal according to EU guidelines for GMP [23]. Manufacturing process was set up in A and B classes (classified according to EN ISO 14644-1), and maximum permitted airborne particle concentration was also monitored taking into account limitations as indicated above [29]. Aseptic conditions were monitored using methods such as settle plates, volumetric air samples, and surface sampling [29].

2.6. Collection and Storage of the BM Cells. From May 2009 to July 2011, 8 patients suffering for end-stage refractory myocardial ischemia have been authorized by our local Ethic Committee to receive as compassionate therapy

direct intramyocardial injections of ATMP-CD133 according to Italian national laws. The collection of BM was performed after obtaining written informed consent from the patient. Patients' clinical profile is depicted in Supplemental Table 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/473159. Bone-marrow blood has been collected from patient's posterior iliac crest in a sterile fashion. Filled syringes were then transferred in a bag containing heparin (final concentration $\geq$ 5 U/mL). Needles were finally removed by a direct pressure around the collection area. Bag containing fresh bone marrow sample has been weighted, labeled, and shipped to the cell factory. Bag's shipping was performed at controlled and recorded temperature (+4°C/+20°C) using a rigid box according to defined GMP SOPs. The time limit for bag delivery has been set at 36 hours from collection. Production process was started immediately after material reception without storage.

2.7. ATMP-CD133 Manufacturing Process. The production of ATMP-CD133 was performed the day before injections in a semiclosed system (CliniMACS Miltenyi Biotech, GmbH, Germany) according to manufacturer's instructions. Briefly, mononucleated cells (MNCs) were isolated from BM by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare Life Sciences, UK). After washing with phosphate-buffer saline containing 0.5% (v/v) HSA (PBS-HSA) and centrifugation at 600 ×g for 15 minutes, cells were resuspended into 25 mL volume with PBS-HSA containing 3.5 mL of anti-CD133 antibody (Miltenyi Biotec, GmbH, Germany) conjugated with dextran-magnetic microbeads and 1.5 mL of human IgG. Cells were incubated for 30 min at room temperature under gentle agitation. After washing (600 ×g, 15 minutes), cells were resuspended in 100 mL of PBS-HSA, counted, and characterized for immunophenotype and $CD133^+$ endothelial progenitors were selected using the CliniMACS Magnetic Separation device (Miltenyi Biotec, GmbH, Germany). Both $CD133^+$ and $CD133^-$ fractions were finally collected in separated bags. Each fraction undergone quality control checks such as cell count, immunophenotype, and viability. Magnetically selected $CD133^+$ cells were then resuspended in X-VIVO15 (Lonza, Switzerland) and stored at +4°C overnight in a 50 mL polypropylene tube (drug substance). The day after, release tests such as cell count, immunophenotype, and viability were repeated before lot releasing. In addition, sampling for endotoxin and sterility tests were performed. Moreover, an aliquot of $CD133^+$ cells was stored in liquid nitrogen for retesting in case of required investigation and/or for research purposes. Remaining cells were washed (600 ×g, 10 minutes) to eliminate X-VIVO15 and resuspended in 10 mL physiological saline plus 5% of HSA. The ready-to-use final product ATMP-CD133 was then shipped, under controlled conditions according to SOPs, to the destination site for clinical use. An outline of manufacturing process as well as relevant quality control steps is reported in Figure 1.

2.8. Process Quality Control (QC) Tests. Less than thirty days before the collection of BM, patients were checked for the absence of infective agents such as HIV-1/2, HBV (HBsAg, anti-HBc, and HBs), HCV (anti-HCV-Ab), Treponema pallidum (V.D.R.L.), and HTLV-1/2, according to Directive 2006/17/CE. If serology test for viral infection was confirmed to be negative, starting material, defined as fresh BM sample, was collected and sent to the GMP facility where it was checked, approved, and processed. Similarly, all raw materials and reagents, either biological or disposable, were verified for their compliance to be used in the GMP manufacturing process [23]. QC tests were performed throughout the manufacturing steps both for drug substance (in-process controls, IPC) and medicinal product (final-product controls, FPC) in order to ensure continuous monitoring. In addition, two aliquots of ATMP-CD133 have been stored in liquid nitrogen for retesting in case of required investigation or for research purposes. Thus the final amount of cells assigned to the patient is determined based on these procedures.

Details on each IPC and FPC assessed during the whole process, are described in Supplemental Table 2. Specifically, IPC1, IPC6, IPC9, and FPC1 were fixed to test cell count, viability and immunophenotype on BM, preselection fraction, postselection positive fraction, and final product, respectively. Moreover, endotoxin, sterility, and mycoplasma were assessed on final product (FPC2, FPC3, and FPC4).

Cell count was determined by trypan blue dye exclusion method, viability was performed by Propidium Iodide staining, and immunophenotyping was performed by multiparametric flow cytometry as previously described [26]. Briefly, fluorochrome-conjugated monoclonal antibodies were used in order to determine $CD133^+$ cells purity. Characteristics and combination of antibodies are reported in Supplemental Table 3. At least 3.5×10^5 cells were incubated for 20 minutes, at +4/+8°C with fluorescent reagents in PBS, then washed by centrifugation, resuspended in PBS, and analyzed using a flow cytometer (FACS Calibur, Becton Dickinson, CA, USA). A hierarchical gating strategy was adopted to determine purity of $CD34^+/CD133^+$ cells as previously reported [26]. Endotoxin detection was performed by Limulus Amebocyte Lysate (LAL) Test (Lonza) according to recommendations of European Pharmacopoeia (E.P.) 2.6.14. Sterility and mycoplasma detection were assessed in outsourcing according to recommendations of E.P.2.6.1 and E.P.2.6.7, respectively. Finally, to characterize the impurities of the final medicinal product, we reviewed in 2 thawed representative samples the complete immunophenotype of the $CD133^-$ fraction testing the presence of B (CD19), T (CD3), and NK lymphocytes (CD56), as well as granulocytes (CD15) and myeloid-monocytes cells (CD14).

2.9. ATMP-CD133 Release Criteria. Batches were processed and released according to specification described in Gaipa et al. [26]. Briefly, the release criteria for ATMP-CD133 were purity ≥50%, viability ≥70%, cellularity $\geq 1 \times 10^6$, sterile, endotoxin <0.5 EU/mL, and mycoplasma absence. List of release test and specifications are summarized in Supplemental Table 4.

2.10. Stability Test. Stability of ATMP-CD133 was assessed both for drug substance and medicinal product. The stability

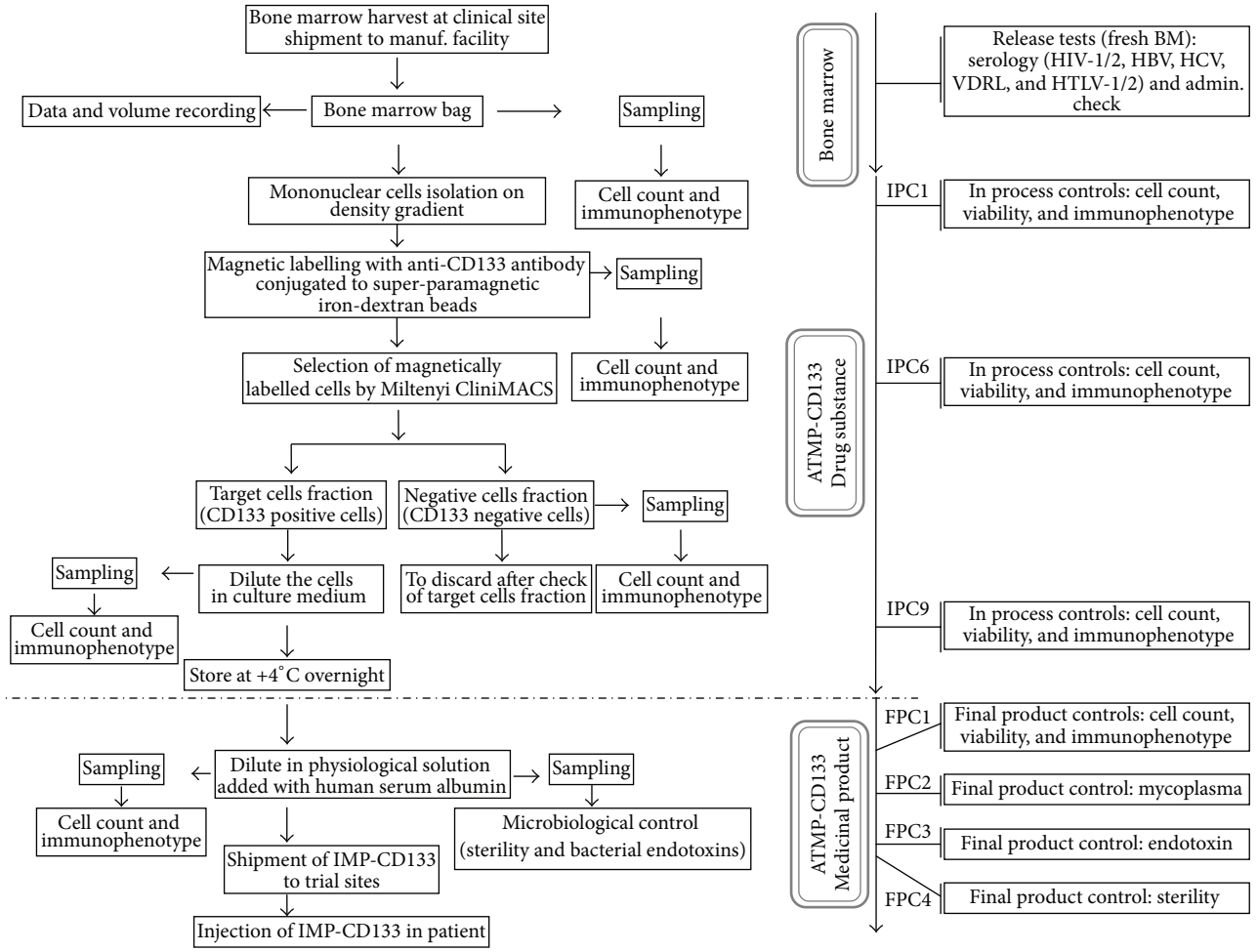

Figure 1: Manufacturing process from the drug substance to the medicinal product. The figure summarizes using a flowchart the entire process from the collection of the patient's bone marrow to the injection of the medicinal product back to the patient.

of the drug substance was validated maintaining cells in X-VIVO15 for 14 hours (h) at controlled temperature between +2 and +8°C. On the other hand the stability of the medicinal product was validated at different time points (6 h, 9 h, and 12 h) maintaining cells in physiological saline + 5% HSA. Viability test was evaluated before and after aforementioned storage conditions to confirm the ATMP-CD133 stability.

3. Results

3.1. Media Fills. Collectively we performed 9 media fill procedures to assess both initial validation (3 batches) and periodic revalidation (6 batches) during the study period (from November 2008 to December 2011). All media fill batches resulted sterile and was compliant for growth promotion analysis. Results demonstrated that both the procedure and personnel involved were able to maintain and guarantee aseptic conditions during all phases of manufacturing process (data not shown).

3.2. BM-Derived CD133$^+$ Cells Collection, Purification, and Recovery. A total of 8 BM-derived CD133$^+$ cell samples were prospectively manufactured from May 2009 to July 2011. Volumes of BM collections ranged between 228 mL and 420 mL (median 278.50 mL). The mean timespan from collection to reception was 59 minutes ± 21.19 (standard deviation, SD). After cell selection with CD133 monoclonal antibody, the median purity of CD133$^+$ cells was 90.60% (range 81.40%–96.20%), largely above the acceptance criteria (≥50%), as described in Table 1. For each batch, viability tests have been performed for both starting materials and final product. Final product viability ranged between 96.03% and 99.97% (median 99.87%), exceeding the minimum threshold of 70% established in our previous experience [26] (see Table 1). Absolute number of CD133$^+$ cells during manufacturing process was highly variable among samples. The number of CD133$^+$ cells in BM samples varied among patients from 15.40 to 180.69 × 10^6 (median 55.35 × 10^6). After density gradient separation of mononuclear cells, a median of 64.89% of CD133$^+$ cells was recovered (range 38.25%–85.21%). CD133$^+$ median recovery after immunomagnetic selection was 30.05% (range 18.37%–56.77%) as referred to prior CliniMACS selection and 20.62% (range 7.69%–34.98%) as referred to BM starting material, respectively.

Table 1: Purity and viability of $CD133^+$ cells (%) obtained in 8 batches of ATMP-CD133.

Batch #	% $CD133^+$		Cell viability	
	Starting material	Final product (purity)	Starting material	Final product
PTC-CD133-052	1.87	96.2	95.83%	96.03%
PTC-CD133-055	3.39	81.87	96.83%	97.70%
PTC-CD133-068	0.86	94.61	95.27%	99.87%
PTC-CD133-085	1.18	89.80	87.67%	99.91%
PTC-CD133-090	0.41	81.40	96.62%	99.75%
PTC-CD133-098	0.90	91.40	93.62%	99.95%
PTC-CD133-109	1.21	93.64	96.28%	99.97%
PTC-CD133-116	0.90	81.94	96.29%	99.86%
Mean	1.34	88.86	94.80%	99.13%
Standard deviation	0.93	6.21	3.06%	1.47%
Median	1.04	90.60	96.06%	99.87%
Minimum value	0.41	81.40	87.67%	96.03%
Maximum value	3.39	96.2	96.83%	99.97%

Table 2: Recovery of $CD133^+$ cells.

Batch #	Starting material	Pre-CliniMACS selection*	Post-CliniMACS selection	
	Absolute number of $CD133^+$ cells ($\times 10^6$)	Cell recovery 1 (%)**	Cell recovery 2 (%)#	Cell recovery 3 (%)§
PTC-CD133-052	180.69	50.29	18.37	9.24
PTC-CD133-055	123.68	38.25	20.10	7.69
PTC-CD133-068	38.13	85.21	30.05	25.61
PTC-CD133-085	44.89	78.99	20.69	16.34
PTC-CD133-090	15.40	70.22	35.45	24.89
PTC-CD133-098	65.81	51.23	30.05	15.40
PTC-CD133-109	97.54	68.17	51.20	34.90
PTC-CD133-116	40.61	61.62	56.77	34.98
Mean	75.84	63.00	32.83	21.13
Standard deviation	54.94	15.78	14.40	10.65
Median	55.35	64.89	30.05	20.62
Minimum value	15.40	38.25	18.37	7.69
Maximum value	180.69	85.21	56.77	34.98

*Preselection is referred to cells obtained after Ficoll centrifugation.
**Cell recovery from starting material.
#Cell recovery from pre-CliniMacs selection.
§Cell recovery from starting material.

The presence of unwanted cells in the final product (checked in two batches) was very low (less than 2%; data not shown). A detailed summary of absolute number as well as recovery of $CD133^+$ cells through each manufacturing phase is described in Table 2 for each batch.

3.3. Results of ATMP-CD133 Release Controls. Eight out of 8 batches resulted to be fully compliant according to the predefined acceptance criteria and they were released by a qualified person. Table 3 reports a summary of the release data obtained in all manufactured ATMP-CD133 batches. A representative example of $CD34^+/CD133^+$ cells characterization is illustrated in Figure 2, representing a simplified gating strategy adopted to determine purity of $CD34^+/CD133^+$ cells.

3.4. Drug Substance Stability. $CD133^+$ cells viability after overnight storage in X-VIVO15 has been systematically tested on each batch as shown in Table 4. An overnight incubation has been scheduled as necessary because cell production and quality test required several hours and they generally can be completed not before 7.00 pm. The surgical procedure was then scheduled the day after BM harvesting and processing. The overnight storage in X-VIVO has been validated for maintenance of cell viability without addition of any cytokines. This procedure allowed us to maintain the composition of the ATMP-CD133 untouched, while limiting the risk of zoonosis and/or adventitious virus contamination related with the use of FBS. Viability was found in compliance with the acceptance criteria of ≥70% for all tested batches. These data allow to confirm that drug substance, resuspended

Table 3: Summary of release data in 8 ATMP-CD133 batches.

