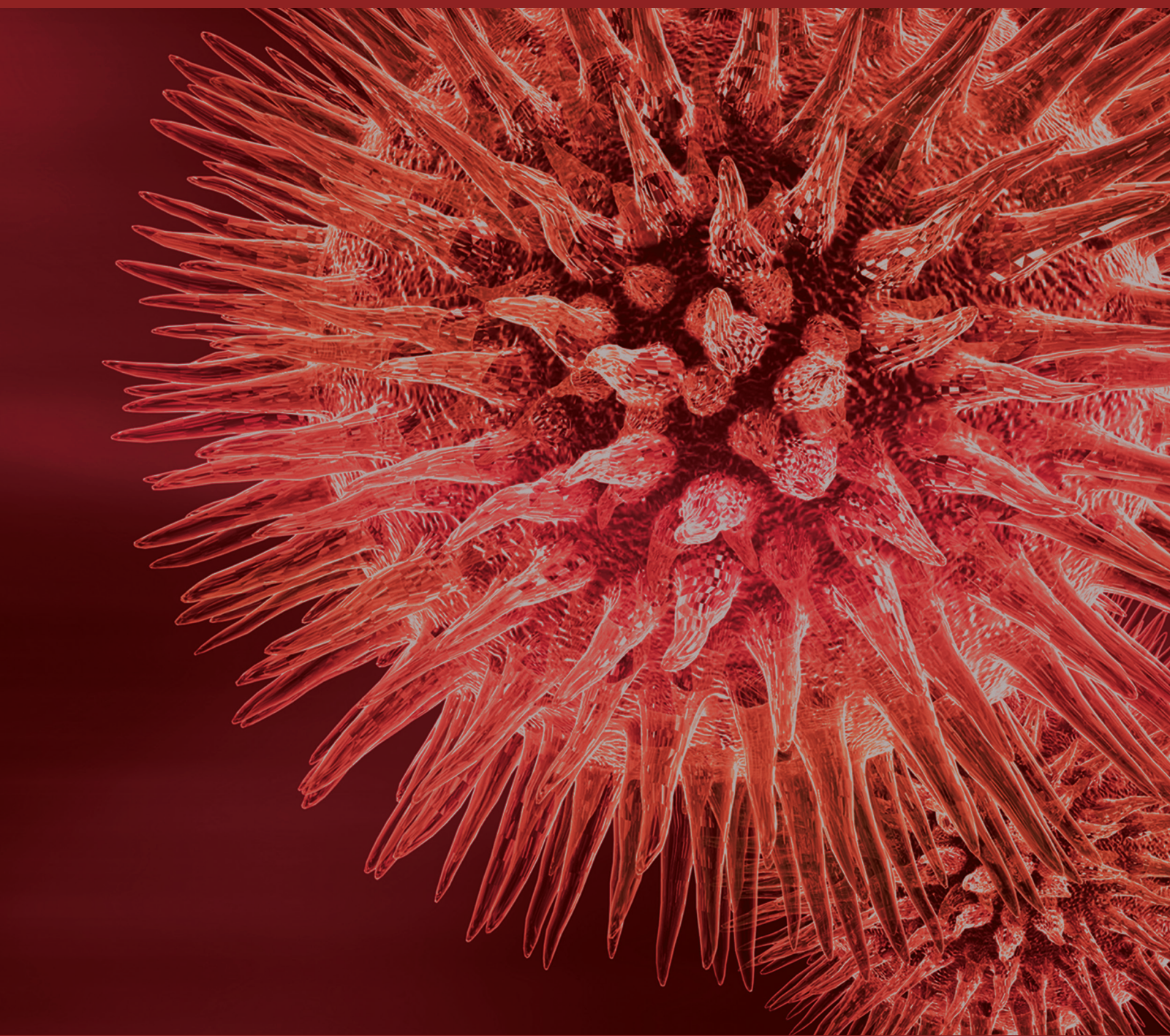
BioMed Research International

Novel Biomarkers and Treatments of Cardiac Diseases

Guest Editors: Dayue Darrel Duan, Hua Zhu, and Renzhi Han



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Contents

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Editorial

Novel Biomarkers and Treatments of Cardiac Diseases

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Cardiac diseases are mainly caused by malfunction of or injuries to the hearts. Although significant advances have been made during the past decades to improve the successful rate of treatments of these diseases, they still remain the top leading cause of morbidity and mortality in the world. Currently, cardiac diseases are defined according to the traditional system- or organ-based classification and the identification of diagnostic and therapeutic biomarkers has been focused on the heart. Therefore, the "golden standard" biomarkers are mainly cardiac muscle-related. For example, the conventional troponin (cTn) has been widely utilized for the diagnosis of acute myocardial infarction in the clinics. However, multiple limitations exist with clinical applications of these types of biomarkers. The ELISA based cTn detection assay is time consuming, and the dynamics of the biomarkers are not sensitive enough to represent the development of the diseases. Clearly, there is an urgent need for identifying sensitive, specific biomarkers of different types of cardiac diseases and development of new therapies for this unmet medical need. In this special issue, we have assembled a series of articles of reviews, perspectives, and original contributions from experts in current research of novel biomarkers and treatments of cardiac diseases in both basic research and clinical practice.

In the effort to develop more sensitive cardiac specific troponin-based diagnosis for heart diseases, the highly sensitive troponin T (hsTnT) was found to offer an excellent diagnostic performance due to its high sensitivity and negative predictive values compared with conventional troponin (cTn) test. In this special issue, the potential clinical use of hsTnT for diagnosing perioperative myocardial infarction (PMI) was discussed. In addition, it was discovered that hsTnT combined with advanced oxidation protein products (AOPPs) could be useful for monitoring myocardial function of cirrhotic patients with chronic hepatitis C virus infection.

Other than the circulating troponins, many other proteins have been tested recently as biomarkers for cardiac diseases. For example, caveolin-1, a membrane protein, has been found in blood circulation and might serve as a novel biomarker for idiopathic pulmonary artery hypertension. Serum level of pentraxin-3, a member of pentraxin family, is a long-term independent predictor of prognosis of patients with chronic heart failure.

In addition to circulating proteins, other circulating molecules have also been discovered as biomarkers in many disease settings. MicroRNAs are small RNAs with ~22 nucleotides in length that can regulate specific messenger RNAs (mRNAs). They present in both tissues and blood circulation and can be used as biomarkers of cardiac diseases [1]. In one research article of this special issue, N. Li et al. found that miR-1183 and miR-1299 in both tissue and plasma can serve as biomarkers for rheumatic heart disease (RHD). Recently, microRNAs have been found in extracellular vesicles (EVs) and can be altered in association with cardiovascular diseases.

Some other molecules have also been investigated for the value of biomarkers in cardiac diseases. For example, alteration of preoperative and postoperative plasmatic endogenous ouabain (EO) has been linked to patients with higher risk of morbidity and mortality after cardiac surgery.

The differential expressed genes (DEGs) in human epicardial adipose tissue (EAT) can serve as biomarkers as well as therapeutic targets for treatment of cardiovascular diseases. A research article in this special issue also demonstrated that activation of endocannabinoid system, as evidenced by elevation of cannabinoid receptors, infiltration of leukocytes and mononuclear cells, is associated with persistent inflammation in human aortic aneurysm. As a novel therapeutic strategy for the treatment of cardiac diseases, *Withania somnifera* leaf extract, one of the most valuable herbs in the traditional Indian systems of medicine, can effectively treat isoproterenol-induced oxidative damage in rat myocardium.

Taken together, the studies assembled in this special issue represent several important paradigm shifts in the area of biomarkers for the diagnosis, prognosis, and treatment of cardiac diseases. The identification of biomarkers for the diagnosis and treatment of cardiac diseases should not be limited to the organ- or tissue-specific molecules (proteins or nucleotides). The systematical nature of pathogenesis and pathophysiology of cardiac diseases requires broader vision and strategies outside the organ- or system-based box [2]. The fast advance in the application of genome-wide association study (GWAS) [3], phenome-wide association study (PheWAS) [4, 5], and epigenome-wide association study (EWAS) [6], as well as big-data technology provides more powerful platforms and paradigms for the new definition of cardiac diseases, which will be based on not only genetic makeups and molecular variations but also environmental impacts. The identification of systematically integrated biomarkers is certainly a crucial step towards precision medicine [7] to optimize diagnosis and treatment of cardiac diseases that takes into account individual differences in molecular makeups, life styles, and environmental impacts.

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Review Article

Potential Diagnostic and Prognostic Biomarkers of Epigenetic Drift within the Cardiovascular Compartment

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Biomarkers encompass a wide range of different measurable indicators, representing a tangible link to physiological changes occurring within the body. Accessibility, sensitivity, and specificity are significant factors in biomarker suitability. New biomarkers continue to be discovered, and questions over appropriate selection and assessment of their usefulness remain. If traditional markers of inflammation are not sufficiently robust in their specificity, then perhaps alternative means of detection may provide more information. Epigenetic drift (epigenetic modifications as they occur as a direct function with age), and its ancillary elements, including platelets, secreted microvesicles (MVs), and microRNA (miRNA), may hold enormous predictive potential. The majority of epigenetic drift observed in blood is independent of variations in blood cell composition, addressing concerns affecting traditional blood-based biomarker efficacy. MVs are found in plasma and other biological fluids in healthy individuals. Altered MV/miRNA profiles may also be found in individuals with various diseases. Platelets are also highly reflective of physiological and lifestyle changes, making them extremely sensitive biomarkers of human health. Platelets release increased levels of MVs in response to various stimuli and under a plethora of disease states, which demonstrate a functional effect on other cell types.

1. Introduction

Biomarkers encompass a wide range of different measurable indicators, including protein, polysaccharides, and nucleic acids, and are distinct, both quantitatively and qualitatively, from common circulating biomolecules, such as albumin. They represent a tangible link to physiological changes occurring within the body and ideally enable diagnostic and/or prognostic evaluations to be determined with respect to disease. Accessibility, sensitivity, and specificity are significant factors in biomarker suitability. The initiation and progression of atherosclerosis are traditionally measured by internal intima-medial thickness (IMT) [1]. However, detection of inflammatory processes through use of biomarkers may facilitate earlier diagnosis well before the clinical threshold and enable preventive remedial action to be taken. A number of commonly used indicators exist, and their wide use in the clinical setting reported in literature indicates large consensus of their efficacy. Nevertheless, new biomarkers continue to be discovered, and questions over appropriate biomarker selection and assessment of their usefulness as reliable indicators of disease remain. In this context, the emerging field of epigenetics has enormous potential as biomarkers of cardiovascular risk. By analysing environmentally induced changes in chromosomes and monitoring these alterations over time, determination of the epigenetic "drift" is possible. Such data have the potential to provide novel therapeutic targets for vascular-associated chronic illnesses and initiate novel drug-development pipelines.

2. CVD and Problems Posed

Cardiovascular disease (CVD) is an umbrella term for a class of diseases involving the cardiovascular system and

consequently includes heart failure, arrhythmia, angina, hypertension, high cholesterol, and stroke amongst others. Vascular inflammation is a key contributor to CVD etiology, with inflammation and CVD risk factors closely interrelated. CVD risk factors, such as hypertension, and hypercholesterolemia are associated with higher circulating concentrations of inflammatory biomarkers [2]. Types of CVD are varied and complex; hence there are multiple pathological mechanisms through which disease may arise. Atherosclerosis is the most common of the vascular inflammatory diseases. It is defined as chronic, specifically affecting arterial blood vessels, and caused by dynamic dysfunction of the endothelium. Endothelial dysfunction is regarded as the earliest detectable indicator of CVD and is used as an independent predictor of future disease occurrence [3].

Dysregulation of endothelial homeostasis is characterised by reduced availability of nitric oxide and is an acknowledged marker of early atherogenesis [4] and a multistage process. Following injury, the resulting immune response leads to inflammation at the site. Macrophage cells are typically the first type to respond. They act by secreting proteins (cytokines and chemokines) in order to recruit additional immune cells to the site of injury [5]. Vascular Cellular Adhesion Molecule 1 (VCAM1) and Intracellular Adhesion Molecule 1 (ICAM1) are other important mediators in cell-cell and cell-matrix interactions during inflammation and resultant aberrant immune response. As such, they may all act as "biomarkers" of inflammation. However, their use is relatively broad.

Biomarkers of associated inflammatory responses yield an alternative/indirect means of detection. Oxidative stress, for example, is widely associated with atherosclerosis, myocardial infarction, and so forth and is known to play a critical role in the genesis and continuance of inflammation through interruption of normal cell signaling mechanisms [6]. As such, a host of related biomarkers have been employed, including high-sensitivity C-reactive protein (C-RP) and interleukin-1, interleukin-6, and interleukin-10 [7, 8].

A recently published longitudinal study conducted over a twenty-year period investigated the relationship between subclinical atherosclerosis and a host of biomarkers including cytokines/adipokines, thrombosis, and adhesion molecules. IMT measurement was used as control and conducted over four time-points during the investigative period. The test group consisted of 886 individuals with type 1 diabetes. Logistic regression models carried out by the team suggest that individual biomarkers were not predictive of/associated with subclinical atherosclerosis. However, composite scores of acute-phase reactants, cytokines/adipokines, and thrombolytic factors were nevertheless associated with higher levels of atherosclerosis at Epidemiology of Diabetes Interventions and Complications (EDIC) year 12 [1].

3. Predictor Potential versus Risk Assessment and Robustness

While biomarkers of other diseases may be derived from surgical biopsy, in terms of detecting vasculature-derived disease, blood represents perhaps the simplest and most logical of sources from which to obtain biological markers. Analysis of blood and bodily fluids in general has emerged as a form of "liquid biopsy." This has primarily been used in the field of cancer diagnosis but equally holds promise for diagnosis of other conditions.

One of the major difficulties in identifying suitable biomarkers is determining those which independently signal a change within the system, without becoming overwhelmed by the myriad of processes surrounding them. Sensitivity and specificity are paramount [9]. Numerous signalling molecules, such as inflammatory cytokines, are proposed as important factors related to atherosclerotic pathogenesis. Tumor necrosis factor alpha (TNFα), for example, is widely used as a marker of vascular inflammation [10, 11]. Cung et al. [12] acknowledge that it is readily capable of activating endothelial cells (ECs). However, they state that, due to the presence of thousands of other factors, the discrete assessment of individual cytokines, such as TNFα or CD40, may fail to capture the global inflammatory stimulus that the blood conveys to the endothelium. As such, they believe that the complex composition of blood results in a tangible gap between biomarker and clinical outcome. A similar sentiment is shared in another recent publication, where the presence of interferons due to the presence of disease inhibited biomarker interpretation [13]. This conflicts with the use of nuclear interferon-inducible-16 (IFI16), which acts as DNA sensor in inflammasome signalling, and may also function as a suitable inflammatory biomarker [14]. It has previously been used as a biomarker of cognitive dysfunction [15]. Nevertheless, this is a serious challenge to the usefulness of blood-based biomarkers [12]. If traditional markers of inflammation are not sufficiently robust in their specificity, then perhaps alternative means of detection may provide more information.

Data from studies carried out by Brindle et al. [16] has shown that traditional factors, such as smoking and diet, lack accuracy and may even result in overestimation of disease risk in low-risk populations and *vice versa*. Involvement of hereditary factors may represent a more significant agent in both vascular remodelling and disease development. Indeed, with regard to coronary artery disease (CAD), genome-wide association studies have led to the identification of a large number of single nucleotide polymorphisms (SNPs) associated with increased risk of developing the disease [17]. However, each of these approaches is based on the fundamental principle that genetic determinants lie within the static DNA code of individuals; the hypothesis being that a person may, in effect, be more susceptible to succumbing to disease based on their unique genetic profile. Nevertheless, the genetic code of individuals has long been known to be flexible and adaptable. Such adaptations may occur in the long term through evolution or may occur as a more direct reaction to various external stimuli by means of up/downregulation of genes in order to best meet the adaptive demands of certain situations.

4. Epigenetics, Aging, and Chronic Disease

Biomarkers discussed thus far are predominantly diagnostic in nature. However, the emerging field of epigenetics may hold enormous predictive potential. Owing to the existence

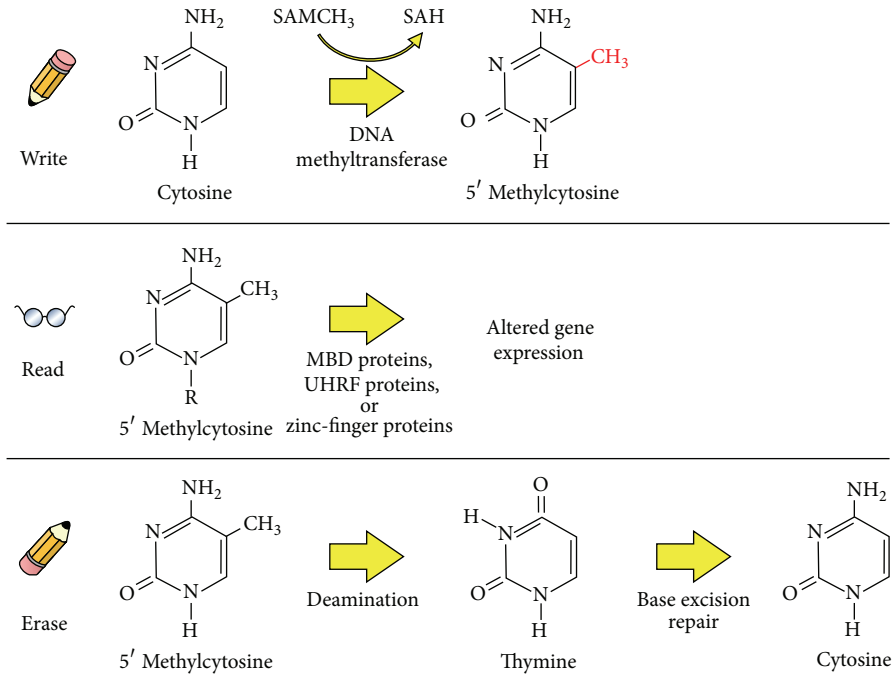

Figure 1: DNA methylation (writing, reading, and erasing) represents one of a number of means through which epigenetic modification of DNA may occur. Methyltransferase enzymes serve to methylate cytosine residues, particularly in CpG islands. Methyl-CpG-binding domain (MBD) proteins, Ubiquitin-like, containing PHD and RING finger domain (UHRF) proteins or zinc-finger proteins may each play a role in reading methylated DNA. Deamination of 5′ methylcytosines to form thymine residues that are subsequently removed via the base excision repair mechanism is one way that methylated bases may be restored.

of numerous conflicting interpretations of what the term meant, epigenetics was finally defined in 2008 as "a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence" [18]. DNA methylation, the covalent addition/removal of methyl groups, is an example of such independent modification. Histone protein modification is an area of some debate and had been considered not to be epigenetically regulated [19], though current thinking places it firmly within the epigenetic realm [20]. More recently, noncoding RNA (ncRNA) has also been included as major players in the epigenetic domain, with microRNA (miRNA) recognised as having an important regulatory function. Importantly, epigenetic modifications persist within the cell [19].