Release test	Results				Acceptance criteria
	Mean	Standard deviation	Median	Range	
Purity (%)	88.86	±6.21	90.60	81.40–96.20	≥50%
Viability (%)	99.13	±1.47	99.87	96.03–99.97	≥70%
Cellularity ($\times 10^6$)	10.37	±9.08	9.69	2.85–30.84	$\geq 1.0 \times 10^6$
Sterility	Sterile (8/8 batches)				Sterile
Endotoxin	<0.5 EU/mL (8/8 batches)				<0.5 EU/mL
Mycoplasma	Absent (8/8 batches)				Absent

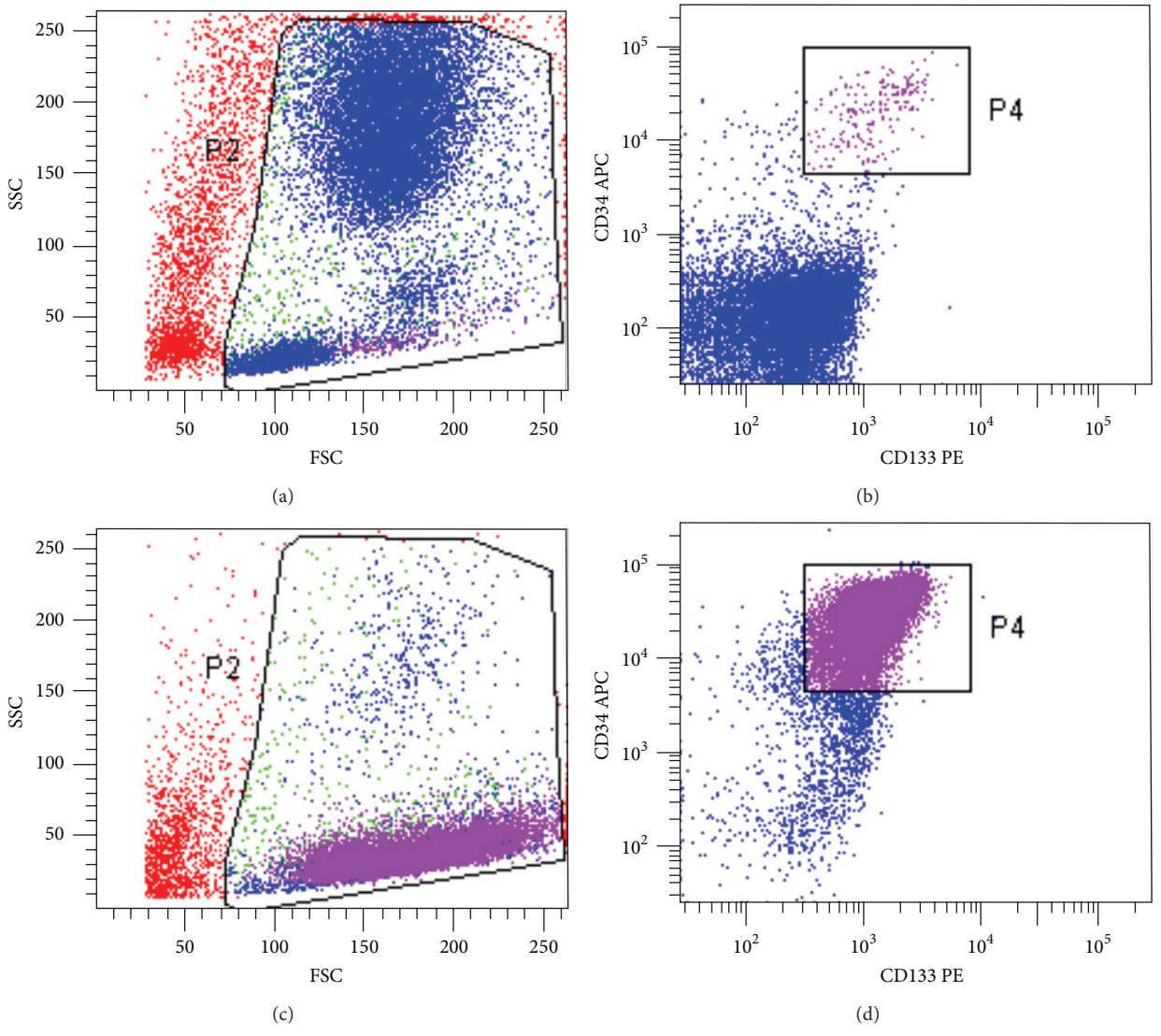

Figure 2: Flow cytometric analysis of $CD133^+$ cells. Representative example of purity testing as assessed by flow cytometric immunophenotyping. Upper panels (a and b) indicate the BM cell bulk before CliniMACS selection and lower panels (c and d) indicate the positive fraction of cells obtained after CliniMACS selection and overnight storage. Briefly population of interest is initially gated by a dual light scatter dot plot (panels a and c); $CD34^+/CD133^+$ double positive cells before and after selection are then identified and calculated by dual fluorescence dot plot analysis as indicated in panels (b) and (d), respectively.

Table 4: Stability of ATMP-CD133 during overnight storage in X-VIVO15.

Batch #	Time of overnight storage (hours:minutes)	Cell viability (% of live cells)
PTC-CD133-052	12:15	96.03
PTC-CD133-055	13:55	97.70
PTC-CD133-068	12:43	99.87
PTC-CD133-085	13:37	99.91
PTC-CD133-090	12:35	99.75
PTC-CD133-098	13:05	99.95
PTC-CD133-109	13:15	99.97
PTC-CD133-116	13:49	99.86

in X-VIVO15 and storage overnight at +2/+8°C, is stable for at least 14 h, which represents the time lapse before resuspending cells in physiological saline + 5% HSA. A summary of drug substance stability results is reported in Table 4.

3.5. Medicinal Product Stability. Stability was assessed retrospectively in 3 out of 8 batches. To this purpose, we thawed ATMP-CD133 frozen samples stored in liquid nitrogen at −196°C. After thawing $CD133^+$ cells were resuspended in physiological saline + 5% HSA and maintained up to 12 h. As reported in Table 5, the expected cell viability of at least 70% was obtained at any time point with values fulfilling the established threshold (mean 92.41% ± 1.22%). In addition the medicinal product demonstrated maintaining the expected immunophenotype up to 12 h, as shown in Table 5.

3.6. Partitioning of ATMP-CD133 Final Product. The final amount of cells shipped for clinical use was determined based on QC tests detailed in Table 6 in which a summary of cell partitioning is reported. The median of total cells after CliniMACS selection and overnight storage in X-VIVO15 was 9.69×10^6 (range 5.16×10^6–40.14×10^6). As described in FPC test (see Supplemental Table 2), a median of 2.40×10^6 cells (range 1.35×10^6–3.73×10^6) was collected to perform QC tests for batch release whereas the median amount of cells frozen for prospective QC testing was 0.96×10^6 (range 0.54×10^6–5.58×10^6). Hence, the final amount of cells delivered to the clinical site was 6.66×10^6 (range, 2.85×10^6–30.84×10^6).

4. Discussion

In November 2011, CAT/EMA agencies published a scientific recommendation concerning the classification of autologous BM-MNCs and autologous BM-derived $CD133^+$ cells intended for the treatment of postacute myocardial infarction and chronic ischemic heart disease as ATMPs [31]. Specifically, according to these guidelines, ATMP-CD133 comes under the definition of a somatic cell-based medicinal product to be manipulated as a drug. It is worth to note that the development of ATMP needs a consistent validation of cell product safety and potency. Moreover, its employment into a specific clinical setting requires taking into account several pieces of information such as the autologous/allogeneic use, the starting material, and the donors/recipient characteristics. Yet, translation of the ATMP-CD133 in a full-GMP setting needs a complete standardization of manufacturing steps as well as a robust validation of the release criteria. We have previously set preliminary clinical-grade manufacturing conditions to select cord blood derived-$CD133^+$ cells for cardiovascular regenerative purposes [26]. We provided a first evidence of manufacturing process reproducibility, setting specification for batches release, and the proof-of-principle of both *in vitro* and *in vivo* potency of ATMP-CD133 [26].

However, for a cardiac autologous clinical application, these data needed to be confirmed in the context of BM starting material of autologous origin, possibly taking into an account the specificity of patients as self-donors. Multiple cardiovascular risk factors, including aging, diabetes, and smoking habit, are in fact well known to severely affect levels and biology of PB and BM progenitors [24, 32]. Consequently, the present work aimed to generate the validation of a full GMP-grade manufacturing process to produce BM-derived $CD133^+$ cells for autologous cardiac cell therapy, taking advantage of the BM of patients dealing with refractory ischemic cardiomyopathy as starting material. Given the nature of compassionate therapy of the first-in-man study, no further validation of the ATMP-CD133 potency has unfortunately been allowed by the local Ethical Committee. This experiment will be carried out in the context of ongoing RECARDIO Trial (clinicaltrials.gov, Identifier: NCT02059681).

Based on our preclinical data [26] and the previous pilot clinical experience [33], we believe that the best recipients of ATMP-CD133 are patients with chronic ischemic HF not suitable to conventional treatments showing a significant amount of reversible ischemia in LV territories. In our pilot clinical experience with ATMP-CD133 in no-option angina patients, we were unable to correlate number of cells injected and angina frequency rate, given the low number of patients treated. This issue is however considered as secondary endpoint in the ongoing RECARDIO Trial (clinicaltrials.gov, Identifier: NCT02059681).

As for the manufacturing process reproducibility, the high variability in $CD133^+$ cell number, related to the high heterogeneity of an elder and sick cardiovascular patient population, is probably the major shortcoming of this therapeutic approach [24, 25, 34]. We have previously shown [14] that cellularity varied among published studies using BM-derived $CD133^+$ in a cardiac setting from 1.5 up to 16.9×10^6. In the recent CARDIO133 trial [3], Nasseri et al. have reported a cell infusion range of $3–9.1 \times 10^6$, with a mean of 5.1×10^6 cells. The relatively low number of cell injected has been advocated by the authors as a possible cause of a limited therapeutic effect. In our process, we were able to purify a mean of 10.37×10^6 (range 2.85×10^6–30.84×10^6) of BM-derived $CD133^+$ cells, thus increasing the number of cells available per patient. A possible partial explanation for this difference may be the more stringent standardization of BM volume collection, thus reducing the high variability among samples described in CARDIO133 trial.

Table 5: Stability of $CD133^+$ cells in physiological saline (+5% HSA) at 4°C in three ATMP-CD133 batches.

Batch #	Parameter	Time points			
		*T*0 (thawing)	*T* + 6 h	*T* + 9 h	*T* + 12 h
PTC-CD133-098	Viability (%)	97.00	91.68	90.76	91.07
	Purity (% $CD133^+$ cells)	92.76	nd	92.73	90.59
PTC-CD133-116	Viability (%)	90.72	94.23	94.07	93.47
	Purity (% $CD133^+$ cells)	84.69	nd	85.98	85.86
PTC-CD133-068	Viability	88.70	90.00	94.70	92.68
	Purity (% $CD133^+$ cells)	89.15	nd	89.41	87.89

nd: not done.

Table 6: Summary cell recovery according to processing phase.

Batch #	Number of total cells* after selection ($\times 10^6$)	Number of cells sampled for all QC test ($\times 10^6$)	Number of frozen cells for QC retesting ($\times 10^6$)	Number of cells shipped for clinical use ($\times 10^6$)
PTC-CD133-052	20.63	3.55	4.5	12.58
PTC-CD133-055	8.16	2.18	0.54	5.44
PTC-CD133-068	7.98	1.35	0.79	5.84
PTC-CD133-085	6.92	1.84	0.58	4.5
PTC-CD133-090	5.16	1.62	0.69	2.85
PTC-CD133-098	11.22	2.62	1.12	7.48
PTC-CD133-109	40.14	3.73	5.58	30.84
PTC-CD133-116	17.82	2.77	1.62	13.43
Mean	14.75	2.46	1.93	10.37
Standard Deviation	11.61	0.87	1.97	9.08
Median	9.69	2.40	0.96	6.66
Minimum Value	5.16	1.35	0.54	2.85
Maximum Value	40.14	3.73	5.58	30.84

*Number of cells obtained after CliniMACS selection and overnight storage in X-Vivo15.

Notably, in-process critical steps are also present throughout the sequential passages of product manipulation in which an amount of cellular loss is strictly related to the adopted purification protocols. Two critical steps (i.e., Ficoll centrifugation and CliniMACS selection) are responsible for a median cellular loss of 35.11% (range 14.79%–61.75%) and 69.95% (range 43.23%–81.63%), respectively. The ATMP-CD133 recovery has then been improved step by step during productions, reaching a cellular loss as low as 35.38% and 43.23% in Ficoll and CliniMACS selection, respectively. It is worth to note that we always achieved a number of cells fitting the threshold for clinical use, regardless of the cell amount for QC testing. As described in Table 6, a median of 3.36×10^6 of ATMP-CD133 cells is needed to perform QC tests. Moreover, considering the minimal manipulation of BM to select $CD133^+$ cells and the absence of an extensive expansion of cells, mycoplasma testing will be omitted from standard release criteria in our current protocols (see FPC2 Supplemental Table 2), thus saving 1×10^6 $CD133^+$ cells *per* batch injectable to the patient.

As for other relevant batch release criteria, as compared to our previous protocol [26], we have further increased the process efficiency, improving cell purity from 50% to 70% and viability from 70% to 80%. Thus, the final batch formula adopted for ATMP-CD133 in the RECARDIO trial [27] is defined as follows: purity ≥ 70%, viability ≥ 80%, cellularity between 1 and 12×10^6 cells, sterile, and endotoxin-free. For both purity and viability criteria a median value ≥ 90% has been obtained. Our standard quality parameters, purity and viability thresholds, are in agreement with data from previously published clinical trials using $CD133^+$ cells in a cardiologic setting, which adopted thresholds of at least 70% and 80% in cell purity and viability, respectively [4, 11, 35, 36]. As for cell dose, according to our first-in-man experience and data available in literature [4, 13, 37], we agreed about safety purposes with the competent Italian regulatory body (Istituto Superiore di Sanità, ISS) to set at 10×10^6 the highest $CD133^+$ cells dose. Considering ≥80% as minimal purity threshold obtained in our preparations, the final total ATMP-CD133 cell maximum number has been then established at 12×10^6. As suggested by ISS, the minimal cell dose has been set at 1×10^6 in line with published studies in the same clinical setting [6, 9–12, 38]. Importantly, this cell range has been verified as sustainable in our GMP conditions.

Finally, according to ATMP recommendations, to ensure the highest quality consistency as well as safety of the final

medicinal product, we have provided a specific characterization and monitoring of product impurities for each ATMP-CD133 batches produced. In summary, in this work we have optimized the ATMP-CD133 manufacturing process. However, in this protocol, Ficoll separation and CliniMACS selection are the most critical manipulation steps, representing a bottleneck for total cell recovery. Technological improvements are currently under investigation to overcome these limitations.

5. Conclusions

In conclusion, this work has been prepared to provide the full-GMP compliant manufacturing validation of bone marrow-derived human $CD133^+$ cells to be used as ATMP for cardiac cell therapy. This work may represent a platform for any future study of cell therapy with $CD133^+$ progenitors in the cardiologic field.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Daniela Belotti and Giuseppe Gaipa have contributed equally to this work as co-first author; Elisa Gambini and Giulio Pompilio have contributed equally to this work as co-last author.