Following methylation and/or histone N-terminal modification (acetylation, deacetylation, phosphorylation, and ubiquitylation), the epigenome is altered. Effect on gene transcription is nuanced and may vary depending on context. Regulation of gene expression was originally believed to occur through alteration of transcriptional start sites (TSSs). Methylated regions have been linked with particular chromatin features associated with transcriptional repression, especially H3K9me3 and methyl-CpG-binding protein 2 (MECP2). Methylation in other regions of the genome is increasingly accepted as having functional relevance and presents fundamentally more dynamic patterns of methylation than those discovered in TSSs [21]. There, for example, methyl-binding domain (MBD) proteins may serve to coalesce DNA, which, as with TSSs, then recruit histone deacetylases and effectively silence that sequence. Current research indicates that CpG methylation is notably avoided at sites close to TSSs. It has been proposed that those TSSs that are methylated exhibit long-term silencing [21].

In contrast to silencing, transcriptional stimulation may instead occur as a result of methylation. This has been observed largely in regions located away from TSSs, a phenomenon termed the "DNA methylation paradox" [22]. Furthermore, over 50% of vertebrate genes contain short (~1 kb) CpG-rich sequences, with the remaining genome deplete of such regions. In mammalian cells, methylation generally occurs within CpG dinucleotides, typically in cytosine residues that are followed by guanine. Hence, development of detailed epigenome maps in normal and disease states (and thus understanding the distribution of methylation across the genome) is requisite to understanding the function of DNA methylation [21]. The methylation mechanism is highly regulated and, importantly, is a fluid, dynamic, and reversible process, orchestrated through a complex network of epigenetic writer, reader, and erasers (see Figure 1).

Epigenetic drift is the term given to epigenetic modifications as they occur as a direct function with age. While age is a known risk factor for many diseases, age-related methylation has been found to occur differentially at specific sites along the genome. Hence, investigation of epigenetic drift may result in the discovery of key underlying biomarkers of numerous age-related pathologies [23]. Research has shown that tissue-specific age-associated CGs are commonly found outside CpG islands with decreased methylation. Such sites

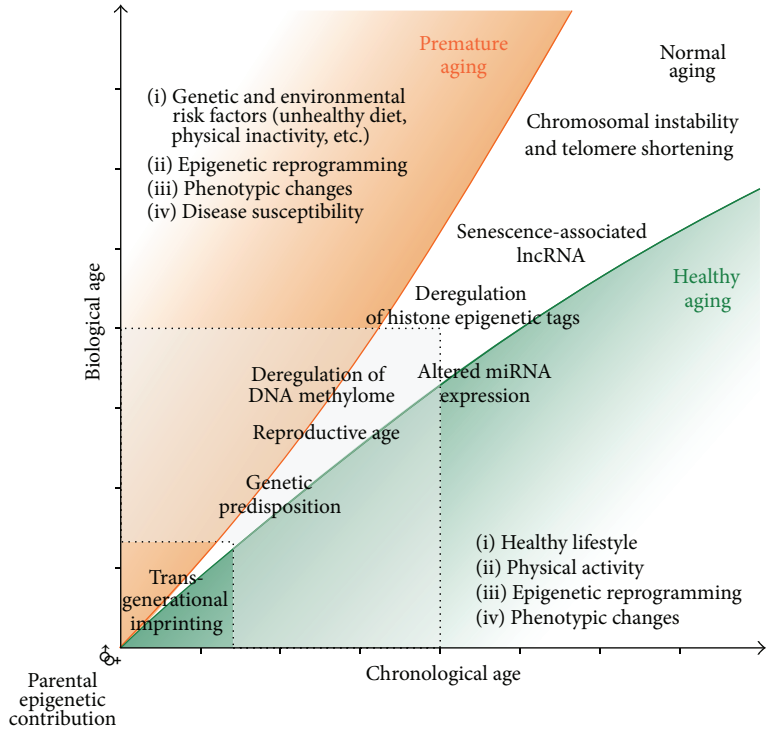

Figure 2: Epigenetic drift over time can result in measurable differences between biological and chronological age. Epigenetic changes have been found to be reflective of lifestyle and may act as functional biomarkers of disease before clinical threshold is reached.

have been linked with higher tissue-specific expression levels compared to those with increased methylation [24, 25]. Some debate has arisen as to the complexity of the methylation mechanism. Recent studies by Valencia-Morales Mdel et al. [25] favour a common instructive mechanism. However, Day et al. [24] believe a much more complex mechanism than a simple epigenetic drift model is at play. The later theory would suggest that epigenetic methylation could potentially represent a viable type of biomarker. Indeed, DNA methylation-derived measures of aging have been found to predict mortality independently of health status and other known genetic factors [26]. The aging effects of epigenetic drift over time are highlighted in Figure 2.

Physical activity (PA) is a modifiable lifestyle choice, with enormous health and fitness benefits. Regular PA is a known requirement to maintain a healthy cardiovascular system [27] and has been shown to produce a long-term anti-inflammatory effect in vasculature [28]. Recent efforts have been made to assess the effects of exercise as an epigenator in DNA from a variety of tissues. Human skeletal muscle has been found to be influenced by exercise applied as a physiological stressor. Genes previously found to be differentially methylated in type 2 diabetes (PGC-1α, PPAR-δ, TFAM, citrate synthase, and PDK4) were evaluated, and methylation was found to be lower following acute exercise. Muscle-specific transcription factors remained unchanged [29].

Rather than taking baseline single point blood sample measurements, stressing the cardiovascular system (*in vivo* instead of *in vitro* stress models) by prescribed exhaustive exercise or by the use of acute models of physical inactivity may provide much more valuable and conclusive evidence in relation to the body's response to stress. Among the first to employ global DNA methylation pattern analysis was a study investigating the effects of a six-month exercise intervention on DNA methylation in adipose tissue of healthy men. Results demonstrated genome-wide DNA methylation changes, and 17,975 individual CpG sites exhibited altered levels of DNA methylation in response to exercise [30]. Conversely, a separate study by Horvath et al. [31] in adipose tissue using the same methylation change data as a comparator found that such short-term weight loss did not impact the DNA methylation age of the tissue.

Obesity, often associated with sedentary lifestyle, has been demonstrated in another study as an accelerant of epigenetic change. Methylation was found to occur in DNA from liver tissue in direct correlation with body mass index (BMI) [32]. This corresponds with evidence uncovered by Horvath et al. [31], in a comparable study where the relationship between DNA methylation and BMI in a variety of tissues was examined, including liver. The authors were able to discern and validate age acceleration in this tissue. Importantly, results highlighted tissue-specific changes, indicating that individual CpG sites may be unsuitable for global comparisons between differing tissue types. Instead, an aggregate approach, validated across cells, complex tissues, and organs, was proposed and used in the analysis. Furthermore, the authors call for caution in the use of BMI as a measure of adiposity, instead, citing muscle mass in association with methylation as a more suitable measure of epigenetic aging for this type of research.

5. Arterial Remodelling and Blood

Blood vessels are readily capable of remodelling/altering themselves in response to hemodynamic prompts associated with variations in blood flow. Significant variations of flow pattern occur, such as that produced in response to exercise or sedentary behaviour. In some cases, these may be pathological flow types that lead to aberrant remodelling and development of disease status. Calls were recently made for deeper investigation into possible correlation between sedentary behaviour and vascular function, particularly in children [3]. A number of studies used the example of the relationship between sedentary television viewing time and cardiovascular risk. Gabel et al. [33] investigated the relationship between sedentary television viewing time in children (aged 7–10 years) and inflammatory and endothelial function biomarker levels. Here, the team found that following adjustment for age, weight, sex, and so forth, each additional hour per week of television viewing was associated with 4.4% (95% CI: 2.1, 6.7) greater C-RP and 0.6% (0.2, 1.0) greater VCAM-1. The authors advocate a more rigid and longer study to fully assess this association. Nevertheless, the relationship between sedentary lifestyle and inflammation was clearly drawn. As such, any further investigation could potentially involve epigenetic markers.

The complex composition of blood as a challenge to biomarker efficacy, discussed previously, was examined in a recent publication by Yuan et al. [23], where whole blood tissue was analysed for epigenetic drift. Key amongst the findings was the fact that the majority (80%) of epigenetic drift observed in blood was independent of variations in blood cell composition. When adjusted for composition, results demonstrated a reduction in epigenetic drift attributable to the increase in the granulocyte : lymphocyte ratio, with a concomitant enrichment of age-hypermethylated CpG islands. This is a significant boost for the use of epigenetic biomarkers, where traditional types have been found to succumb to sensitivity and specificity issues.

6. Circulating Biomarkers: Microvesicles, miRNA, and Platelets

There can be no doubt that markers of endothelial dysfunction and epigenetic screening may be of prognostic value in detection of disease. In the immediate future, however, microvesicles (MVs) may represent a more feasible opportunity to translate potential prognostic biomarkers to clinical practice. These secreted vesicles permit reprogramming of recipient cells and are easily obtained in bodily fluids [34].

Different types of MVs are released by mammalian cells into the extracellular space, including blood. Critically, they carry membrane and cytosolic components [35] and maintain both a similar topology and antigenic signature to the parent/donor cell and yet may be extracted from sites distal to the parent cell within the body via blood. MVs are found in plasma and other biological fluids in healthy individuals. Altered MV profiles may also be found in individuals with various diseases [36]. They may be released under a variety of different circumstances, for example, when cells undergo the natural processes of apoptosis or necrosis or when injured. MVs may be divided into one of the three categories, outlined in Table 1.

Due to their origin from different cell types, their differential mechanism of biogenesis, and resultant variable composition, MV effector function is believed to be similarly varied. As such, it is believed that microvesicles represent detectable units involved in intercellular exchange. Indeed, particular biomarkers have been associated with particular types of vesicles; for example, the presence of CD80 and CD86 is indicative that exosomes found in plasma may be of antigen presenting cell (APC) origin [37]. In terms of the vasculature, endothelial cell-derived microparticles (MPs), therefore, display characteristic antigen markers that allow for their identification and detection. An interesting aspect of MP surface marker research is the requirement of specific techniques to generate MPs that carry specific markers. For example, work carried out by Takahashi et al. [38] included generation of aortic EC-derived MPs bearing VE-cadherin in response to TNFα stimulation. Neither hydrogen peroxide (H_2O_2) nor cigarette smoke extract (as the study also looked at pulmonary vascular ECs) treatment of the same cell type yielded a similar result. Furthermore, the relationship between expression levels of particular antigens and levels detectable on MPs did not appear to directly correlate.

Examinations on circulating endothelial- and platelet-derived MPs have been conducted. Findings suggest that levels of circulating endothelial and platelet MPs correlate to the size of myocardium at risk in patients with ST-elevation myocardial infarction. This is indicative of the fact that they reflect the severity of endothelial injury and platelet activation during myocardial ischemia [39].

As with cytokines, discussed previously, the sheer number of MVs present in vasculature from all cell types may present an overarching masking effect that renders individual effects difficult to perceive. Of interest is research carried out by Garnacho-Montero [40] on the reliability of circulating cell-free DNA (cf-DNA) concentrations, compared to C-RP, procalcitonin (PCT), and eosinophil count, in infection diagnosis in patients with systemic inflammatory response syndrome. Results showed that cf-DNA levels did not correlate with C-RP or PCT in septic patients. Those with acute myocardial infarction (known to increase cf-DNA levels) were excluded from the study. No research has so far been conducted with regard to the reliability of cf-DNA as an indicator of vascular inflammation.

7. MicroRNA

Perhaps one of the most important components of MPs is miRNA. These are short noncoding RNA molecules, approximately 22–25 nucleotides in length [41], the actual length of which is defined by the specific argonaute involved in its genesis [42]. Since their discovery in 1993, during experimentation on the nematode, *Caenorhabditis elegans* [43], the field of research has expanded to encompass genome-wide studies. Current opinion hails miRNA as molecules of vast regulatory potential [44]. Target identification/characterisation remains

Table 1: Microvesicle class based on size, origin, and composition.

Microvesicle type	Size (μm)	Formation	Internal composition	External composition
Exosome	<0.1	Exocytosis	Cytoplasm, protein, miRNA, mRNA	
Microparticle	0.1–1.0	Cell stress/stimuli Cell membrane asymmetry and cytoskeletal deregulation	Cytoplasm, protein, miRNA, mRNA	Plasma membrane and surface proteins of parent cell
Apoptotic body	>1.0	Apoptosis	Cytoplasm, protein, miRNA, mRNA, nuclear fragments	

a significant challenge, given the vast numbers of miRNAs so far discovered.

Nevertheless, a number of potential circulatory miRNA markers have been determined. Of those investigated thus far, mir-155 has been revealed as an important herald of inflammatory response. Analysis of both *in vitro* and *in vivo* models has revealed mir-155 to be involved in a negative feedback loop. Impeding mir-155 resulted in downregulation of inflammatory cytokines, IL-6 and TNFα. Absence of these cytokines served to impede atherosclerotic development. Significantly, mir-155 is postulated to be responsible for post-translational regulation of the MAPK pathway by targeting MAP3K 10 [45].

8. Platelets Act as Biomarkers

Circulating blood platelets are 2–4 μm sized anucleate fragments of precursor cells called megakaryocytes. Their production is arguably the most elegant and distinct developmental process in eukaryotes [46]. An obvious advantage of using platelets as biomarkers of human health and disease is their fast, simple, and minimally invasive accessibility from whole blood in large numbers, representing the second most abundant cell type in blood. Platelets are also highly reflective of physiological and lifestyle changes, making them extremely sensitive biomarkers of human health. Although anucleate, platelets retain a sophisticated repertoire of messenger RNA, miRNA, and protein which contribute to primary (adhesion, activation, secretion, and aggregation) and alternative (immune regulation, inflammation, RNA transfer, and tumour metastasis) functions [47].

Typical biomarkers of platelet health include identification of primary platelet function changes in activation, aggregation, and secretion. A multitude of techniques, most notably flow cytometry (to measure glycoprotein activation-dependent changes, exposure of granule membrane proteins, platelet-leukocyte aggregate, etc.), aggregometry, and Enzyme-Linked Immunosorbent Assay (ELISA) have previously been used to identify these changes. Lack of standardisation between labs and preactivation of platelets due to methodological pitfalls are major problems when analysing platelet-derived biomarkers, warranting a pressing requirement for reliable platelet biomarkers [48].

9. Platelet miRNA as Epigenetic Biomarkers

It has been well documented that activated platelets also release the most abundant cell-derived MVs, accounting for 70–90% of circulating peripheral MVs. These MVs harbour an array of cell components including protein, DNA, and RNA and can act as a network of communication by selectively interacting with target cells. Platelets release increased levels of MVs in response to various stimuli and under a plethora of disease states including CVD, cancer, and neurodegenerative diseases.

10. Platelet miRNA Has a Functional Effect on Other Cells

The modern "omics" revolution enables simultaneous quantification of hundreds of molecules from a single sample and may be more reflective of platelet function changes, providing novel biomarkers for a range of diseases [47]. Platelets provide considerable contribution to the circulating miRNA pool and their harboured miRNAs have been labelled as markers of mature megakaryocyte miRNA [49]. As platelets are anucleate and have a limited lifespan, platelet-derived miRNA could represent an ideal marker of temporal and modifiable epigenetic drift [48].

Furthermore, studies by Laffont et al. [50] and Gidlöf et al. [51] demonstrate the functionality of platelet miRNA, whereby functional complexes of miR-223 and Argonaute 2 protein (Ago2) packaged in MVs from activated platelets could modulate expression of targeted endothelial cell mRNA transcripts FBXW7 and EFNA1. This same complex has also been shown to reduce expression levels of another transcript, the insulin-like growth factor 1 receptor in endothelial cells, and promote human umbilical vein endothelial cell (HUVEC) apoptosis [52]. This type of platelet MV-cell interaction is illustrated in Figure 3.