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Research Article

Heart Rate Variability in Shift Workers: Responses to Orthostatism and Relationships with Anthropometry, Body Composition, and Blood Pressure

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In order to investigate the response of heart rate variability (HRV) components to postural change and their association with cardiovascular risk factors in shift workers, a cross-sectional study with 438 Brazilian males rotating shift workers was done. Anthropometric, body composition, and clinical measures were collected. Electrocardiogram was recorded for 3 minutes, in the supine and orthostatic position, and HRV components were extracted. Descriptive analyses showed that mean values of body mass index, waist circumference (WC), waist-to-height ratio, visceral fat area (VFA), and blood pressure (BP) were higher than the reference values. In the regression model, age, WC, VFA, and systolic BP showed negative association with HRV components. These findings suggest the need for determining effective strategies for the evaluation and promotion of health among shift workers focused on the altered variables.

1. Introduction

Heart rate variability (HRV) is a standard noninvasive method that assesses the action of the autonomic nervous system (ANS) on the heart based on variations in the RR interval between consecutive heartbeats [1]. Analysis of HRV on electrocardiogram allows for the decomposition of elements that assess the ANS function on the heart period [2]. A reduction in HRV, and more particularly in vagal modulation, could be considered an indicator of an imbalance in autonomic function and could be associated with an increased risk of morbidity and mortality independent of the presence of well-established risk factors, such as obesity, smoking, and sedentary lifestyle [3–5].

The continuous interplay of sympathetic and parasympathetic activity is crucial to increase or reduce heart performance under different circumstances. Under general physiological conditions, parasympathetic activity is more intense in the resting state and during repairing functions, and sympathetic activity is more intense in situations requiring the mobilization of energy [4]. For instance, during postural change (from supine to orthostatic position), blood pooling occurs in lower parts of the body, which is a normal stimulus that triggers an increase in sympathetic activity in the heart

and the vessels, with a consequent rise in blood pressure [6]. Such behavior has been found in some studies, which showed that sympathetic activity was greater in the orthostatic position (90°) and parasympathetic activity prevailed in the supine position (0°) [7, 8]. According to an important review, HRV at rest (wake-sleep and activity-rest) is reduced in shift workers compared to nonshift workers [9]; however, little is known about the causes of these alterations in the shift workers and besides that there is not any study investigating the cardiac autonomic response to postural change in this sample.

Obesity tends to be a strong risk factor for cardiovascular morbidity and mortality in young, middle-aged, and older persons. It has been suggested that ANS dysfunction is an important mediator in the development of obesity associated disease and insulin resistance, although the nature of the link between adiposity and insulin sensitivity is still unclear [10]. Shift work has been associated with deregulation of the circadian rhythm, which could change psychological functions, dietary and social habits, and ANS function and thus contribute to the high risk of cardiovascular diseases [11]. Therefore, it is relevant to investigate the relationship between the HRV components and cardiovascular risk factors (age, obesity, and hypertension) in shift workers.

Therefore, the aims of the present study were to investigate the response of HRV components to postural change and the association of HRV components with age, blood pressure, and obesity indices (anthropometric and body composition variables) in Brazilian shift workers. The hypotheses underlying the present study were as follows: (1) shift workers exhibit deviations in obesity indices, blood pressure, and HRV components relative to reference values; (2) shift work affects the baroreflex mediated response to postural change (transition of supine position to orthostatic position); and (3) HRV components exhibit negative associations with obesity indices, blood pressure, and age. This study has some relevant points: the sample homogeneity (Brazilian, male, rotating shift, and mine worker) and multiple variables collected on the same sample (HRV components, obesity indices, and blood pressure).

2. Materials and Methods

2.1. Study Design and Sample. This cross-sectional study was conducted with 438 adult Brazilian males who were older than 18 years and worked in shifts. Working in shifts, according to the International Labour Office (ILO) [12], is defined as a "method of organization of working time in which workers succeed one another at the workplace so that the establishment can operate longer than the hours of work individual workers" and can be classified into fixed shift (working time can be organized in two or three fixed shifts: the early, late, and night shifts) or rotating shift (workers might be assigned to work shifts that vary regularly over time: from a shift in the morning, to one in the afternoon, to one at night) [12].

The participants of this study performed rotational shift work as operators of iron ore extraction modern machines without noise exposure. The work regimen included a six-hour shift followed by 12-hour rest. The work shifts and rest periods were rotated, and after each fourth shift the rest period was longer, 36 hours, after which a new cycle began. The full work cycle included four consecutive shifts, in this sequence: 7:00 pm to 1:00 am, 1:00 pm to 7:00 pm, 7:00 am to 1:00 pm, and 1:00 am to 7:00 am.

The study was conducted in the morning of the day with the longest rest period from September 2011 to March 2012. The study complied with the Declaration of Helsinki and was approved by the ethics committee of the institution. All volunteers signed an informed consent form.

2.2. Anthropometric Variables. The volunteers' weight, height, and circumferences were measured by trained professionals with volunteers in the orthostatic position and wearing light clothing. The waist circumference (WC) was measured at the mid-point between the last costal arch and the iliac crest [13]. The hip circumference (HC), which was used to calculate the waist-to-hip ratio (WHR), was measured at the level of the greater trochanter [13]. The neck circumference (NC) was measured immediately below the laryngeal prominence [14]. The WHR and the waist-to-height ratio (WHtR) were calculated by dividing the WC by the hip circumference and the WC by the height, respectively [13]. The body mass index (BMI) was calculated by dividing the body weight by the squared height [13].

2.3. Body Composition Variables. The body fat mass in kilograms (BFkg) and percentage (BF%) and the visceral fat area (VFA) were calculated by means of segmental tetrapolar bioelectrical impedance using a body composition analyzer InBody model 720 (Biospace Co. Ltd. Factory, Korea) [15].

2.4. Clinical Variables. Blood pressure was assessed using an automated digital sphygmomanometer HEM705CP (Omron, Japan) on the right arm with volunteers in the sitting position and after a five-minute rest period. The measurement was performed three times with one-minute interval between measurements [16]. The three measurements of the systolic (SBP) and diastolic (DBP) blood pressure were computed and their means were calculated.

2.5. Heart Rate Variability. One PC-compatible computer controlled data acquisition for electrocardiographic parameters using the WinCardio (Micromed, version 4.8.2.8) software program. Electrocardiographic recordings were collected at a sampling frequency of 1000 Hz. An off-line peak detection algorithm (derivative plus threshold) was used to estimate fiducial R-wave points, after which the series was screened by hand and corrected for artifacts. Successive RR intervals were estimated in milliseconds and were converted to heart rate (HR) in beats per minute.

The HRV components were extracted for 3 min in the supine position and for 3 min in the orthostatic position using time domain analysis and frequency domain analysis. The time domain analysis measured changes in the intervals between successive normal RR intervals over time. The parameter used in this study was the root mean square

of successive RR interval differences (RMSSD) that reflects parasympathetic activity [2]. The frequency domain method is a spectral method for analysis of the tachogram that provides basic information about how power (variance) distributes as a function of frequency [2]. The components considered in the RR power spectrum in this study were as follows.

(i) High frequency (HF), from 0.15 to 0.4 Hz, reflects parasympathetic activity [2].

(ii) Low frequency (LF), from 0.04 to 0.15 Hz, could reflect sympathetic activity or a combination of sympathetic and parasympathetic activity [2]. More recently studies have proposed that LF reflects only the parasympathetic activity [17, 18].

(iii) LF/HF reflects sympathetic and parasympathetic balance [2].

Data processing followed the recommendations of the Task Force of the European Society of Cardiology and the North American Society of Pacing Electrophysiology [2]. MATLAB software (KARDIA) was used to analyze cardiac parameters [19].

2.6. Procedure. After the employees arrived at the research laboratory and signed the informed consent form, a medical interview was performed to collect information on the use and dosage of medications. Next, body weight, height, WC, HC, NC, BFkg, BF%, and VFA were measured.

Subsequently, after 5 min of resting, blood pressure was measured three times and finally a six-minute electrocardiogram (ECG) was recorded, with three minutes in the supine position and three minutes in the orthostatic position. Although all 12 leads were recorded, only peripheral (or bipolar limb) lead II was used for ECG processing. The employees had fasted for 12–14 hours.

2.7. Statistical Methods. The description of variables was presented as median, percentile, and interquartile ranges according to normality as tested by the Shapiro-Wilk test.

After logarithmic normalization of HRV variables, the Wilcoxon test was used to establish whether there were significant differences between the HRV components (logHR, logRMSSD, logHF, logLF, and logLF/HF) in the supine and orthostatic positions.

Spearman's correlation test was used to investigate the presence of a correlation between each HRV component as assessed in the supine position and obesity indices (anthropometric and body composition variables), blood pressure, and age. These correlations were performed only with HRV components in the supine position because these values are more stable and accurate than those in the orthostatic position [2].

Multiple regression models were fitted to investigate the influence of anthropometric and body composition variables, blood pressure, and age (independent variables) on each HRV component as assessed in the supine position (dependent variables). Analysis of residuals of each model was performed to assess validity of assumptions of normality, homoscedasticity, and independence between observations. The statistics Cook distance and variance inflation factor (VIF) were used to identify outliers and to check for possible multicollinearity.

Our analyses considered the wide range of medications reported as modifiers of HRV [20]. Two different physicians classified the medications used by each shift worker, and, in the event of disagreement between them, a third physician was consulted. Different groups of medicines are known to affect HRV in different ways and so, in order to identify the possibility of drug bias, all the analyses were redone after exclusion of workers who were taking medications that could influence the evaluated data.

The data were analyzed using Statistica 7.0 (StatSoft Inc.) and R Development Core Team (2013) software. The significance level was established as 0.05.

3. Results

3.1. Sample Characteristics. The present study comprised 438 individuals working in rotational shifts. Their age, anthropometric, body composition, and clinical characteristics are described in Table 1. The median age of the sample was 34 years. The median values of BMI, WC, WHtR, VFA, SBP, and DBP were higher than reference values [13–16].

3.2. Response of Heart Rate Variability Components to Postural Change. Comparison of HRV components between the supine and orthostatic positions showed increases in the median HR, LF, and LF/HF and reductions in the median RMSSD and HF in the orthostatic compared to the supine position (Table 2).

3.3. Relationship between Heart Rate Variability Components and Age, Anthropometric, Body Composition, and Clinical Variables. HR exhibited a positive correlation with all investigated variables except age. RMSSD and HF, which are related to parasympathetic activity, exhibited negative correlations with age, BMI, WC, NC, WHR, WHtR, BFkg, VFA, SBP, and DBP. LF, which is related to sympathetic and parasympathetic activity or to baroreflex function, exhibited negative correlations with age, BMI, WC, WHR, WHtR, and NC. The LF/HF ratio, which is related to autonomic balance, exhibited positive correlations with age, SBP, and DBP. All correlations are described in Table 3.

The fitted regression models showed that HRV components were influenced by age, WC, VFA, and SBP (Table 4). More specifically, VFA and SBP contributed to the increase of HR. VFA and age contributed to the reduction of logRMSSD. Age and WC explained the reduction of logHF and logLF. Finally, age and SBP explained the increase of logLF/HF.

Ninety-three of the 438 employees were using some type of medication. Of those 93, 27 were using one of the following groups of medicines, which were not identified as HRV modifiers: proton pump inhibitors, corticosteroids, nonsteroidal anti-inflammatory drugs, oral anticoagulants, and/or statins. The other 66 were using at least one of the following groups, which can potentially influence HRV [20]: antihypertensive drugs (thiazide-type diuretic; calcium

Table 1: Sample characteristics.

Variables	Median (p25; p75)	Quartile range
Age (years)	34.00 (31.00, 39.00)	8.00
Anthropometric		
Height (m)	1.74 (1.70, 1.79)	0.09
Body weight (kg)	80.80 (73.60, 89.30)	15.70
Body mass index (kg/m^2)	26.70 (24.40, 29.20)	4.80
Waist circumference (cm)	92.00 (85.50, 97.50)	12.00
Hip circumference (cm)	102.50 (98.30, 107.00)	8.75
Neck circumference (cm)	39.25 (37.50, 41.15)	3.65
Waist-to-hip ratio	0.89 (0.86, 0.93)	0.07
Waist-to-height ratio	0.53 (0.49, 0.56)	0.07
Body composition		
Total body fat (%)	23.55 (19.20, 28.20)	9.00
Total body fat (kg)	18.95 (14.60, 24.30)	9.70
Visceral fat area (cm^2)	121.25 (95.40, 147.70)	52.30
Clinical		
Systolic blood pressure (mmHg)	131.67 (122.67, 141.33)	18.70
Diastolic blood pressure (mmHg)	82.33 (76.00, 88.33)	12.30

Table 2: Medians of heart rate variability (HRV) variables in the supine and orthostatic positions.

HRV variables	Supine position Median (p25, p75)	Orthostatic position Median (p25, p75)	p value
HR (bpm)	65.9 (59.7, 71.9)	82.3 (74.2, 90.2)	<0.0001
RMSSD (ms)	34.3 (23.9, 50.1)	19.9 (13.3, 30.1)	<0.0001
HF (ms^2)	81.9 (35.7, 168.7)	26.1 (10.5, 62.1)	<0.0001
LF (ms^2)	103.4 (60.8, 186.4)	120.6 (62.4, 224.1)	0.005
LF/HF	1.3 (0.7, 2.5)	4.7 (2.5, 7.9)	<0.0001

HRV: heart rate variability; HR: heart rate; RMSSD: root mean square of successive RR interval differences; HF: high frequency; LF: low frequency; LF/HF: ratio of low frequency and high frequency. *Rawdata.* The Wilcoxon test was made using log transformation values, but, for ease of interpretation, all medians (plus p25, p75) are given in the original units.

Table 3: Spearman correlation coefficients (Rho) between heart rate variability measures (supine position) and age, anthropometric, body composition, and clinical variables.

Variables	log HR	log RMSSD	log HF	log LF	log LF/HF
Age	−0.04	**−0.27***	**−0.24***	**−0.17***	**0.13***
Anthropometric					
Body mass index (kg/m^2)	**0.18***	**−0.14***	**−0.14***	**−0.12***	0.05
Waist circumference (cm)	**0.22***	**−0.17***	**−0.16***	**−0.15***	0.05
Neck circumference (cm)	**0.19***	**−0.15***	**−0.14***	**−0.13***	0.04
Waist-to-hip ratio	**0.20***	**−0.18***	**−0.15***	**−0.15***	0.05
Waist-to-height ratio	**0.21***	**−0.15***	**−0.15***	**−0.14***	0.05
Body composition					
Total body fat (%)	**0.15***	−0.06	−0.07	−0.03	0.03
Total body fat (kg)	**0.17***	**−0.09***	**−0.11***	−0.08	0.03
Visceral fat area (cm^2)	**0.24***	**−0.13***	**−0.11***	−0.09	0.04
Clinical					
Systolic blood pressure (mmHg)	**0.21***	**−0.13***	**−0.14***	−0.002	**0.16***
Diastolic blood pressure (mmHg)	**0.18***	**−0.16***	**−0.16***	−0.06	**0.11***

log HF, natural logarithm of power in the high frequency range; log HR, natural logarithm of the heart rate; log LF, natural logarithm of power in the low frequency range; log LF/HF, natural logarithm of the ratio LF (ms^2)/HF (ms^2); log RMSSD, natural logarithm of the root mean square of successive RR interval differences; $*$ represents $p < 0.05$.

Table 4: Equations using regression analysis for cardiac variables.

Regression models	$\beta \pm$ SE	p value	R^2 adjusted
HR			
Constant	47.3 ± 4.172	<**0.001**	
Visceral fat area (cm^2)	0.0459 ± 0.01109	<**0.001**	8.4%
Systolic blood pressure (mmHg)	0.101 ± 0.03195	**0.002**	
log RMSSD			
Constant	2.018 ± 0.067	<**0.001**	
Age (years)	−0.010 ± 0.001	<**0.001**	10.3%
Visceral fat area (cm^2)	−0.0008 ± 0.0002	**0.002**	
log HF			
Constant	3.098 ± 0.242	<**0.001**	
Age (years)	−0.018 ± 0.003	<**0.001**	8.0%
Waist circumference (cm)	−0.005 ± 0.002	**0.016**	
log LF			
Constant	2.85 ± 0.193	<**0.001**	
Age (years)	−0.009 ± 0.002	**0.001**	4.5%
Waist circumference (cm)	−0.005 ± 0.002	**0.006**	
log LF/HF			
Constant	−0.652 ± 0.181	<**0.001**	
Age (years)	0.008 ± 0.002	**0.001**	4.3%
Systolic blood pressure (mmHg)	0.003 ± 0.001	**0.004**	

log HF: natural logarithm of power in the high frequency range; log HR, natural logarithm of the heart rate; log LF: natural logarithm of power in the low frequency range; log LF/HF: natural logarithm of the ratio LF (ms^2)/HF (ms^2); log RMSSD: natural logarithm of the root mean square of successive RR interval differences. Numbers in bold represent $p < 0.05$.

channel blocker; angiotensin-converting enzyme inhibitor; angiotensin receptor blocker; and beta-blockers), anticholinergics, beta2-agonists, antidepressants, anxiolytics, sedative/hypnotics, insulin, oral hypoglycemic agents, and/or levothyroxine. After excluding these 66 employees, we repeated the statistical analyses (data not shown) and noted that the significant results found for the total 438 employees remained. This allowed us to rule out the possibility of drug bias on the findings reported in this study.

4. Discussion

The results of the present study pointed to the presence of cardiovascular risk factors in the sample of Brazilian male rotating shift workers, as shown by anthropometry, body composition, and blood pressure, whose median results were higher than the corresponding normal values. In addition, these alterations were shown to correlate with changes in HRV components in the supine position, indicating increased sympathetic and reduced parasympathetic activity. However, the responses of HRV components to the postural change, a baroreflex mediated response, were adequate.

Obesity and hypertension, in addition to other comorbidities such as dyslipidemia and diabetes, are disorders that are frequently found in shift workers [21–23]. In the present study, median values of BMI, WC, WHtR, and VFA, which are indicators of obesity, and SBP and DBP, which are related to hypertension, were above the reference values [13–15], thus corroborating the reports in the literature. The constant presence of those disorders in shift workers is mainly associated with environmental and behavioral changes, as well as deregulation of biological rhythms and the lifestyle to which they are exposed, and these factors may exert a direct influence on their dietary habits, physical activity, sleep quality, mental health, and time available for social interaction [11, 22, 23].

Our findings showed that HRV components (HF, LF, and LF/HF) were qualitatively lower compared to the mean values in a review study with healthy adults [24]. In general, the HRV components vary within a wide range in male shift workers: HR, 58.2 to 82.8 bpm; LF, 323.7 to 1,212 ms^2; RMSSD, 29.9 to 70.2 ms; HF, 181.2 to 558.6 ms^2; and LF/HF, 1.6 to 3.4 [21, 23, 25, 26]. Values for LF and HF in the present study were substantially lower than the minimum when compared qualitatively with the values described above. The reason for that discrepancy may be associated with the methodological particularities of the various studies, including shift regimen (permanent or rotating), ECG signal processing (application of Task Force guidelines), and sample characteristics, including age and the presence of cardiovascular risk factors.

Nevertheless, the cardiac autonomic response to postural change was adequate, as the values corresponding to parasympathetic components (RMSSD and HF) were higher in the supine compared to the orthostatic position. Those results corroborate HRV at rest as a marker of parasympathetic control of the heart [4]. An increase in LF and LF/HF in the orthostatic position relative to the supine position is controversial [17]. Cysarz et al. [6] and Porta et al. [8] found that the magnitude of tilt interferes with the behavior of LF and LF

and LF/HF increased at a 90° tilt; the same increase occurred in the present study with voluntary postural change. To our knowledge, this is the first study to investigate the postural changes in a sample of shift workers. Probably, because postural change is a basic process of cardiac autonomic control, mediated by the baroreflex response, the postural control continued to function properly despite the fact that HRV components in the supine position were found to be lower than expected for healthy adults [24].

Use of RMSSD and HF as indicators of parasympathetic activity is well established [1, 2]. However, the meaning of LF, and consequently also of LF/HF, is still a subject of debate [6, 17, 18, 27]. According to classic studies, LF represents mainly the cardiac sympathetic modulation [5, 28]. Nowadays, some authors have proposed that LF is related to baroreflex function. According to Goldstein et al. [18], with or without adjustment for HF power or respiration, LF power seems to provide an index not of cardiac sympathetic tone but of baroreflex function once manipulations and drugs that change LF power or LF/HF do so not by affecting cardiac autonomic outflows directly but by affecting modulation of those outflows by baroreflex. Piccirillo et al. [27] showed, in a canine experimental acute myocardial infarction model, that, during congestive heart failure, the reduction of LF power and LF/HF ratio probably reflect diminished sinus node responsiveness to autonomic modulation or an abnormal baroreflex function. In another study of the same authors, they showed that LF and HF power were significantly lower seven weeks after acute canine myocardial infarction than at baseline [29]. Other authors have proposed that LF is predominantly associated with parasympathetic activity based on the findings: increases in HF and LF occur after the use of drugs that specifically enhance the cardiac vagal tone; there is similarity of LF values between individuals with cardiac sympathetic denervation and normal baroreflex sensitivity; some validated measures of sympathetic control of the heart, such as plasma epinephrine levels and cardiac norepinephrine spillover, are not correlated with LF [17, 18]. Therefore, more studies are necessary to clarify the origins and clinical significance of LF.

Among the variables that exhibited a correlation with HRV, the following remained significant following multiple linear regression analyses: age exhibited negative correlations with logRMSSD, logHF, and logLF and a positive correlation with LF/HF; WC exhibited negative correlations with logHF and logLF; VFA exhibited a negative correlation with logRMSSD and a positive correlation with HR; and SBP exhibited positive correlations with HR and LF/HF. These results indicate that, in the present sample, age, WC, VFA, and SBP were the variables with the strongest influence on HRV.

Similar to our study, Kim et al. [26] also observed that greater age is associated with lower HRV, parasympathetic activity, and baroreflex sensitivity in particular.

The findings about the WC and VFA could indicate that higher values of these anthropometric and body composition parameters are associated with less activity of HRV, specially the components that reflect parasympathetic activity. Similar results were reported by Ramos and Araújo [30], who found that cardiac vagal components decrease in parallel with increases in BMI, the sum of skinfold measurements (reflecting total body fat), and WC. Additionally, other studies found a relationship between WC, BF, and WHR and HRV vagal components [5, 26, 31].

With regard to blood pressure, SBP and DBP exhibited positive association with HR and LF/HF. Yue et al. [32] assessed the HRV in hypertensive and nonhypertensive patients and found increased sympathetic and decreased parasympathetic activity in hypertensive patients, compared to normotensive patients. In the same line, Thiyagarajan et al. [33] found that young adults with prehypertension had decreased cardiovagal modulation (HF, RMSSD, and SDNN), increased LF/HF ratio, and elevated cardiovascular risk factors comparable to the normotensive group.

Some limitations must be discussed about our findings. First, as the sample is composed only of male shift workers with the same work arrangement of alternating shifts, comparisons of gender and shift work specificities (permanent or alternating, clockwise or counterclockwise, etc.) could not be performed. Second, although the employees had similar environmental and work conditions (they were all operators of iron ore extraction machinery working in air-conditioned trucks with insulation against sound, water, and dust), data on behavioral factors (e.g., eating habits, physical activity, sleep-wake pattern, and smoking) was not collected. This information could be useful in determining potential mediators of the associations found. Third, we did not measure breathing during the electrocardiographic recordings. Although it is known that breathing (respiratory frequency and volume) potentially influences the HRV variables, Penttilä et al. [34] demonstrated that RMSSD values are not affected by the respiratory pattern. In the present study, both parasympathetic variables collected (HF and RMSSD) showed the same statistical tendencies; therefore, we can infer that the lack of breathing register most likely did not bias the study results.

5. Conclusion

Our findings showed that the cardiac autonomic response to postural change in shift workers was adequate. Nevertheless, they exhibited alterations in the assessed anthropometric, body composition, clinical, and HRV variables compared to the reference values. Regression analysis showed that age, obesity (WC and VFA), and SBP account for a significant part of the reduction of HRV components in this population. Even when taking into consideration the full complexity and variety of factors that may be involved in those alterations, the need to provide comprehensive healthcare to shift workers is undeniable, including specific occupational strategies to minimize the impact of these cardiovascular risk factors on their health and ensure good quality of life.

Conflict of Interests

The authors declare no conflict of interests.

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Research Article

The Potential of GMP-Compliant Platelet Lysate to Induce a Permissive State for Cardiovascular Transdifferentiation in Human Mediastinal Adipose Tissue-Derived Mesenchymal Stem Cells

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Human adipose tissue-derived mesenchymal stem cells (ADMSCs) are considered eligible candidates for cardiovascular stem cell therapy applications due to their cardiac transdifferentiation potential and immunotolerance. Over the years, the in vitro culture of ADMSCs by platelet lysate (PL), a hemoderivate containing numerous growth factors and cytokines derived from platelet pools, has allowed achieving a safe and reproducible methodology to obtain high cell yield prior to clinical administration. Nevertheless, the biological properties of PL are still to be fully elucidated. In this brief report we show the potential ability of PL to induce a permissive state of cardiac-like transdifferentiation and to cause epigenetic modifications. RTPCR results indicate an upregulation of Cx43, SMA, c-kit, and Thy-1 confirmed by immunofluorescence staining, compared to standard cultures with foetal bovine serum. Moreover, PL-cultured ADMSCs exhibit a remarkable increase of both acetylated histones 3 and 4, with a patient-dependent time trend, and methylation at lysine 9 on histone 3 preceding the acetylation. Expression levels of p300 and SIRT-1, two major regulators of histone 3, are also upregulated after treatment with PL. In conclusion, PL could unravel novel biological properties beyond its routine employment in noncardiac applications, providing new insights into the plasticity of human ADMSCs.

1. Introduction

Advances in stem cell therapy for treating cardiovascular diseases have been hampered by the complexity to understand the modality by which stem cells are committed to the cardiovascular lineage once injected and what molecular mechanisms underlie stem cell regenerative potential. In combination with the transdifferentiation ability toward the cardiac lineage, stem cell candidates should have also additional attributes such as high achievable yield, reproducible in vitro expansion, and immunotolerance. Among those adult stem populations under clinical investigation, mesenchymal stem cells (MSCs) have been reported to show all the above-mentioned features [1–4]. Particularly, adipose tissue-derived MSCs (ADMSCs), which originate from a noncardiac tissue source, have been successfully employed as a tool for cardiac stem cell-based therapy and for myocardial infarct treatment [5–7].

ADMSCs are routinely expanded in platelet lysate (PL), a hemocomponent enriched with growth factors and cytokines already employed as a substitute for foetal bovine serum (FBS) in cell culture and for treating those medical applications where repair, regeneration, and neoangiogenesis are desirable [8–10]. Interestingly, few studies have recently suggested PL as a candidate for cardiovascular tissue engineering purposes, due to its ability to enhance both matrix production and remodelling [11]. In addition, 5-azacitidine and PL-cultured ADMSCs have been demonstrated to possess high ability to transdifferentiate towards the cardiac phenotype similarly to FBS [12] and to promote migration and differentiation of murine cardiac fibroblasts [13]. Therefore, PL could represent an interesting tool for cardiac repair potential of ADMSC cultures.

We have already described both the suitability of the GMP-compliant PL to enhance the biological properties of a novel tissue source of human ADMSCs derived from the mediastinal depots [8] and their ability to transdifferentiate towards a cardiac-like phenotype upon exposure to a niche-like microenvironment such as that produced by cardiospheres [14], a 3D in vitro model of spontaneous cardiac microtissue [15–19].

In this brief report, we show for the first time the ability of a GMP-compliant PL (Mesengen kindly provided by Futura Relife, Publication number WO/2013/042095) to potentially promote a permissive state of cardiovascular commitment in human mediastinal ADMSCs and to cause epigenetic modifications mainly based on acetylation of histones 3 and 4.

2. Materials and Methods

2.1. Isolation and Expansion of Human Mediastinal ADMSCs. Human mediastinal ADMSCs have been isolated from patients undergoing thoracic surgery, as previously described [8, 14]. Informed consent was obtained from all subjects before the procedure. Experiments have been conducted in compliance with the Tenets of the Declaration of Helsinki. Briefly, after enzymatic digestion with trypsin-EDTA/1 mg/mL collagenase (Aurogene, Rome, Italy, Cat. number AU-X0930-100; GIBCO, Monza, Italy, Cat. number 17100), cells were divided into equal amounts and seeded in complete medium (DMEM-low glucose/1% PenStrep/1% glutamine/1% nonessential amino acids, all from Biowest, Nuaillé, France) supplemented either with 10% GMP-compliant PL (Mesengen kindly provided by Futura Relife S.r.l. Publication number WO/2013/042095) or FBS (Sigma-Aldrich, St. Louis, MO, USA), at a density of 4000 cells/cm^2. Nonadherent cells were removed after 3 days. Cell count and viability were evaluated by Trypan Blue (Sigma-Aldrich, St. Louis, MO, USA, Cat. number T8154). ADMSC cultures were characterized according to the criteria of the International Society for Cellular Therapy (ISCT) [20]. All experiments were performed at passage 3.

2.2. GMP-Compliant Platelet Lysate Preparation. GMP-compliant PL (Mesengen) was prepared and virally inactivated, as previously described [8, 9]. Briefly, after obtaining informed consent, buffy coats from healthy volunteers were centrifuged and treated with InterSol solution (318 mg Na-citrate $2H_2O$, 305 mg Na 2-phosphate anhydro, 105 mg Na dihydrogen phosphate $2H_2O$, 442 mg Na-acetate $3H_2O$, 452 mg NaCl, 100 mL H_2O, Fenwal Inc., Lake Zurich, Illinois) and subsequently with 20–30% human plasma. Potential pathogens were inactivated by using the Intercept Blood System for Platelets (Cerus Corporation, Concord, California, USA). PL was stored at −80°C for 24 hours before thawing at 37°C for 60 minutes. This procedure was repeated three times to enrich the pool of growth factors. Concentration and sterility of the preparation were determined by the haematology analyzer ABX Pentra DX 120 (Horiba ABX, Montpellier, France) and BACTEC 9240 (Becton and Dickinson), respectively. 5 U/mL heparin was added to cell culture media in order to avoid fibrin gel formation.

2.3. Real Time PCR. Total RNA was extracted using Total RNA Extraction kit (Qiagen) according to manufacturer's instructions and then reverse-transcribed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems). The evaluation of the expression of cardiac transdifferentiation genes [14] was assessed by quantitative Real Time PCR [14] using SensiMix SYBR Hi-ROX kit (Bioline) on a 7900HT Fast Real Time PCR System equipped with SDS software (Applied Biosystems) for 40 thermal cycles (95°C for 15 s, 56/58°C for 15 s, and 72°C for 15 s). Primers sequence and annealing temperatures for cardiac markers have been used according to our previous publication [14], whereas p300 and SIRT-1 primers sequences were the following: p300 forward → GGTCAAGCTCCAGTGTCTCAA; reverse → CCCTGGAGGCATTATAGGAGA; SIRT-1 forward → TGTACGACGAAGACGACGAC; reverse → TTCATCACCGAACAGAAGGTT.

The ΔΔCt method was used for data analysis, with GAPDH as the housekeeping gene and FBS as the reference condition.