11. Platelet miRNA as a Biomarker of CVD

Platelets play a key role in CVD progression where the normal platelet response is modified by enhanced proaggregatory processes and decreased antiaggregatory mechanisms thereby generating a condition of platelet hyperaggregability

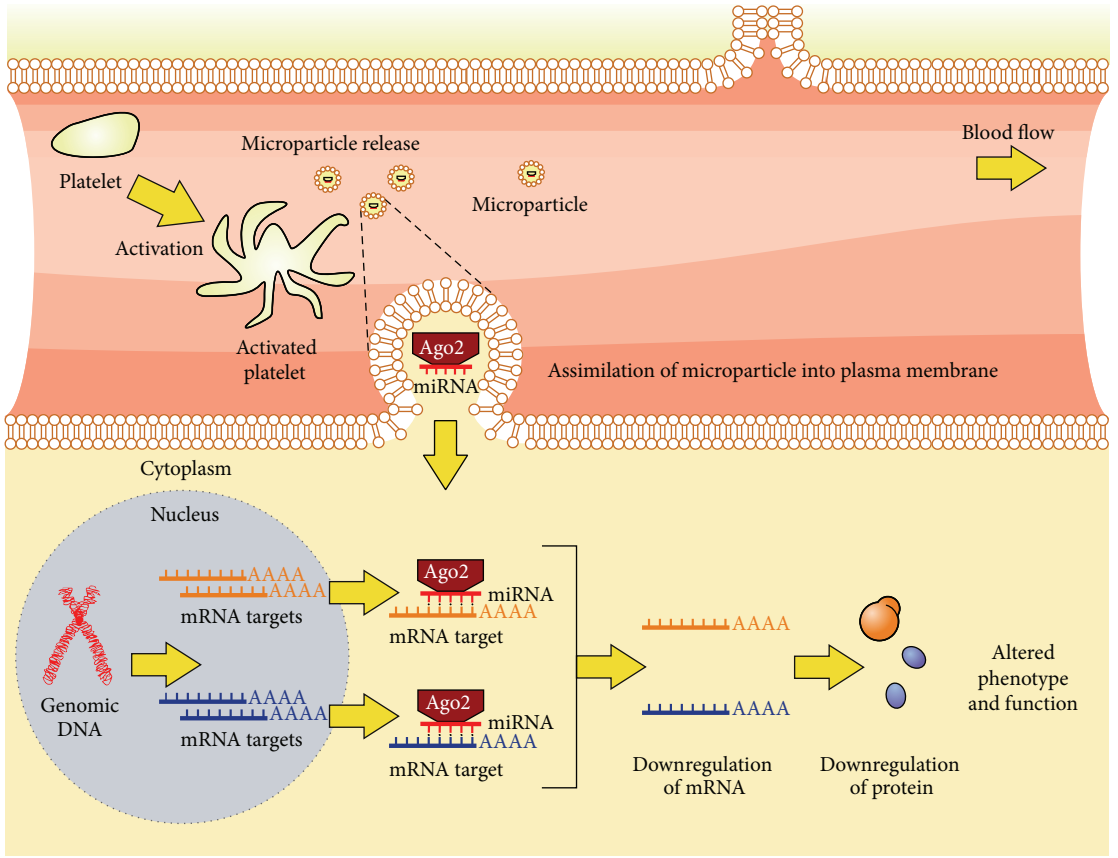

Figure 3: Platelet MVs act as intercellular transporters of functional Ago2-miRNA complexes. Activated platelets release microvesicles packaged with functional Argonaute 2- (Ago2-) miRNA complexes which can be assimilated by human aortic endothelial cells (HAECs). Platelet-derived miRNA accumulates within the cell, regulating expression of endothelial genes at the mRNA and protein level, resulting in altered phenotype and function.

on an acute and chronic level. Following atherosclerotic plaque rupture in severe CVD states, platelets adhere to the disrupted plaque and aggregate to form a prothrombotic surface, subsequently encouraging thrombosis and vascular occlusion. Platelet miRNAs have been differentially implicated in the presence and progression of a range of diseases, including but not limited to various CVDs such as Acute Coronary Syndrome (ACS), Atrial Fibrillation (AF), and coronary artery disease (CAD) [53].

Platelet miRNA profiles are differentially expressed in ACS. In a study by Ward et al. [54], blood was obtained from 13 patients with ACS presenting with either ST Segment Elevation Myocardial Infarction (STEMI) or Non-ST Segment Elevation Myocardial Infarction (NSTEMI). In STEMI, the artery is usually completely occluded as compared to NSTEMI, which displays partial occlusion [55]. Four platelet enriched miRNAs were differentially expressed between groups. Platelet enriched miR-186-5p and miR-342-3p were significantly lower in patients with STEMI as compared with NSTEMI. In contrast, platelet enriched miR-25-3p and miR-221-3p were significantly higher in patients with STEMI as compared with NSTEMI.

Circulating levels of the platelet enriched miR-328 have also been linked to AF in 2445 offspring in the Framingham Heart Study [53]. This miR controls particular genes involved in inflammation, vascular function (ABCG2), cell signalling, and cellular aging (H2AFX), which constitute some of the mechanisms in the development of AF. miR-328 expression was decreased in subjects with AF in comparison to subjects without AF. Furthermore, in a separate investigation, Sondermeijer et al. [56] demonstrated by microarray analysis that platelet-derived miRNAs (miR-624 and miR-340) were significantly elevated in CAD patients compared to healthy individuals.

12. Platelet Mitochondria as a Biomarker

Investigation of metabolic function in platelets for disease diagnosis and prognosis is a new area of translational research [57]. Platelets contain functional mitochondria whose activity is modulated in different pathological states such as CVD [58]. This suggests that platelets can sense metabolic stress and act as surrogate markers of mitochondrial dysfunction in remote inaccessible tissue types [59].

The traditional role of mitochondria in the platelet was considered to supply energy in the form of ATP for primary platelet functions: adhesion, activation, and aggregation. However, novel functions for mitochondria continue

to emerge. Mitochondrial membrane potential ($\Delta\Psi_m$) is reduced in a subtype of platelets known as Collagen and Thrombin Activated (COAT) platelets. Dual activation of platelets with collagen and thrombin results in striking alterations in function and structure. COAT platelets differ from typical "activated platelets" by exhibiting a myriad of features such as phosphatidylserine exposure due to cytoskeletal reorganisation, high microparticle release, and increased levels of fibrinogen on the platelet surface. In addition, mitochondria have been noted to be involved in ATP-controlled thrombotic signalling and platelet apoptosis [60].

13. Platelet mtDNA Methylation

Platelets have a higher degree of ATP turnover than resting mammalian muscle which holds high levels of mitochondria, suggesting a critical role for mitochondria in platelet function and huge requirement for energy during platelet activation. As with nuclear DNA, mitochondrial DNA (mtDNA) has the potential to be methylated by factors such as disease, ageing, and environmental exposure, moderating control of mitochondrial gene expression. In tandem with platelet miRNA biomarkers, simultaneous understanding of epigenetic regulation of mitochondrial genes in platelets is proving crucial to understanding their implication in CVD development. Recently, Baccarelli and Byun [61] investigated platelet mtDNA methylation in a selection of mitochondrial genes including NADH dehydrogenase (*MT-MD5*), cytochrome c oxidase (MT-CO1, MT-C02, and MT-C03), tRNA leucine 1 (MT-TL1), and ATP synthase (MT-ATP6 and MT-ATP8) amongst CVD patients and healthy individuals by bisulfite PCR coupled with pyrosequencing. CVD patients had significantly higher mtDNA methylation than healthy individuals in MT-CO1, MT-C02, MT-CO3, and MT-TL1, genes involved in ATP synthesis.

DNA methylation in platelet mitochondria could be a potential contributor to CVD development through regulation of platelet function. The role of mtDNA methylation in platelet function has yet to be fully determined, but this study is the first to portray platelet mtDNA methylation as being implicated in, and a possible biomarker of, CVD.

14. Conclusion

Initiation of vascular inflammation is a key event in endothelial dysfunction and CVD as a whole. While a small group of markers (ICAM, VCAM, IL-6, cytokines, etc.) have been generally accepted by the scientific community, questions remain regarding the overall biological relevance, as both reporters and effectors of pathophysiological processes.

Epigenetic biomarkers may yield far-reaching prognostic results but, as a technique, is in its infancy. Nevertheless, epigenetic alterations in vasculature have potential to provide novel therapeutic targets for vascular-associated chronic illnesses and initiate novel drug-development pipelines.

Yet, significant challenges remain. Deeper investigation into the physiological roles of MVs *in vivo* is required. A great deal about MVs remains unknown; even their biogenesis is yet to be completely understood. A concerted effort must be made in the direction of MV profiling. Knowledge of miRNA and their associated pathways will prove necessary in order to understand effects on cells and, importantly, unlock their diagnostic and predictive potential. The observation of proinflammatory miRNA upregulation in stimulated MPs suggests that MV miRNA currently presents perhaps the most compelling opportunity for significant progress in the field of vascular research. Platelet miRNA signature may well quantitatively reflect platelet activation *in vivo* and therefore could have huge potential as biomarkers of cardiovascular risk. In addition, correlating platelet physiology and function with its miRNA content may provide new insights into the changes in age and lifestyle-associated epigenetic drift. To this end, it is essential to identify and subsequently interpret miRNA functionality and hence distinguish which of these are most relevant in a clinical setting.

Disclosure

Dr. Ronan Murphy is a Management Committee Member of the EU H2020 COST Action, CM1406, EpiChemBio. Robert G. Wallace and Laura C. Twomey are joint first authors.

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

Kidney Injury Molecule-1 and Cardiovascular Diseases: From Basic Science to Clinical Practice

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Despite the recent findings concerning pathogenesis and novel therapeutic strategies, cardiovascular disease (CVD) still stays the leading cause of morbidity and mortality in patients with renal dysfunction, especially acute kidney injury (AKI). Early detection of patients with impaired renal function with cardiovascular risk may help ensure more aggressive treatment and improve clinical outcome. Kidney injury molecule-1 (KIM-1) is a new, promising marker of kidney damage which is currently the focus of countless studies worldwide. Some recent animal and human studies established KIM-1 as an important marker of acute tubular necrosis (ATN) and reliable predictor of development and prognosis of AKI. Food and Drug Administration (FDA) in USA acclaimed KIM-1 as an AKI biomarker for preclinical drug development. Recent data suggest the importance of monitoring of KIM-1 for early diagnosis and clinical course not only in patients with various forms of AKI and other renal diseases but also in patients with cardiorenal syndrome, heart failure, cardiopulmonary bypass, cardiothoracic surgical interventions in the pediatric emergency setting, and so forth. The aim of this review article is to summarize the literature data concerning KIM-1 as a potential novel marker in the early diagnosis and prediction of clinical outcome of certain cardiovascular diseases.

1. Background

Despite the recent findings concerning pathogenesis and novel therapeutic strategies, cardiovascular disease (CVD) still stays the leading cause of morbidity and mortality in patients with renal dysfunction, especially acute kidney injury (AKI). The term "acute kidney injury" (AKI) represents a wide range of structural and functional renal changes from mild alteration to complete organ failure [1]. RIFLE classification [Risk-Injury-Failure-Loss-End-stage kidney disease (ESKD)] was established by the Acute Dialysis Quality Initiative (ADQI) group in order to supply the obligation for uniform definition, early detection, and grading of AKI. After this, the AKIN criteria were created by the Acute Kidney Injury Network (AKIN) for further refinement of the definition of AKI [1]. The RIFLE classification criteria are shown in Figure 1.

In spite of advances in the understanding of pathogenesis of AKI and progress in classifying different forms depending on the etiology, clinic manifestations, and stages as well as the novel therapeutic strategies, the mortality rate still remains high (approximately 2 million people worldwide pass away because of AKI and its consequences every year) [2–4]. One of the principal reasons for this poor prognosis is too late detection of renal impairment and the preventive strategies are most effective when they are started before oliguria. Serum creatinine is still gold standard of kidney injury although it is well known as an insensitive and unreliable biomarker (e.g., its concentration does not increase significantly until about half of the kidney function is lost) [5, 6].

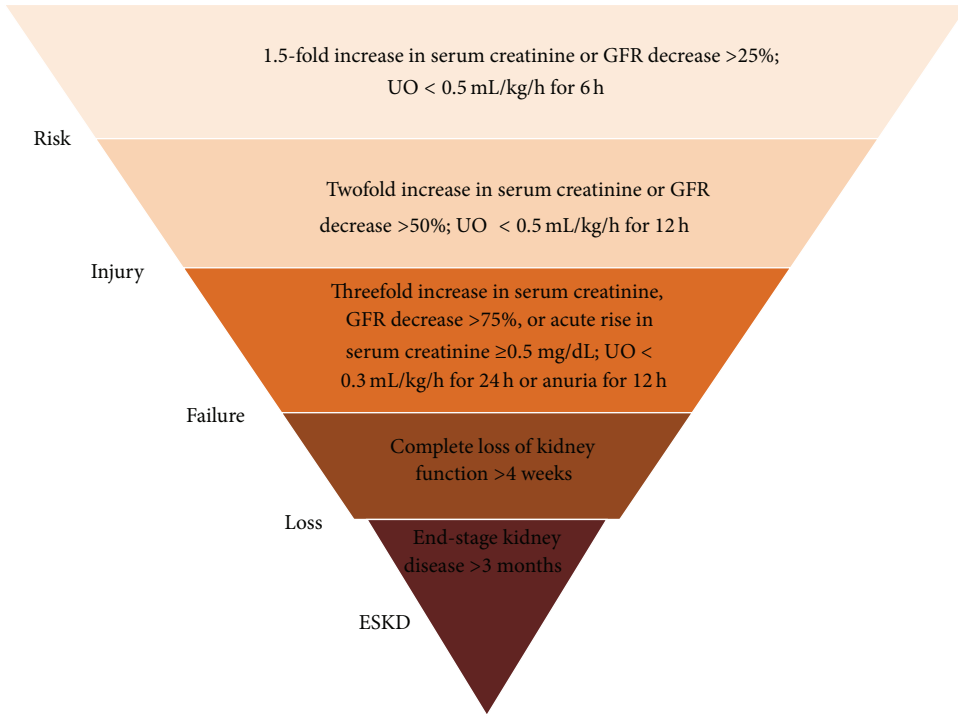


Figure 1: RIFLE classification [Risk-Injury-Failure-Loss-End-stage kidney disease (ESKD)]; GFR, glomerular filtration rate; UO, urine output (modified by [1]).

Considering these data, scientists and clinicians worldwide were making great efforts in the past decade in order to discover and validate novel AKI biomarkers. The search for such biomarker can be specified as "the search for renal troponin I" [7]. The term biomarker (acronym for biological marker) is used to define a characteristic that can be measured and evaluated as normal biological process, pathological process, or pharmacological response to therapeutic intervention [8, 9].

Numerous studies and the previous experience have shown that the ideal marker for AKI should be (1) noninvasive, (2) easily detectable in accessible body samples (e.g., serum or urine), (3) highly sensitive and specific for AKI, (4) rapidly and reliably measurable, (5) capable of early detection, (6) predictor of AKI severity and prognosis, (7) unaffected by other biological variables, (8) inexpensive, and so forth [10].

KIM-1 is one of the most promising, early biomarkers due to its translatability between preclinical and clinical trials. It is believed that this molecule participates in the process of both kidney injury and healing, although precise mechanism of restoration of tubular integrity after injury still remains unclear. In the past 15 years Ichimura and collaborators have published several papers regarding importance and clinical applicability of new biomarkers of acute renal failure [11–15]. Recently, his team has demonstrated that in ischemic injury KIM-1 expression is most prominent in S3 segment (i.e., the segment most susceptible to ischemic injury) [11]. Numerous animal and human studies recognized KIM-1 as an early and reliable predictor of AKI [12, 16, 17].

The aim of this review article is to summarize and discuss the literature data concerning KIM-1 as a potential novel marker in the early diagnosis and prediction of clinical outcome of certain cardiovascular diseases.

2. KIM-1: Molecular Structure

Kim-1 protein is a membrane receptor for human hepatitis A virus (HHAV) and T-cell immunoglobulin and mucin domain containing 4 (TIMD 4). KIM-1 is a single pass type I cell membrane glycoprotein which contains, in its extracellular section, a six-cysteine immunoglobulin-like domain, two N-glycosylation sites, and T/SP rich domain characteristic of mucin-like O-glycosylated proteins. Kim-1 has one transmembrane domain and a short intracellular domain which contains a signaling motif for tyrosine phosphorylation present in the renal form of protein (Kim-1b). The structure of the protein led to the conclusion that it has adhesion properties, but, later on, its diverse biological functions were revealed [18].

Kim-1 gene is located in chromosome 5q33.2. It is widely expressed with highest levels in kidney and testis. It is also expressed by activated CD4+ T-cells during the development of helper T-cell response [21]. The gene is upregulated in the kidney in renal diseases, which was confirmed at the protein level [22]. The reference genome represents an allele that retains MTTVP amino acid (allelic variant with the 5-amino acid insertion at position 158) segment that confers protection against atopy in HHAV seropositive individuals.

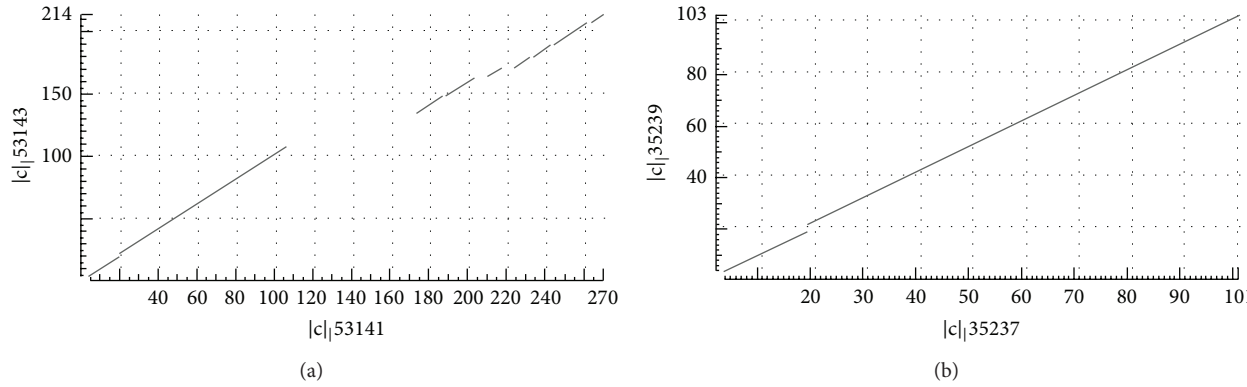


Figure 2: Dot matrix view of human EC Kim-1 domain and rat's EC Kim-1 domain and Ig like V subdomain aligned in BLAST. (a) Human sequence is plotted on x-axis and rats sequence is plotted on y-axis. Several gaps demonstrate the existence of repeated amino acid sequences, which exist only in human ortholog of Kim-1. (b) Human sequence is plotted on x-axis and rats sequence is plotted on y-axis. Single gap shows the two amino acids which are present only in rat's ortholog.