2.4. Immunofluorescence. Human mediastinal ADMSCs cultured in PL or FBS were fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA, Cat. number 158127). After permeabilization with 0.5% Triton X-100 (Sigma) and incubation in blocking buffer (0.2% Gelatin, Sigma), primary antibodies (SMA 1 : 100 Abcam Cat. number ab7817; c-kit 25 μg/mL Abcam Cat. number ab5506; Thy-1 1 : 50 Dianova Cat. number DIA 120; Cx43 1 : 50 Millipore Cat. number MAB3067) were added overnight at +4°C [14]. Secondary antibodies (all Alexa Fluor 488, Invitrogen) were added at room temperature for 2 hours. Images were acquired by fluorescent microscope (Leica, software IAS2000). Nuclei were counterstained by DAPI (1 : 1000, 4′-6′-diamidino-2-phenylindole, powder ≥ 98%; Sigma, St. Louis, MO, USA, Cat. number D9542).

2.5. Acetylated Histones 3 and 4 Quantification. The endogenous levels of acetylated histones 3 and 4 were determined by ELISA assay (PathSCan acetyl-histone 3 and histone 4 Sandwich ELISA kit, Cell Signaling Cat. numbers 7209 and

7238, resp.) according to manufacturer's instructions. Briefly, human ADMSCs after 0, 6, 12, 24, 48, and 72 hours of stimulation with 10% LP were lysed with acid buffer and protein quantified by Bradford method. Equal amounts of protein lysates were incubated on histones 3 and 4 coated microwells. After washing, the acetylated-lysine monoclonal antibody was added, followed by incubation with the peroxidase-linked (HRP) antibody. The substrate for the HRP antibody was then added to develop colour. The absorbance was measured at 540 nm on a 96-well plate reader (Tecan). The magnitude of the optical density is directly proportional to the quantity of acetylated histones 3 and 4. Stimulation with Valproic acid (15 mM for 12 hours) was used as positive control.

2.6. Immunoblotting. Protein expression was analysed by denaturing discontinuous gel electrophoresis (Laemmli Gel Method). Whole extracts were obtained from subconfluent cultures resuspending cells in RIPA buffer (25 mM Tis-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 0,1% SDS, 1% deoxycholate, protease inhibitor (Sigma), and 0,1% mM phenylmethylsulfonyl fluoride). After incubation for 30 min at 4°C the lysate was centrifuged at 9240 rcf for 10 min. Protein concentration was determined by using Bradford reagent (Biorad). Whole extracts (80 μg) were fractionated by 15% SDS-PAGE electrophoresis (acrylamide and bisacrylamide 29 : 1, Biorad) and transferred onto 0.45 m PVDF sheets (Amersham) by using Transfer Blot SD Cell (Biorad) at 15 Volts for 30 min in presence of Towbin buffer (25 mM Tis-HCl, 192 mM glycine pH 8.3, 20% methanol, and 0.1% SDS). Membrane was blocked in 5% nonfat dry milk (Cell Signaling) and incubated overnight at 4°C with primary antibody against bimethylation sites of lysine 9 on histone 3, anti-H3K9m2 (07-212 Millipore), diluted 1 : 1000 in 5% nonfat dry milk. After incubation the sheets were washed in PBST buffer and incubated at room temperature for 1 hour in secondary antibody conjugated to horseradish-peroxidase (Amersham) diluted 1 : 10000. Actin was used as loading control. After ECL assay (GE Healthcare) the membrane was incubated with film specific for protein detection (Kodak).

2.7. Statistical Analysis. Data are presented as mean ± SEM unless specified. The independent sample two-tailed t-test with associated 95% confidence intervals was used to compare any two groups. A p value < 0.05 has been considered statistically significant.

3. Results and Discussion

In order to investigate whether the GMP-compliant PL is able to induce cardiac-like transdifferentiation, we derived three human primary mediastinal ADMSC lines from bioptic samples, expanded in presence of 10% PL (which is the standard concentration employed in vitro) or in presence of standard FBS and differentially characterized as we previously described [8, 14]. At passage 3, we evaluated their cardiac gene expression profile induced by PL, by testing cardiac stem cell/differentiation genes, such as smooth muscle actin

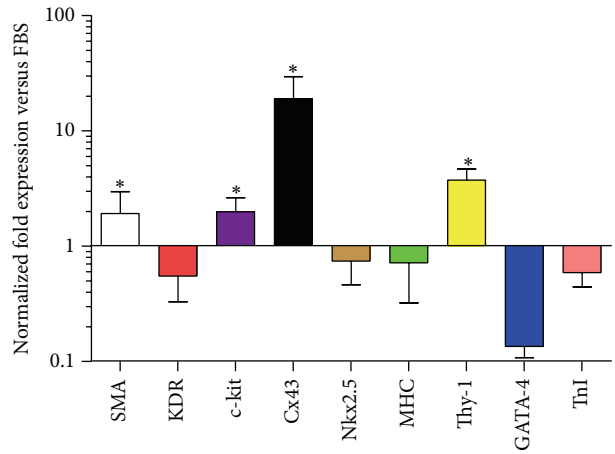

Figure 1: Cardiovascular gene expression profile by RTPCR of ADMSCs stimulated with 10% GMP-compliant platelet lysate. The graph shows a significant upregulation of SMA, c-kit, Cx43, and Thy-1. ADMSCs cultured in FBS were considered as the reference for normalization. $^*p < 0.05$.

(SMA), KDR, c-kit, Connexin 43 (Cx43), Nkx2.5, myosin heavy chain (MHC), Thy-1, GATA-4, and troponin I (TnI). ADMSCs cultures derived from biopsies cultured in FBS were used as control. Results showed a significant upregulation of SMA, c-kit, Cx43, and Thy-1 in PL cultures versus FBS (Figure 1, all $p < 0.05$). Differently, other cardiac markers (KDR, Nkx2.5, MHC, GATA-4, and TnI) were unmodified.

To then verify whether a similar phenotype was also expressed at protein level, we performed immunofluorescence staining for the upregulated cardiovascular markers (SMA, Cx43, Thy-1, and c-kit). Results showed a much more aligned distribution of Cx43, with focal-like areas in PL-cultured ADMSCs, compared to scattered spots in FBS (Figure 2(b)). We also found an enhanced expression of Thy-1 and c-kit (Figures 2(c)–2(e)) compared to FBS (Figures 2(d)–2(f)) but similar positivity for SMA (Figures 2(g) and 2(h)).

The differentiation of ADMSCs toward a cardiogenic phenotype has been described, spontaneously [21], by using 5-azacytidine [22] or by culturing MSCs onto a feeder layer of rat cardiomyocytes [23]. Recently, we have strengthened the biological potential of a novel multipotent stem cell population of ADMSCs derived from the mediastinal depots by demonstrating their plastic commitment to cardiac/endothelial/muscular-like phenotypes upon cardiosphere-mediated paracrine action [14]. Our results confirm such plasticity of human ADMSCs, able to respond to a wide range of stimuli including PL. Nevertheless, cardiosphere and PL-generated microenvironments differently influenced ADMSC cultures. Specifically, both c-kit and Cx43 (a stem cell cardiac precursor marker, albeit highly debated [24], and a late marker of cardiac transdifferentiation, resp.) are upregulated in PL-cultured ADMSCs, but they were not induced by cardiosphere-conditioned media exposure. This indicates the relevance of the specific stimulus on the outcome of the phenotypical commitment, most likely due to the different composition in the type and concentration of soluble factors within the two microenvironments [25–27]. Besides,

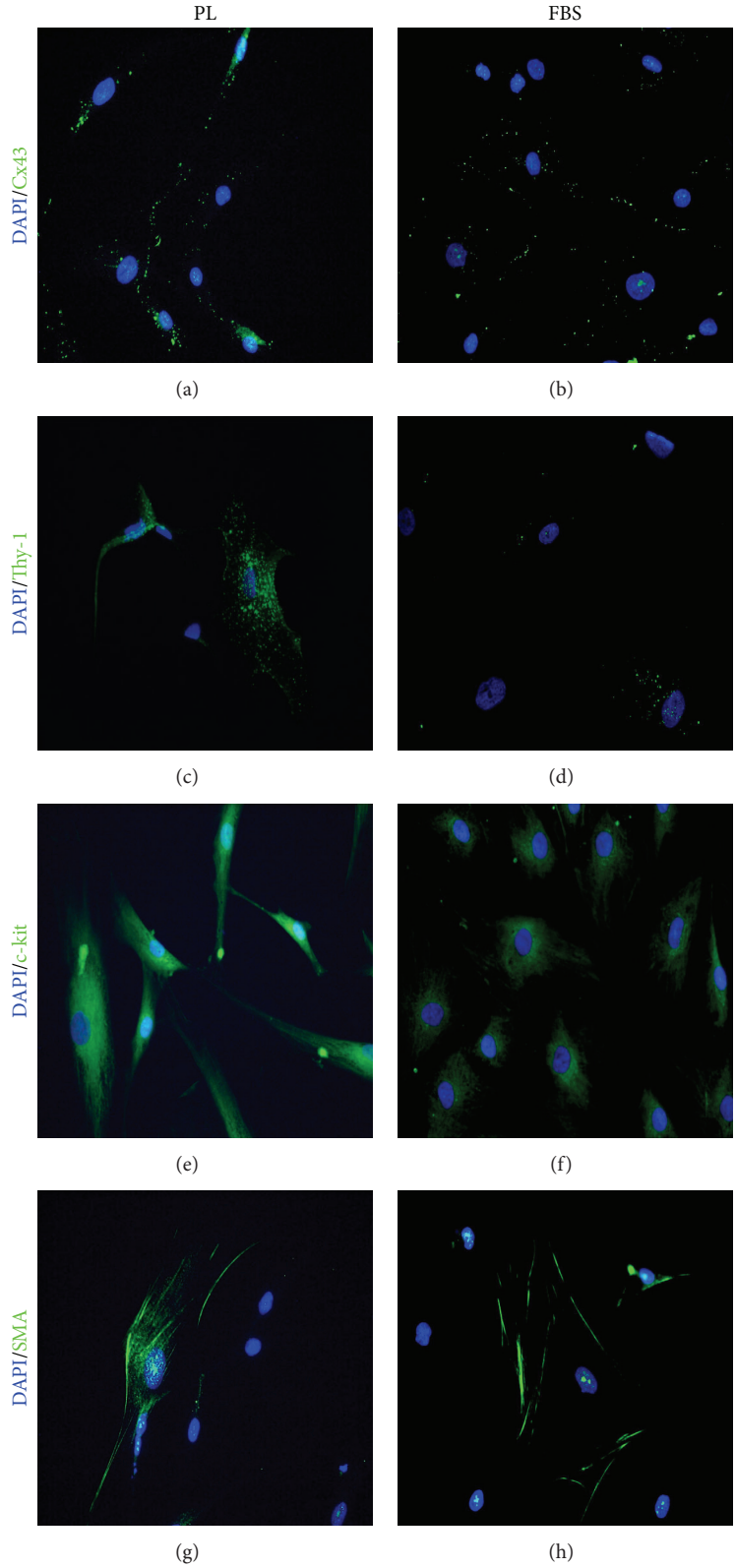

Figure 2: Representative images of immunofluorescence staining for SMA, Cx43, Thy-1, and c-kit. Platelet lysate-cultured ADMSCs displayed positive aligned and focal-like fluorescence for Cx43 (a) and positivity for Thy-1 (c) and c-kit (e). ADMSCs cultured in the presence of FBS showed a scattered distribution of Cx43 (b) and a fainter positivity for Thy-1 (d) and c-kit (f). The expression of SMA was similar in both conditions (g-h). DAPI staining (blue); Cx43, Thy-1, c-kit, and SMA staining: green. PL: platelet lysate. Magnification 20x.

although a different study has already suggested the potential of PL to promote cardiac phenotype of ADMSCs [12], the commitment was driven by a combination of PL and 5-azacytidine (a widely used, although controversial, cardiac transdifferentiation agent [14]), thus masking the real efficacy of PL. The GMP-compliant PL preparations employed in this study are enriched with specific growth factors, including VEGF, TGF-β1, PDGF, and EGF [8, 9], known to improve cell proliferation and migration, the angiogenic process, and collagen deposition and recruitment of circulating macrophages and lymphocytes to the site of injury, thus enhancing tissue regeneration and wound healing [23, 28–31]. In particular, TGF-β1 has been reported to significantly increase the expression of both Cx43 and SMA [32] and to contribute to the remodeling of cardiac fibroblast populations after myocardial infarct [33]. The upregulation of Cx43 and SMA expression levels in ADMSCs upon PL stimulation confirms the role of PL in the cytoskeletal rearrangement and suggests an electrical and metabolic coupling potential between MSCs. To what extent PL could lead to beneficial effects is still to be fully elucidated. For instance, the culture of heart valve tissue constructs in presence of PL is not desirable due to fibrotic effects [34], thus suggesting a selective suitability for cardiovascular applications.

Differently, despite a discrete concentration of VEGF and EGF in the PL preparations used in this study, we have not observed a preferential commitment to the endothelial lineage but to an increase in c-kit gene expression levels, which is normally not expressed by ADMSCs after isolation [8]. However, ADMSCs have been shown to contain a c-kit$^+$ subpopulation, probably derived by the perivascular area and displaying high preservation ability for cardiac progenitor cells [35]. Besides, considering that niches containing a combination of adult cardiomyocytes, allogenic MSCs, and c-kit$^+$ cardiac progenitor cells expressing Cx43-mediated gap junctions have been found in in vivo models [36, 37], we could speculate that the treatment with PL might also promote a protective and feeder-like support role for ADMSCs within the cardiac niche once injected.

Finally, given the involvement of histones 3 and 4 modifications in cardiac differentiation [38], we have assessed whether PL could induce similar epigenetic changes. Accordingly, human mediastinal ADMSC cultures derived from 3 patients were starved for 2 hours and stimulated with 10% PL up to 72 hours and both levels of total acetylated histones 3 and 4 (occurring at lysine residues such as 4, 9, 14, 18, 23, or 27) [39, 40] were screened by ELISA assay. Conditioning with Valproic acid, known to exert histone deacetylase-inhibiting effects, was used as positive control. Results showed a significant increase in histone 3 acetylation in all patients, but with a different time trend, certainly due to their individual genetic asset which influences the biological response to PL. Patient 1 displayed an oscillating trend starting with a prompt upregulation of histone 3 acetylation after 6 hours (Figure 3(a)), whereas Patients 2 and 3 responded with a more constant increase over the time, reaching a peak after 48 hours (Figures 3(c) and 3(e)). A similar trend with regard to acetylated histone 4 was observed in all patients (Figures 3(b), 3(d), and 3(f)). The interpatient variability was also confirmed by the different reactivity to Valproic acid (Figures 3(g) and 3(h)).

Next, we investigated whether p300 and SIRT-1, two main regulators of histone 3 with acetyltransferase [41] and deacetylase activity [42], respectively, were modulated upon PL treatment. To this aim, we specifically focused on Patient 3, who had displayed the lowest reactivity to Valproic acid, thus potentially suggesting a more complex chromatin structure. Results showed that the treatment with PL, but not with FBS, led to increased mRNA expression levels of both p300 and SIRT-1 after 6 hours, rapidly decreasing and fluctuating around the baseline (Figures 4(a) and 4(b)). The discrepancy between the early upregulation of p300 and the late acetylation of histone 3 (Figure 3(e)) could be explained by a possible autoacetylation of the protein, as already observed in cardiac myocytes upon acute stress, leading to the stabilization and accumulation of p300 itself after 24 hours [43]. Moreover, a parallel involvement of SIRT-1 is not surprising, considering that other cardiac-like genes such as MHC, KDR, TnI, and GATA-4 were downregulated (Figure 1), thus suggesting control of epigenetic gene silencing through deacetylase activity. Although the mechanism by which SIRT-1 acts in MSCs is still unclear, the loss of SIRT-1-based deacetylase activity corresponds to deregulated MSC differentiation [44, 45]. Therefore, MSC lineage determination is certainly controlled by both epigenetic histone acetylation and deacetylation mechanisms.