Alternative splicing of this gene results in multiple transcript variants. The related pseudogenes have been identified on chromosomes 4, 12, and 19 [23].

Among mammals the gene for Kim-1 is highly conserved, suggesting its biological importance and low evolutionary plasticity, particularly of its extracellular and intracellular domains, with the conservation score 921. The amino acid sequence of Kim-1 genes, belonging to the *Homo sapiens*, *Rattus norvegicus*, *Mus musculus*, and *Felis catus*, was obtained from the UniProtKB/Swiss-Prot database and aligned by T-COFFEE (tree-based consistency objective function for alignment evaluation) bioinformatic tool [24].

Similarity between human extracellular domain and rat extracellular domain of Kim-1 protein was determined by comparison of amino acid sequences. Using the UniProt database, amino acid sequences of human and rat Kim-1 were accessed by following entries Q96D42 and O54947, respectively. Sequence alignment was performed by using BLAST (Basic Local Alignment Search Tool) [25].

These domains show 57% of sequence identity (E-value $5e - 41$) and most of differences are due to the existence of repeated elements in human EC domain of Kim-1. The aminoacid sequence similarity between human's and rat's Ig like V subdomain of EC domain is 57% (E-value $2e - 39$). Similarity between human and rat Kim-1 EC domain suggests that structural conditions are fulfilled for equalization of ligand binding and overall biochemical similarity. The sequence conservation in both species suggests the significant selective pressure against sequence alteration during evolution of Kim-1 gene. The human anti-EC Kim-1 Mab binds to the same domain of rat's Kim-1, leading to a conclusion that experimental results based on rat's model could be seriously taken into concern for extrapolation to humans [26]. Dot matrix view of human EC Kim-1 domain and rat's EC Kim-1 domain and Ig like V subdomain aligned in BLAST program is shown in Figure 2.

Rat and human cDNAs encoding KIM-1 (KIM-1 in the rat) were identified for the first time using difference analysis between normal kidneys and kidneys exposed to ischemia/reperfusion (I/R) injury followed by regeneration of proximal tubular cells. KIM-1 is found to be expressed at low to undetectable levels in the normal adult rat kidney but is markedly expressed by the epithelial proximal tubular cells in response to ischemic or toxic AKI [13, 18]. Modern molecular cytogenetic techniques (*in situ* hybridization and immunohistochemistry) indicated KIM-1 as a marker of proliferation and regeneration in proximal tubules [14].

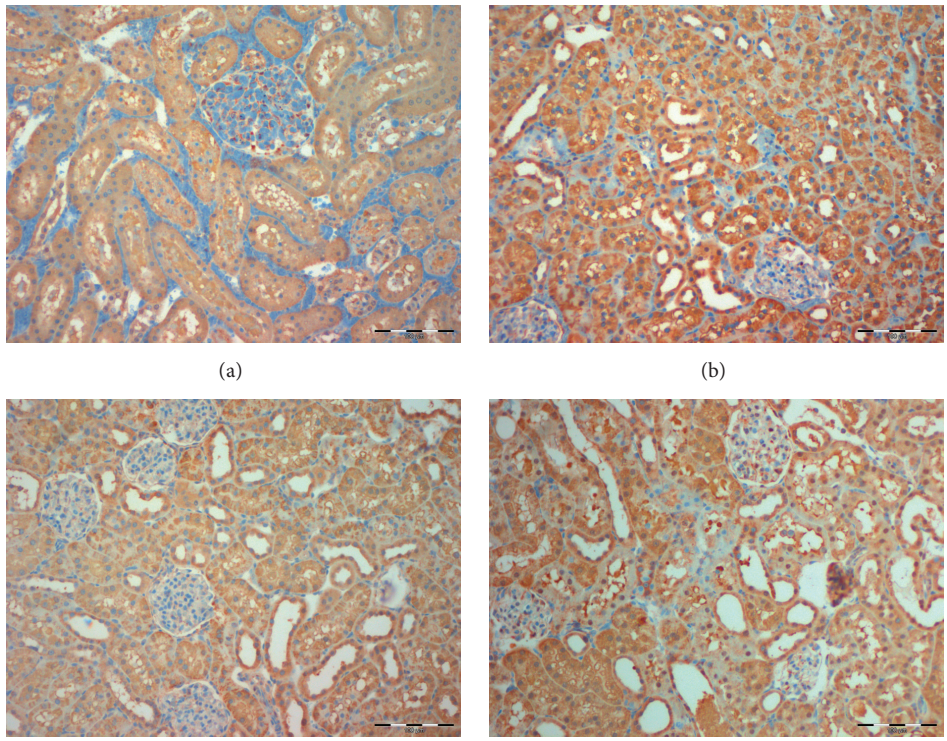
Some later reports showed mechanism of dropping of KIM-1 ectodomain cells into the urine after proximal tubular injury *in vivo* in rats and rodents [11, 27–29].

Besides that, after injury, KIM-1 acts as a phosphatidylserine receptor that shows the ability to recognize and phagocytose dead cells presented in the postischemic kidney [15, 23].

3. KIM-1 in Preclinical Studies

Since the identification of KIM-1 upregulation in the rat model of renal ischemia, additional studies have been performed in order to examine the diagnostic role of KIM-1 in other models of AKI. Vaidya et al. stressed out the importance and practical application of determination of KIM-1 concentration in urine in these kinds of experiments [12]. Ichimura and colleagues examined tissue and urinary KIM-1 expression in a cisplatin-induced nephrotoxicity in rats and proved KIM-1 as a faster and superior marker compared to serum creatinine. A further advance was the development of a sensitive microbead-based KIM-1 ELISA in order to confirm and facilitate the use of urinary KIM-1 as a biomarker of AKI in animal studies [18, 30, 31].

At this moment it is possible to measure KIM-1 concentration in tissue samples and urine and plasma/serum in a simple, rapid, and accurate manner. Some recent studies confirmed KIM-1 as an important biomarker of AKI and acute tubular necrosis (ATN) and showed correlation between its concentration and the degree of renal dysfunction. Renal and urinary Kim-1 correlated with proteinuria and interstitial damage [32].



(a) (b) (c) (d)

Figure 3: The effects of chloroquine (0.3 mg/kg, i.v; 3 mg/kg, i.v.) on renal I/R injury and histological micrographs of renal tissues: KIM-1 staining score. Chloroquine, in dose of 0.3 and 3 mg/kg, i.v., was injected 30 min before ischemia. Control groups, Sham + Saline, and IR + Saline received instead of drug i.v. bolus of 0.5 mL saline only (unpublished data from our laboratory projects: Professor Milica Prostran (ON175023) together with Professor Gordana Basta-Jovanovic (ON175059)). Histological micrographs of renal tissues: kidney sections taken from Sham-operated rats or rats subjected to renal I/R injury. Kidney injury molecule-1 (KIM-1) staining. Original magnification ×200. Figures were randomly chosen from the series of at least 6 experiments (a–d). (a) Sham-operated animals treated with saline only: absence of immunoreactivity for KIM-1. (b) Rats subjected to renal I/R injury, pretreated with chloroquine at 0.3 mg/kg, i.v. 30 min, before ischemia: most of proximal and some distal tubules show mild staining for KIM-1. (c) Rats subjected to renal I/R injury, pretreated with chloroquine at 3 mg/kg, i.v. 30 min, before ischemia: most of proximal and some distal tubules show moderate staining for KIM-1. (d) Rats subjected to renal I/R injury, pretreated with saline only: proximal and distal tubules show moderate to intensive positive KIM staining.

Adjusted for age, gender, and length of time delay between insult and sampling, a one-unit increase in normalized KIM-1 was associated with a greater than 12-fold increase in the presence of acute tubular necrosis (ATN) [16].

In a recent preclinical study, the diagnostic value of urinary KIM-1 significantly exceeded traditional biomarkers (serum creatinine and urea) as predictors of kidney tubular histopathological changes in rats [33].

Recently, research group from our project provided the first evidence that KIM-1 staining scores could be used as an indicator of the therapeutic benefit of different pharmacological agents in the experimental model of renal ischemia/reperfusion (I/R) injury. KIM-1 reliably confirmed that chloroquine affords an acute protective effect on kidney function and morphology [26].

Unpublished data from our laboratory project are presented in Figure 3.

In addition, KIM-1 has been approved by the US Food and Drug Administration as an AKI biomarker for preclinical drug development [34].

4. KIM-1 in Clinical Studies

Subsequent studies in adults suggested that KIM-1 can discriminate patients with different types of acute tubular necrosis (hospitalized patients, critically ill patients, and patients with acute graft rejection) from those without AKI [22]. In hospitalized patients with established AKI, urinary KIM-1 levels predicted adverse clinical outcomes such as dialysis requirement and mortality [35].

A rapid testing method for KIM-1 has been described, yielding semiquantitative results in just 15 minutes [36, 37]. One prospective study has shown that KIM-1 can even predict adverse clinical outcomes in patients with AKI: patients with the highest levels in urinary KIM-1 had the highest odds for dialysis and hospital death [33, 38, 39].