Finally, given the main role of acetylated histone 3 to mediate cardiac differentiation, we asked whether a modulation of the methylation, also known to control mesodermal transdifferentiation in MSCs [46], could occur after stimulation with PL. To this aim, we specifically assessed the bimethylation of lysine 9 on histone 3 (H3K9m2), normally associated with the chondrogenic, osteogenic, and adipogenic transdifferentiation ability of MSCs [46]. Results on Patient 3 have shown a significant increase in methylation at 24 hours after stimulation with PL (Figures 5(a) and 5(b)).

The physiological process of transdifferentiation occurs at gene expression level, influencing stem cell reprogramming and lineage commitment [47, 48]. Particularly, epigenetic modifications by regulation of histone acetylation and methylation have been associated with stem cell fate, determining the modality by which chromatin is more or less accessible at the gene promoters sites [49]. A recent study has demonstrated that distinct gene-specific histone modifications are involved in cardiac differentiation potential of cardiac progenitor cells (CPCs) and that histones 3 and 4 of CPCs$^{Sca\text{-}1+/CD29+}$ are more acetylated at the promoters of cardiac specific genes, thus highlighting that both these two histone proteins are involved in cardiac differentiation [38]. Interestingly, our data highlight that PL is able to induce epigenetic modifications by promoting more transcriptionally active chromatin structures and that the response is patient-dependent.

A further study has reported that the epigenetic cardiac signature in ADSCMs is based on a combination of low levels

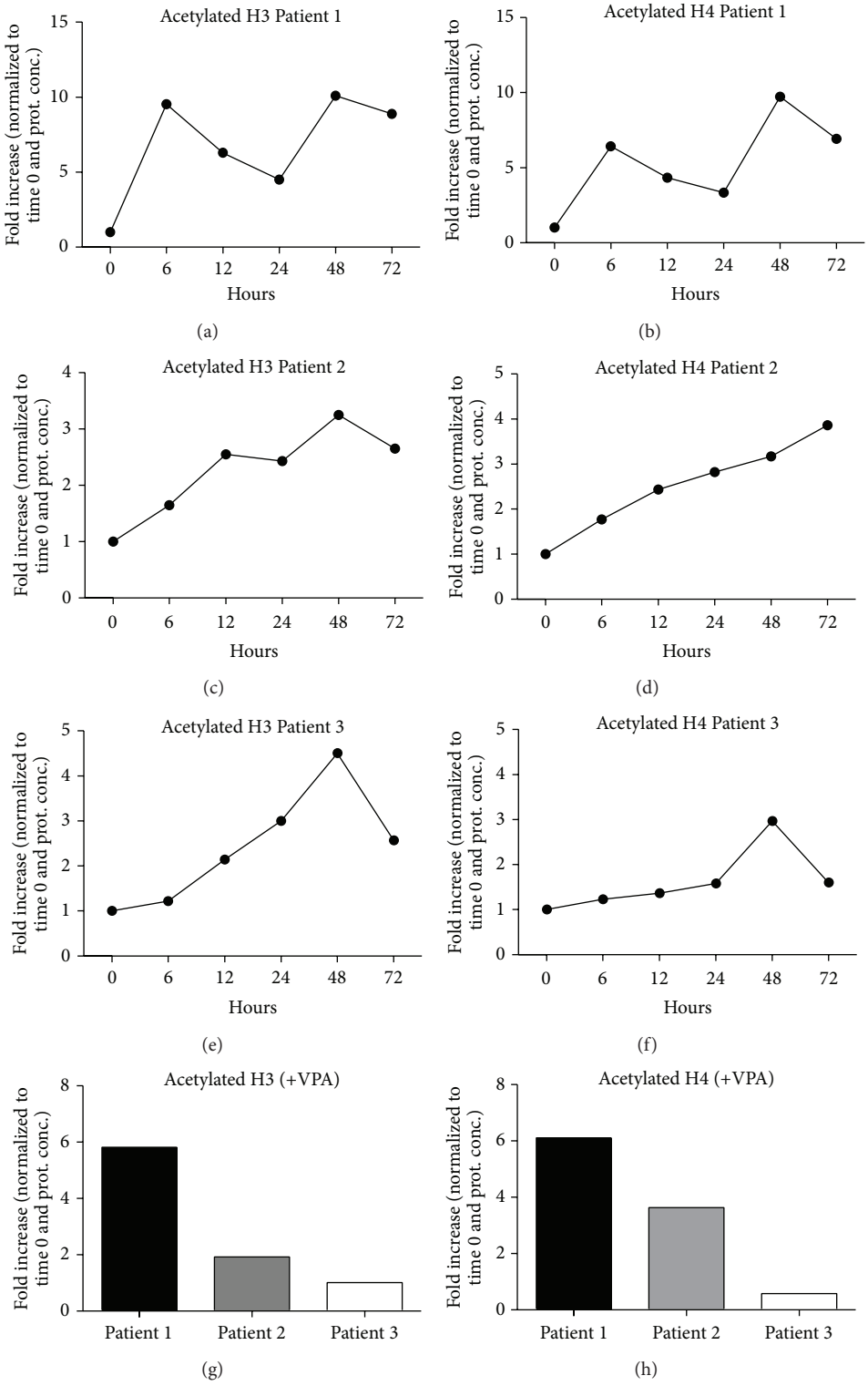

Figure 3: Detection of acetylated histone 3 and histone 4 levels in Patients 1 (a, b), 2 (c-d), and 3 (e-f) up to 72 hours of stimulation with platelet lysate, by Sandwich ELISA Antibody Pair assay. The graphs show an oscillatory response to platelet lysate of Patient 1 and a sustained signal in Patients 2 and 3 for both histones. Levels of acetylated H3 and H4 in Patients 1, 2, and 3 after stimulation with Valproic acid (VPA) showed a response with a different reactivity of the subjects (g-h). Results have been normalized to both total protein concentration and time 0. H3, histone 3; H4, histone 4.

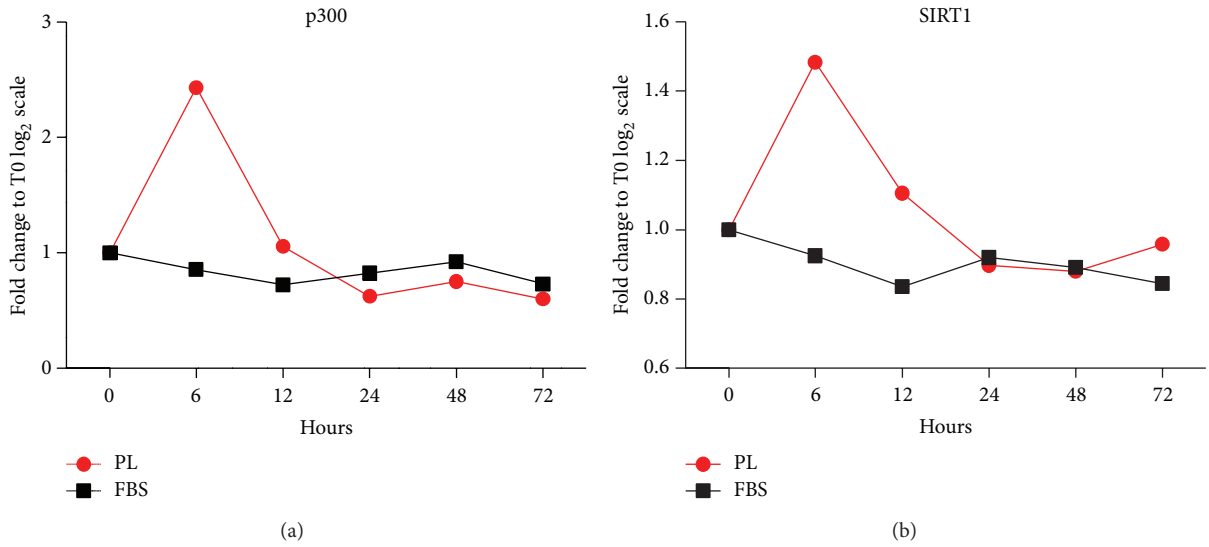

Figure 4: Evaluation of p300 and SIRT-1 gene expression by RTPCR of ADMSCs derived from Patient 3 and stimulated with 10% GMP-compliant platelet lysate. The graphs show that the treatment with platelet lysate but not with FBS is able to increase mRNA expression levels of both p300 (a) and SIRT-1 (b) after 6 hours, rapidly decreasing and fluctuating around the baseline. PL, platelet lysate.

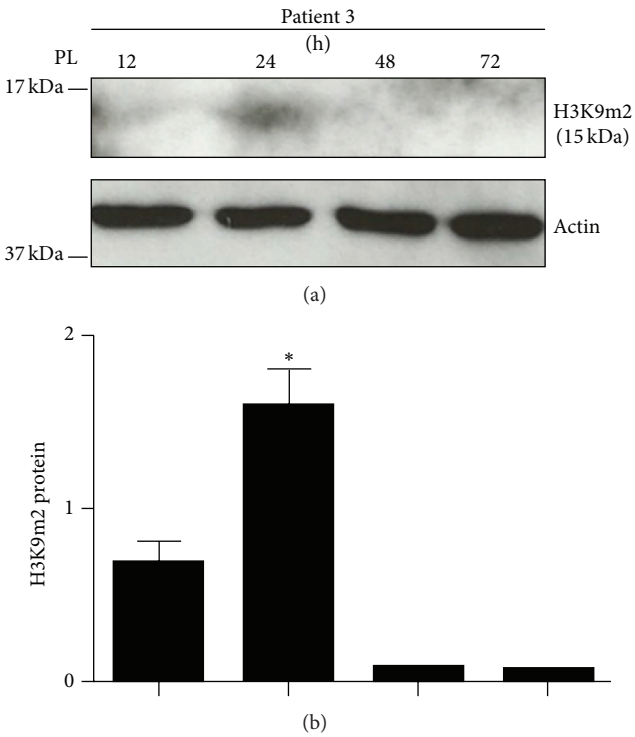

Figure 5: Representative image of the immunoblot of the bimethylation on lysine 9 of H3 (H3K9m2) in Patient 3 (a), where a significant increase at 24 hours was observed (b). $^{*}p < 0.05$; PL, platelet lysate; H3, histone 3.

of total acetylated histone 3 and high levels of trimethylated lysine 27 [50], whereas a different study has associated the same methylation (H3K27) with the osteogenic differentiation and the acetylation of histones 3 and 4 mainly with the cardiac lineage [46]. This clear discrepancy is likely due to the possibility that MSCs derived from different tissue sources do not share similar epigenetic states [22], thus suggesting a consequent different transdifferentiation potential. By conditioning with PL, we also observed a general acetylation of histone 3 peaking at 48 hours preceded by dimethylation of lysine 9. We might speculate that the shift towards the demethylation, which is associated with a condensed state of histone 3 [46], could represent a preliminary and permissive step to switch on the acetylation. In addition, considering the bivalent activity of the chromatin in MSCs where parallel methylation and acetylation can coexist [50] but also the role of the demethylation of lysine 9 on histone 3 both in adipogenic, chondrogenic, and osteogenic transdifferentiation [46, 51, 52] and in the capacity to escape senescence mechanisms [53], we could suggest that PL is able to target a multipotent gene expression potential in ADMSCs, including the cardiac lineage but concurrently preserving a proliferative stem cell pool and the capacity to transdifferentiate into the mesodermal lineage. In other words, the commitment to the cardiovascular and the mesodermal lineage would not be mutually exclusive. Moreover, our data are in line with a further study, showing that the inhibition of histone acetylation interferes with the mesodermal commitment of PL-grown ADMSCs [54], thus confirming that the acetylation of histone 3 can bear a bivalent function in inducing both cardiac and mesodermal commitment. Since stem cell fate is not solely determined by epigenetic modifications, the functional consequence of the potential ability of PL to induce either cardiovascular or mesodermal transdifferentiation will eventually depend on the tissue microenvironment surrounding the ADMSCs.

We cannot still conclude that the potential of PL is sufficient to the final cardiac transdifferentiation in ADMSCs but only that it is able to provide a permissive state, likely functional upon specific cardiac/vascular signals. Besides, we could assume that the chromatin remodelling observed is

due to a general effect caused by the synergic action of the numerous growth factors within the PL or simply that other nonepigenetic mechanisms, such as posttranslational histone modifications, are involved. Moreover, a proper comparison with a nonadipose tissue-derived MSC population and with FBS would allow us to understand whether or not the effect is specific to both ADMSC and PL.

The possibility to precondition MSCs prior to in vivo injection with PL (containing numerous angiogenic and muscular growth factors but many inflammatory cytokines as well) could represent an advantage compared to other differentiation methods in modulating the immune response. Accordingly, in order to enhance MSC cardiac commitment and therapeutic potential, PL could substitute the numerous current attempts to design cocktails of soluble molecules and factors [55, 56] by a more standardized, safe, and reproducible method. The numerous intrinsic biological effects of MSCs could be maximized and potentiated, including the feeder-like support potential of MSCs on cardiac myocytes, considering the ability of MSCs to secrete TGF-β and its discrete amount in PL preparations that may allow cardiac myocytes to reenter the cell cycle [55]. The dual ability of PL to both simultaneously expand and commit ADMSCs to the cardiac-like lineage could be also useful to overcome the low retention of engrafted MSCs observed within the cardiac tissue after in vivo injection [57], compensated by a higher number of injected cells. Besides, it has been extensively demonstrated that the cardiac differentiation potential of other different stem cell types (bone marrow derived c-kit$^+$ and monocytes, fibroblasts, or cardiomyocytes) is limited as they only provide either the angiogenic or the muscular component [58]. From this perspective, only cardiac resident progenitor cells could represent a better suitable option than MSCs [14, 59, 60]; however their scarcity and difficult retrievability in cardiac tissue limit their employment, albeit even combined cell therapy protocols have been suggested [61]. Considering the intrinsic ability of ADMSCs to differentiate into multiple cell types including muscle cells [62] and to induce neovascularization in the injured heart [63], the treatment with PL may be biologically significant on multiple levels in MSCs.

4. Conclusions

Understanding the mechanisms controlling the cardiac transdifferentiation process in ADMSCs is essential to boost MSC unique beneficial properties. The possibility to explore PL as a cardiac transdifferentiation permissive agent could suggest a novel therapeutic approach beyond its routine employment in noncardiac applications and provide new insights into the biology of human mediastinal ADMSCs.

Conflict of Interests

The authors declare no conflict of interests. Luca Pierelli only retains the inventorship of the patent concerning platelet lysate in regenerative medicine (Patent Italy-RM2011A000500-23.09.2011-IT-Frati L, Frati G, Nuti M, Pierelli L. Platelet lysate, uses, and method for the preparation thereof-Lysat de plaquettes, ses utilisations et son procédé de préparation, Applicants: Sapienza University of Rome [it/it]; (it). Futura Stem Cells sa [ch/ch]; (ch) Pub. number WO/2013/042095, International Application number PCT/IB2012/055062).

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Research Article

Using Multicriteria Decision Analysis to Support Research Priority Setting in Biomedical Translational Research Projects

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Translational research is conducted to achieve a predefined set of economic or societal goals. As a result, investment decisions on where available resources have the highest potential in achieving these goals have to be made. In this paper, we first describe how multicriteria decision analysis can assist in defining the decision context and in ensuring that all relevant aspects of the decision problem are incorporated in the decision making process. We then present the results of a case study to support priority setting in a translational research consortium aimed at reducing the burden of disease of type 2 diabetes. During problem structuring, we identified four research alternatives (primary, secondary, tertiary microvascular, and tertiary macrovascular prevention) and a set of six decision criteria. Scoring of these alternatives against the criteria was done using a combination of expert judgement and previously published data. Lastly, decision analysis was performed using stochastic multicriteria acceptability analysis, which allows for the combined use of numerical and ordinal data. We found that the development of novel techniques applied in secondary prevention would be a poor investment of research funds. The ranking of the remaining alternatives was however strongly dependent on the decision maker's preferences for certain criteria.

1. Introduction

The difficulty of developing biomedical discoveries into new medical technologies or therapies has been widely recognized and is often referred to as the "bench-bed gap" or the "valley of death" [1, 2]. Translational research aims to bridge this gap by integrating the societal needs identified at the bedside with the research done at the bench. It encompasses the entire value chain from basic biomedical research, through epidemiology, clinical testing, product development, policy and regulatory compliance, and marketing. As a result, the overall success of a translational research project is determined by a multitude of technological, clinical, economic, and regulatory factors. All these factors need to be considered when evaluating which of the available research strategies are most likely to yield innovations that will eventually gain widespread adoption in daily clinical practice. This makes priority setting for translational research a complex problem that requires decision makers to gather and synthesize expertise from different fields. Without the use of a formal decision support method, it is generally impossible to simultaneously consider all aspects of such a decision problem, making it likely that too much emphasis is put on a single outcome of the translational research process. In such a setting, the use of multicriteria decision analysis (MCDA) can assist in structuring the problem and in making the decisions justifiable and replicable, thereby increasing accountability for public resources spend [3].

In the context of government-sponsored technology development programs, MCDA has previously been applied to support the selection of research and development projects across different industries and focus areas [4, 5]. However, these applications are not directly portable to research priority setting in biomedical translational research projects as the healthcare industry has specific properties that were not addressed in these studies. In particular, healthcare markets are heavily regulated and public provision of goods and services plays an important role in these markets. These characteristics impose rather strict constraints with respect to market penetration and price setting that already need to be considered early during the translational research process. In this paper, we demonstrate how these aspects can be

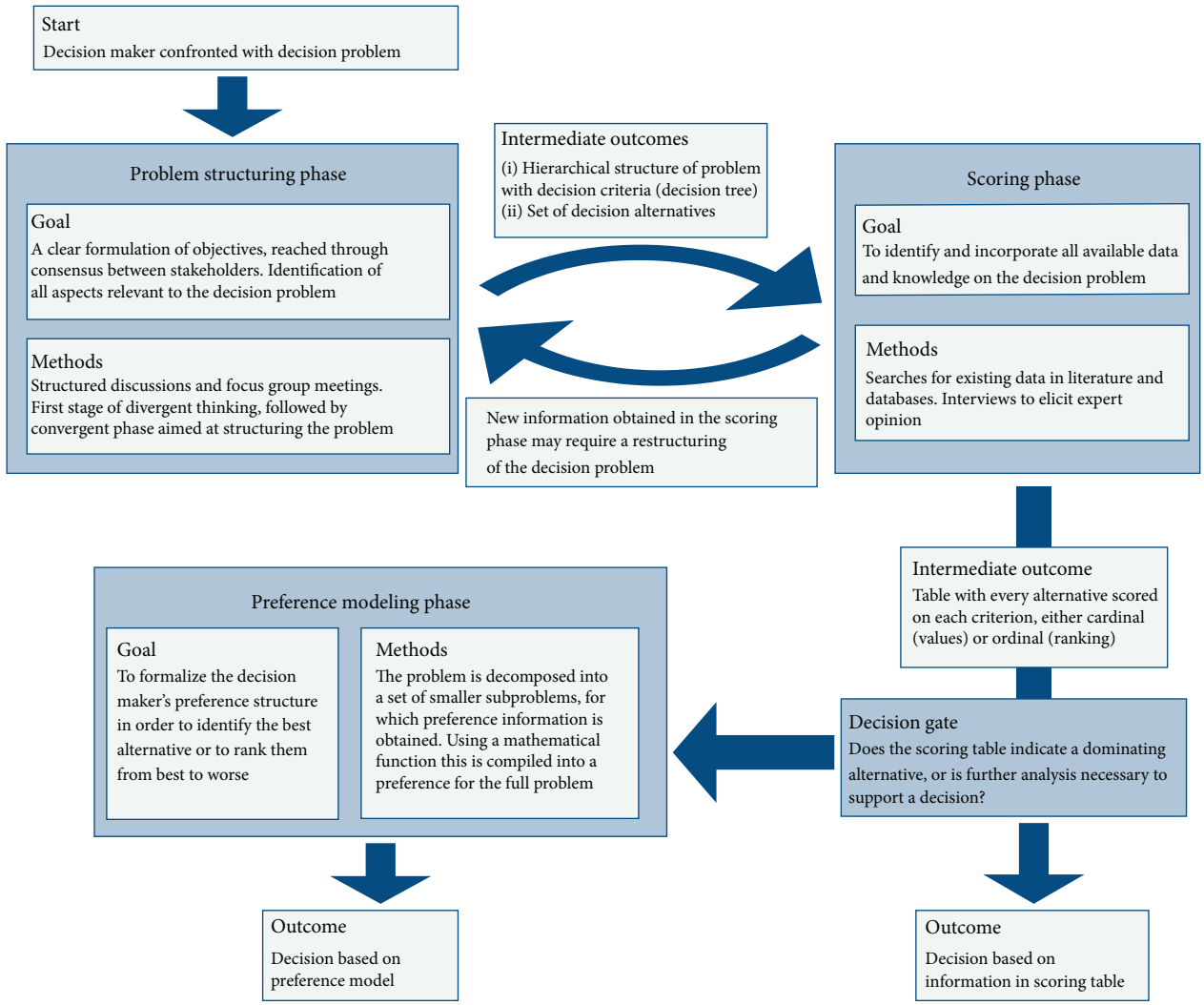

Figure 1: Schematic overview of the application of multicriteria decision analysis for priority setting.

incorporated in a formal way by using MCDA for priority setting at the start of a translational research project. We illustrate this by means of a case study conducted within the context of a translational research project aimed at the prevention of type 2 Diabetes Mellitus (DM2) and its related complications.

2. Application of MCDA to Research Priority Setting in Biomedical Translational Research Projects

Research priority setting for biomedical translational research is a complex problem that requires decision makers to consider a multitude of technological, clinical, economic, and regulatory factors. In such situations, the use of a formal decision support method encourages the incorporation of views and knowledge from experts in different parts of the value chain of biomedical research, thereby reducing the possibility that at later stages in the product development process problems are encountered that in hindsight could already have been foreseen at the start of the project. It can also ensure that all available information related to the decision problem is incorporated into the decision making process, thereby reducing the chance that the decision focuses too much on a single or narrow set of aspects of the problem. Within the framework of MCDA, this is achieved by sequentially going through the following three phases: problem structuring, scoring of the alternatives against the criteria, and preference modeling (Figure 1). Each of these phases is briefly described in the subsections below.

2.1. Problem Structuring. During problem structuring, the different stakeholders involved in the decision making process express their knowledge and views on the context of the decision problem as well as their objectives regarding the decision. Several formats and tools have been proposed to support this idea generation process, including "Post-It" sessions and various checklists and other aids to thinking such as adopting different perspectives and identifying barriers and constraints [3]. This divergent mode of thinking

is followed by a convergent phase of idea structuring, in which ideas are clustered and aggregated to arrive at a set of decision alternatives (if not yet clearly defined at the start of the process) and a set of criteria against which these alternatives are to be evaluated. Depending on the decision context, the definition of these criteria can to an extent be informed by objective knowledge of relevant cause-and-effect mechanisms from scientific literature or other sources. However, the criteria should reflect the objectives of the relevant decision makers and therefore should be derived from discussions with the decision makers. Knowledge from outside the decision maker group can be incorporated into these discussions but should never dictate criteria by itself. The output of the problem structuring phase is often a value tree. This is a graphical representation of the hierarchical ordering of the criteria.

2.2. Scoring of the Alternatives against the Criteria. The next step is to score the alternatives against these criteria, which is done at the lowest level of the value tree. For some criteria (e.g., cost), it may be possible to assess the performance of the alternatives numerically, whereas, for others (e.g., quality), it may only be feasible to obtain an ordinal ranking of the alternatives or to allocate them to verbally defined levels of performance (e.g., poor, reasonable, and excellent). How the alternatives are scored against the criteria differs from decision context to decision context and depends, amongst others, on the amount of data (e.g., results from observational and/or experimental studies, output from mathematical models, or expert opinion) that is available at the start of the decision making process and on how many resources one is willing to invest in the collection of more precise measurements. As the information obtained in the scoring phase can change the perspective on the decision problem, it might be necessary to revert to the problem structuring phase in order to incorporate these new insights in the decision context. If this is not the case, the end of the scoring phase concludes the formal specification of the decision problem.

Based on the information in the scoring table, it is sometimes possible to identify one or more alternatives for which there is at least one other alternative that performs better on all of the criteria included in the decision problem. As it is never optimal to select one of these dominated strategies, they can safely be eliminated from the set of decision alternatives. If there is sufficient budget to fund all the remaining strategies, the decision problem is solved, meaning that the multicriteria decision making process can be ended after the scoring phase. If not, the set of decision alternatives needs to be further reduced by making value trade-offs among the performance levels on the different criteria. In such situations, the use of preference modeling can assist in formalizing the decision makers' preference structures, thereby reducing the chance that the decision focuses too much on a single aspect of the decision problem.

2.3. Preference Modeling. At the research priority setting stage of a translational research project, the amount of developmental uncertainty surrounding the conceived product concepts is usually still enormous. As a result, a full quantitative assessment of the expected clinical and economic benefits from each of the identified decision alternatives is generally not yet possible. It is therefore likely that for some of the criteria the data in the scoring table are solely based on expert opinion. As experts are often more comfortable with producing rankings (e.g., the number of competitor products is larger for alternative A than for alternative B) than with providing exact numerical estimates (e.g., there are 10 competitor products for alternative A and 6 for alternative B), it is important that such ordinal data can be accommodated in the preference modeling phase. For this reason, we will focus in this section on describing SMAA-O [6], a variant of the stochastic multicriteria acceptability analysis (SMAA) method [7, 8] that has been developed for decision problems where the data for some or all criteria is ordinal.

In SMAA-O, it is assumed that the decision maker's preference structure can be represented by means of a mathematical function $v(x)$ that is constructed in such a way that alternative i is preferred over alternative j if and only if $v(x^i) > v(x^j)$, where x^i denotes the column of the scoring table associated with alternative i. To simplify the construction of $v(x)$, it is generally assumed that the criteria satisfy the independence conditions for applying the additive value function $v(x, w) = w_1 v_1(x_1) + \cdots + w_n v_n(x_n)$, where n is the number of criteria and w_k is the weight attached to criterion k. The partial value functions $v_k(x_k)$, normalized so that the worst possible score on each criterion is assigned a value of 0 and the best possible score is assigned a value of 1, reflect the relative desirability of the different levels of achievement on the individual criteria. For numerical criteria, it is usually assumed that equal size ranges on the measurement scales represent the same amount of value to the decision maker, resulting in partial value functions that are linear. For ordinal criteria, the use of such a linear mapping between scale values and partial values is however not directly suitable as the distance between ranks on an ordinal scale is not known. In SMAA-O, this problem is dealt with by randomly assigning the scale values on the ordinal scale to partial values between 0 and 1, in such a way that the rank order between the scale values is maintained. Different ordinal to partial value mappings may translate into a different ranking of the decision alternatives as the overall value associated with each of these alternatives may change. This uncertainty is captured by the *rank acceptability indices* b_i^r, which describe the fraction of Monte Carlo iterations for which alternative i is ranked at place r. The *pairwise winning indices* c_{ij} describe the fraction of Monte Carlo iterations for which alternative i is ranked at a higher place than alternative j. Missing or imprecise information with respect to the values of the weights can be handled in a similar way by sampling the weight vector from a uniform distribution in the feasible weight space induced by the available preference information.

3. Case Study

3.1. Decision Problem. The prediction and early diagnosis of diabetes and diabetes-related cardiovascular complications

(PREDICCt) project of the Center for Translational Molecular Medicine (CTMM) was initiated to enhance the possibilities for prevention of DM2 and associated complications through the development of methodologies for molecular diagnostics and molecular imaging of novel biomarkers associated with the development of DM2 and its related complications. DM2 is a complex disease with many genetic, environmental, and behavioral determinants as well as biological pathways involved. Additionally, it is a chronic disease that takes a long time to develop. As a result, there are many different possible target applications for novel diagnostic and imaging techniques. Not all target applications are however equally likely to achieve the objectives of the project to the same extent. As a result, a decision had to be made on the priority setting for the investment of available resources.

3.2. Problem Structuring

3.2.1. Methods. Several discussion sessions were held with various researchers from the PREDICCt project. During these discussions multiple perspectives on the decision problem were suggested by participants and discussed in the group. Based on these discussions, a set of alternatives was defined. The business plan of CTMM, in which the stakeholders in the project expressed their views and interests, served as the starting point to define a set of criteria. All statements concerning objectives were isolated from the business plan and subsequently ordered and grouped.

3.2.2. Results. As the main aim of the PREDICCt project was the prevention of DM2 and associated complications, the decision alternatives were defined in the scope of the preventive medicine framework. Preventive medicine is often classified in three different levels. Primary prevention targets those in whom the disease is not yet present, with the aim to provide interventions to prevent the disease from manifesting. Secondary prevention targets those who have the disease but are not yet symptomatic, aiming to reduce the morbidity through early treatment. Tertiary prevention is aimed at those who are diagnosed with the disease and enables the provision of interventions limiting further morbidity caused by complications. Complications of DM2 are an important aspect in this case, as most of the burden of the disease is caused by these complications [9]. There are two distinct categories of complications: microvascular (diabetic nephropathy, neuropathy, and retinopathy) and macrovascular (coronary artery disease, peripheral arterial disease, and stroke) [10]. These two categories of complications have distinct approaches to prevention, diagnosis, and care. Therefore, it was considered important to make a distinction between tertiary prevention aimed at microvascular complications and tertiary prevention aimed at macrovascular complications. The 4 alternative research approaches identified for the development of a novel biomarker technology in DM2 were thus as follows:

A biomarker technology applied to the general population to

(1) select individuals eligible for interventions aimed at preventing or delaying the onset of DM2 (primary prevention),

(2) identify those with undiagnosed diabetes in order to initiate treatment earlier (secondary prevention).

A biomarker technology applied to the population of diagnosed DM2 patients to

(3) select those that would benefit from interventions aimed at preventing or delaying *microvascular* complications (tertiary prevention),

(4) select those that would benefit from interventions aimed at preventing or delaying *macrovascular* complications (tertiary prevention).

The structuring of objectives from the business plan resulted in the identification of four main objectives: reduce the burden of disease, reduce healthcare costs, increase economic activity, and obtain a high academic profile.

The profile of academic output is to a large extent determined by the novelty and quality of scientific work presented. This is not directly related to the decision alternatives at hand, meaning that a high academic profile could be obtained no matter what alternative is chosen. This objective was therefore not considered relevant for the purpose of the present analysis. For the other three objectives, we conducted a literature review and a brainstorming session to identify a set of factors that are important determinants of these objectives and to identify potential barriers and constraints that hinder their achievement. This resulted in the value tree depicted in Figure 2.

In the healthcare technology market, the commercial potential of a product is dependent on its clinical value and its impact on the downstream healthcare consumption. The extent of this relation is determined by the level of regulation, which differs between jurisdictions as well as between different parts of the healthcare system. For highly regulated parts of the healthcare system, the impact of these factors on a technology's commercial potential can be assessed quantitatively by conducting a headroom analysis [11]. The rationale behind this approach is that the estimated change in health effects and healthcare costs, both direct and indirect, resulting from the implementation of a new technology determine the value of the technology for society, and thereby the maximum device-related cost which the use of this new product will still be reimbursed. As this cost provides an upper-bound for the price that the producer can charge for its product (the principle of value-based pricing), the amount of headroom available is a suitable proxy for the commercial potential of a new medical technology. The upper arm of the value tree therefore consisted of the following 3 determinants of the commercial headroom available: the decrease in downstream healthcare cost, the increase in quality-adjusted survival, and the cost of the intervention associated with the diagnostic or prognostic test. The 3 criteria forming the lower arm of the value tree captured the likelihood that the availability of a more accurate diagnostic or prognostic test will trigger changes in how the healthcare system currently operates.

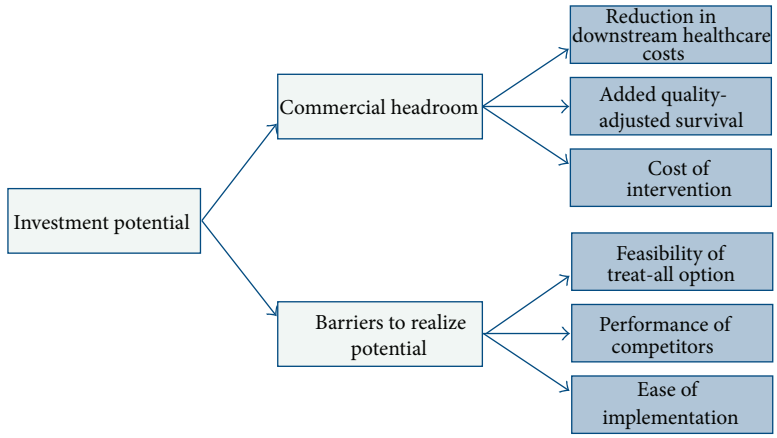

Figure 2: Value tree of overall and lower-level objectives of the public-private partnership.

The feasibility of a treat-all option indicated the added value of the ability to treat specific patients as opposed to treating all patients. This provided an indication of the value stemming from better discrimination or prediction. Furthermore, the existence of high-quality competitor technologies, or lack thereof, was considered a major driver for the success of a novel technology to gain market share. Lastly, not all decision alternatives were considered equal in terms of the accessibility of the market and the ease of implementation in the clinical protocol. Technologies that readily fit within the practice as outlined by current guidelines can be implemented with relative ease. Contrarily, those that require a major change in clinical or public health protocols, for example, the initiation of a universal screening program, cannot fulfill their potential until such changes are established.

3.3. Scoring of the Alternatives against the Criteria

3.3.1. Methods. For each of the decision alternatives, quantitative estimates of the decrease in downstream healthcare costs, the increase in quality-adjusted survival, and the intervention costs were available in the literature. The performance of the decision alternatives on these criteria was therefore expressed numerically. The performance on the other 3 criteria is strongly dependent on the type of technology developed and can therefore not be quantified at this stage. We therefore used expert opinion to formulate an ordinal ranking of the decision alternatives with respect to these criteria.

3.3.2. Results. The complete scoring matrix is shown in Table 1. Estimates of the effects of primary prevention of diabetes and tertiary prevention of macrovascular complications on the reduction of downstream healthcare costs, gain of quality-adjusted survival, and the costs of interventions were based on a modeling study [12]. For the primary prevention scenario, a lifestyle intervention program in obese individuals was modeled, and for the tertiary prevention of macrovascular complications, a multifactorial treatment scenario combining intensive glycemic control, cholesterol-lowering treatment, and antihypertensive treatment was modeled. Estimates of the reduction in downstream healthcare cost, gain of quality-adjusted survival, and the costs of interventions for tertiary prevention of microvascular complications were based on a study that modeled the results of intensive blood glucose control and use of ACE-inhibitors on nephropathic complications [13]. As studies have found that secondary prevention of DM2 has little to no effect on downstream healthcare costs and quality-adjusted survival, the performance of this alternative on these two criteria was set equal to 0 [14]. However, in case screening is performed and patients are discovered, they will be treated. Therefore, the treatment costs of diabetes patients without complications were included [15].

Two main aspects contributed to the ranking of the feasibility to treat-all criterion: the budget impact and lack of implementation of existing cost-saving interventions. Primary and secondary prevention were ranked as highest and second highest as providing treatment to all individuals eligible for screening would not be feasible due to budget impact reasons. Within tertiary prevention, the microvascular complication alternative was ranked lowest as cost-saving interventions are readily available there but not yet fully implemented [13]. The barriers to implement such interventions must therefore first be overcome before the improved risk stratification possibilities can be implemented. Considering the performance of existing competing technologies, secondary prevention was ranked lowest. There, the diagnosis of diabetes itself cannot be improved as the disease is defined on measurements with the gold standard (glucose measurements). Additionally, there are numerous prescreening tools available that perform well and cost little (risk questionnaires) [16]. As a result of the latter, primary prevention was ranked second lowest. On the contrary, such risk stratification tools are hardly available and perform less well, for microvascular complications and to a lesser extent macrovascular complications. Lastly, the primary and secondary prevention settings of diabetes would necessitate some form of screening. Such a public health program could take years before being realized. This entails a serious problem for the implementation of any biomarker technology.

Table 1: Scoring of the decision alternatives against the evaluation criteria.

	Preference direction	Primary prevention	Secondary prevention	Tertiary microvascular prevention	Tertiary macrovascular prevention
Reduction in downstream healthcare costs	Increasing	€ 658M	€ 0	€ 73M	€ 312M
Added quality-adjusted survival	Increasing	€ 280K	€ 0	€ 1K	€ 80K
Cost of related intervention	Decreasing	€ 792	€ 663	€ 155	€ 561
Feasibility of treat-all option		2	1	4	3
Performance of existing tests		3	4	1	2
Ease of implementation		2	2	1	1

As diagnosed diabetes patients regularly consult a physician, access to the patient is less problematic in the case of tertiary prevention.

3.4. Preference Modeling

3.4.1. Methods. The partial value functions for the numerical criteria were obtained by linearly rescaling the criteria measurements to the interval [0, 1], with the values of 0 and 1 assigned to the worst and best levels of performance on these criteria, respectively. The rankings of the alternatives on the ordinal criteria were randomly mapped to partial values between 0 and 1 consistent with these rankings by using the SMAA-O method. With respect to the weights, we specified three scenarios. First, we considered a base case scenario in which no additional constraints on the values of the weights were incorporated. The results of such a preference-free analysis can be used to eliminate alternatives that always fall short to at least one other alternative, irrespective of the decision maker's preferences. Second, we considered a scenario where a large commercial headroom was considered more important than avoiding barriers to realize potential, implying that $w_1+w_2+w_3 > w_4+w_5+w_6$. Lastly, we considered a scenario where the previous preference statement was reverted, implying that $w_1 + w_2 + w_3 < w_4 + w_5 + w_6$. All analyses were conducted in R (version 3.0.1) using the smaa (version 0.1.1) and hitandrun (version 0.2.2) packages that are available from CRAN.

3.4.2. Results. For the preference-free analysis (Figure 3), we found that secondary prevention has a very low (<0.05) first rank acceptability index, making it unlikely to be optimal for any decision maker. The optimality of the three remaining strategies was however strongly dependent on the decision maker's preferences. Primary prevention was very likely to be the best alternative when maximizing the commercial headroom available is considered more important than minimizing the barriers and constraints to utilize this headroom (Figure 4). This is confirmed when looking at the pairwise winning indices, which show that the probability that primary prevention is preferred over tertiary prevention of microvascular complications, the second best alternative when improvement of commercial headroom is favored, is 61% (Table 2). Contrarily, tertiary prevention of microvascular complications and tertiary prevention of macrovascular complications were clearly the preferred strategies when having to deal with lesser obstacles is preferred over potential higher gains in terms of the objectives stated by the stakeholders (Figure 5). However, as is shown by the pairwise winning indices for this scenario (Table 3), the provided preference information with respect to the values of the weights was not precise enough to further discriminate between these two remaining strategies.

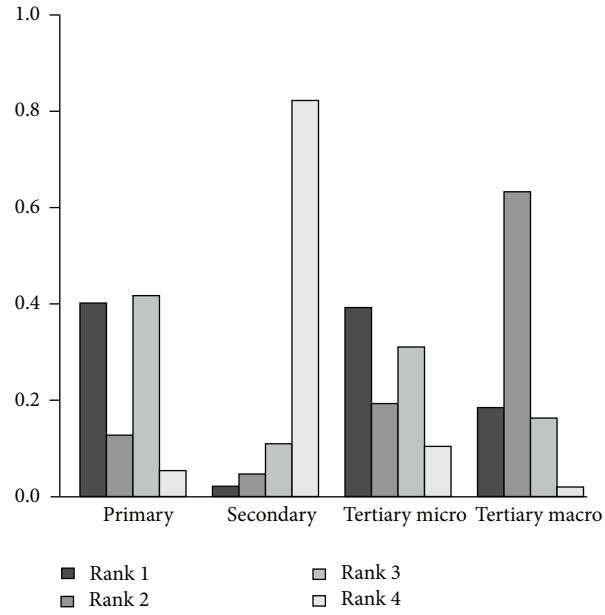

Figure 3: Rank acceptability indices for the base case scenario.

4. Discussion

Priority setting for translational research is a complex problem that requires decision makers to gather and synthesize expertise from different fields. In this paper, we have shown through a case study how this process can be supported in a formal way by applying MCDA.

The complete value chain in biomedical innovation poses a complex and multifaceted problem for priority setting. Additionally, ethics, public opinion, and politics come into play when dealing with a healthcare setting. Under these

Table 2: Pairwise winning indices when improvement of commercial headroom is favored.

	Primary prevention	Secondary prevention	Tertiary microvascular prevention	Tertiary macrovascular prevention
Primary prevention		0.96	0.61	0.65
Secondary prevention	0.04		0.07	0.02
Tertiary microvascular prevention	0.39	0.93		0.45
Tertiary macrovascular prevention	0.35	0.98	0.55	

Table 3: Pairwise winning indices when avoidance of barriers is favored.

	Primary prevention	Secondary prevention	Tertiary microvascular prevention	Tertiary macrovascular prevention
Primary prevention		0.88	0.35	0.31
Secondary prevention	0.12		0.18	0.12
Tertiary microvascular prevention	0.65	0.82		0.48
Tertiary macrovascular prevention	0.69	0.88	0.52	

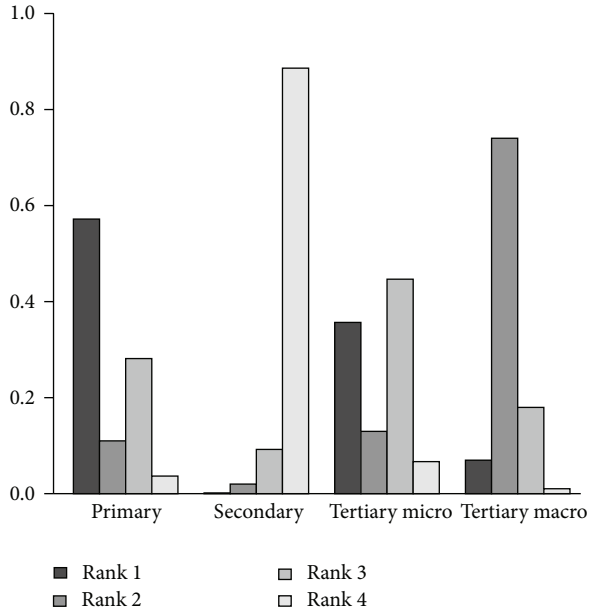

Figure 4: Rank acceptability indices when improvement of commercial headroom is favored.

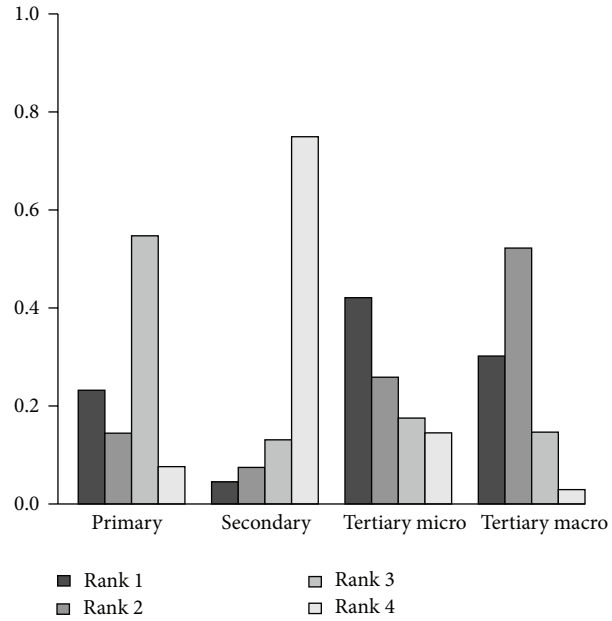

Figure 5: Rank acceptability indices when avoidance of barriers is favored.

conditions, informal decision making will lead to the use of intuitive and heuristic approaches as a decision maker is unable to grasp the full complexity and trade-offs in a decision [17]. Informal decision making will therefore depend to a large extent on who is appointed to make the decision and what the background expertise of the decision maker (or group of decision makers) is, which would be undesirable in case of large investments or investments of public funds. The problem structuring phase of MCDA helps to overcome this by encouraging the incorporation of expertise exogenous to the decision makers. In our case study, this led to the integration of two different perspectives on the decision problem: that of the commercial headroom (based on the improvement in diagnostic power of new technologies over existing ones) and that of the barriers that new technologies would face to access the market. After the scoring phase it became apparent that the development of novel methods to measure biomarkers that can be used in secondary prevention of DM2 was certainly an unattractive research objective. If decision makers were willing to invest in all remaining three alternatives, the priority setting process could be stopped after this phase. However, in order to explore under which preferences the remaining alternatives would be most attractive, we proceeded with the preference modeling phase. A preference of decision makers for the maximization of commercial headroom made the development of novel methods to measure biomarkers used in primary prevention the most attractive strategy. Alternatively, investing in novel

methods to measure biomarkers for tertiary prevention of microvascular and macrovascular complications was optimal in case a safer strategy with fewer obstacles, but less gain, would be preferred.

Early health economic modeling—the process of performing an initial assessment of the costs and health effects associated with a new medical technology before the technology has been fully developed—has recently been suggested as a tool to inform new product development within translational research projects [18–20]. However, given that such calculations require very strict assumptions about how a new technology performs in a specific clinical setting, this approach cannot yet be applied when specific biological targets still need to be identified. Other softer approaches such as SMAA-O are therefore required to support research priority setting at the start of a translational research project, where outcomes are generally too uncertain to make a full quantitative assessment of the expected return-on-investment meaningful. Using MCDA for priority setting at the beginning of a research project can facilitate decision making further on in the research and development process. For example, the data during the scoring phase can serve as input for quantitative approaches such as headroom analysis for product investment decision making [11] and value-based pricing for market access [21]. We therefore see SMAA-O or similar MCDA methods as a new instrument in the early health technology assessment toolbox, being one to be used at the very start of translational research projects.

A strength of the SMAA-O methodology that we employed in our case study is the possibility to combine ordinal and numerical scoring of the alternatives. This allowed us to make full use of the large amount of data available in the scientific literature on costs and health burden related to DM2, while still being able to incorporate expert judgment on aspects for which no data was available. A limitation of our study is that, apart from the scenarios considered, we did not elicit any preference information on the weights from the decision makers. Ordinal and ratio constraints on the weights can however easily be incorporated in a SMAA analysis by utilizing efficient weight generation techniques such as hit-and-run sampling [22].

We have demonstrated in this paper how the priority setting in translational research may be approached by applying MCDA. Future research is needed to fully assess the applicability of this method at the very start of a translational research project. Nonetheless, we are confident that we have already made a convincing case for formal decision making in priority setting in translational research. Our report may serve as a guide for future decision makers, ultimately making the approach common practice.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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