Additional studies have confirmed that KIM-1 urinary concentration is upregulated in various kidney diseases including diabetic nephropathy, focal glomerulosclerosis, membranoproliferative glomerulonephritis, IgA nephropathy, and even renal cell carcinoma [40].

Recent data suggest the importance of monitoring this marker for early diagnosis, prognosis, and the therapy effects not only in patients with various forms of AKI and other renal diseases but also in patients with heart failure after cardiopulmonary bypass, various forms of cardiorenal syndrome, cardiothoracic surgical interventions in the pediatric emergency setting, and so forth.

In the next paragraph, we will discuss the importance of determining KIM-1 markers in certain clinical entities.

5. KIM-1 in Cardiorenal Syndrome

Cardiorenal syndrome (CRS) commonly represents complex interaction between heart and kidneys in which acute or chronic dysfunction in one organ may induce acute or chronic dysfunction in the other organ [41]. The pathophysiology of this clinical entity includes reduced renal perfusion, increased venous pressure, and activation of multiple neurohormonal systems, although whole process is still not completely understood [42, 43].

Five different subtypes of CRS have recently been proposed: type 1, acute cardiorenal syndrome (acute impairment of heart function leads to kidney injury and/or dysfunction), type 2, chronic cardiorenal syndrome (chronic heart diseases lead to kidney injury and/or dysfunction), type 3, acute renocardiac syndrome (acute impairment of kidney function leads to heart injury and/or dysfunction), type 4, chronic renocardiac syndrome (chronic kidney disease leads to heart disease and/or dysfunction), and type 5, secondary CRS occurring in systemic disorders (e.g., sepsis, diabetes mellitus, and amyloidosis) simultaneously causing both cardiac and renal dysfunctions [44–49].

Clinical outcome of this syndrome remains poor with a high mortality rate, partly because of delayed diagnosis (approximately 24 h after the event). For this reason, numerous studies are currently underway to confirm the clinical utility of the new biomarkers. In one of them, Damman et al. recently confirmed KIM-1 as an excellent predictive marker for the detection of acute tubular injury in patients with chronic heart failure (HF) after the suspension and the reintroduction of diuretic therapy. KIM-1 levels increased significantly as early as 8 h after diuretics were stopped, remained elevated within three days, and then returned to normal levels as early as 4 h after furosemide was resumed [50]. In this study KIM-1 defeated other markers (such as NGAL and N-acetyl-β-D-glucosaminidase (NAG)) and showed how changes in volume status can lead to subclinical tubular injury that may be undetected by traditional biomarkers.

Also, it is shown that urinary KIM-1 was also associated with increased risk of death or hospitalization, independent of GFR in patients with chronic heart failure [51]. Still, for more precise role of KIM-1 in early detection and/or evaluation of therapy in CRS, it is necessary to conduct more comprehensive evaluation.

6. KIM-1 in Cardiac Surgery-Associated AKI

Cardiac surgery-associated acute kidney injury (CSA-AKI) often includes coronary artery bypass grafting (CABG), surgery for valvular disease, and congenital heart surgery reportedly occurring in 30%–40% of cases (according to some authors, CRS could be defined as a particular type of type 1 cardiorenal syndrome for which no clear understanding of pathogenesis exists [52]).

It represents the second most common cause of AKI in the intensive care units and an independent predictor after cardiac surgery [53–55]. Hemodynamic and inflammatory factors that lead to oxidation from reactive oxygen species represent major determinants in poor prognosis of cardiac surgery-associated AKI [56].

Recent studies confirmed that increases in serum creatinine concentration are observed too late (usually within 48 h) and have significant impact on mortality rate in these cases [54, 57, 58]. This finding was one of the reasons to start with examination of novel biomarkers in clinical trials in CSA-AKI.

Some of the first investigations were carried out on patients undergoing CBP where KIM-1 levels increased significantly at both 2 hours and 24 hours after operation in patients with AKI [59, 60]. Similar results were found in a small case-control study of 40 pediatric patients following CPB [61].

Koyner et al. compared the ability of several biomarkers to predict the progression of kidney damage in patients with elevated serum creatinine concentration levels who underwent cardiac surgery. KIM-1 was shown as a predictor of secondary importance in these situations [62]. Conclusion of the research conducted by Hall and colleagues was the fact that urine NGAL had the best results, followed by KIM-1 and IL-18 [63]. Arthur and associates evaluated ability of 32 AKI biomarkers to predict declining of renal function in patients with AKIN stage 1 AKI after cardiac surgery. Although they found IL-18, independently, as a best performer, it was demonstrated that combination of KIM-1 and IL-18 was much more accurate in prediction [64].

Another study measured KIM-1, NAG, and NGAL in 90 adults undergoing cardiac surgery. The values of area under the curve (AUC) in prediction of AKI immediately and 3 h after operation were 0.68 and 0.65 for KIM-1, 0.61 and 0.63 for NAG, and 0.59 and 0.65 for NGAL, respectively. Combining the three biomarkers enhanced the sensitivity of early detection of postoperative AKI compared with individual biomarkers: the AUCs for the three biomarkers combined were 0.75 and 0.78 [65].

Recently, it was shown that preoperative KIM-1 urinary level is able to predict the development of AKI in adults undergoing cardiac surgery [62, 66]. Also, KIM-1 showed potential of being a good predictor of development of AKI in pediatric cardiorenal injuries in emergency settings. For example, Han et al. found that urinary KIM-1 detected AKI before serum creatinine in a cohort of children undergoing cardiopulmonary bypass (CBP) [67]. Krawczeski et al. proved evidence that KIM-1 at 12 h following CPB independently correlated with CPB time and risk adjustment for congenital heart surgery score (RACHS-1) [68].

Contrary to previous research, Hazle et al. did not confirm KIM-1 as a good prognostic factor in children. They measured urinary levels of few novel biomarkers (neutrophil

gelatinase-associated lipocalin (NGAL), interleukin-18 (IL-18), kidney injury molecule-1 (KIM-1), and cystatin C) pre- and postoperatively in infants younger than 6 months of age to predict outcomes following congenital heart surgery. It was shown that KIM-1 poorly differentiated patients with either good or poor outcomes and was, therefore, removed from further analysis [69].

7. KIM-1 in Myocardial Infarction

Progressive decline in renal function coexists with myocardial infarction (MI), although mechanisms underlying its dysfunction are poorly understood. The mortality of these patients is high (it is assumed that 20% of hospitalized patients with acute MI have renal impairment and around 25% of them die during hospitalization) [70–72]. The pathogenesis may include an inflammatory response after MI and various cytokines, such as IL-6, TNF-α, IL-1β, and transforming growth factor-β (TGF-β), which appear to be major contributors to renal fibrosis [73–75].

Additionally, neurohormonal activation and hemodynamic disturbance (mainly the renin-angiotensin-aldosterone and sympathetic nervous system activation) that have been demonstrated in both humans and animals after acute MI may affect cardiac pump function leading to systemic hypotension and hypoperfusion of all organs, including kidney [76–79].

In a recent study, conducted by Lekawanvijit and colleagues in a rat MI model, they examined potential mechanisms of development of renal changes by monitoring time-course renal functional, structural, and molecular changes following acute MI. They showed kidney injury molecule-1-positive staining in the tubules of experimental animals just one week after MI and concluded that KIM-1 may be a potentially useful kidney injury biomarker for early detection and monitoring of disease progression [80].

8. KIM-1 in Organ Transplantation

Acute graft dysfunction provoked by immunological or ischemic injury leads to severe obstacles. In this sense, finding markers that could predict potential organ donors, early posttransplant periods, and long-term follow-up represent a crucial step in further studies [81].

Another potential application of, for example, kidney-injury-specific biomarkers is for guiding decisions on when to initiate renal replacement therapy (RRT). It is well known that KIM-1 values increase in acute graft rejection, but its role in delayed graft function (DGF) is still obscure [82].

Recently, one small study linked urinary KIM-1 as a positive predictor of 14-week and 1-year posttransplantation serum creatinine. KIM-1 values were measured in tissue and urine in 20 brain death kidney donors before organ removal and these were compared with living donors before nephrectomy. Tissue KIM-1 mRNA was 2.5-fold and urinary KIM-1 was twofold higher in brain death donors when compared with living donors [83].

First prospective study that has examined the relationship of preimplantation tissue KIM-1 expression with immediate and long-term graft function was conducted by Schröppel et al. They measured KIM-1 RNA and protein expression in preperfusion biopsies of 30 living-donor and 85 deceased-donor kidneys and correlated the results with histologic and clinical outcomes after transplantation. Their results showed that tubular KIM-1 expression correlated with eGFR at the time of organ procurement but did not correlate with the incidence of DGF [84].

A recent study has explored urinary biomarkers in 63 renal transplant recipients who showed decline of renal function. Urinary KIM-1 expression is marked as significant predictor of prognosis in these patients (group with high KIM-1 expression has significantly worse graft survival) [85].

Besides that, it is important to point out that KIM-1 is not the most useful biomarker in prediction of DGF. Peake et al. showed in their study that urinary levels of KIM-1 increased after transplantation peaking at 24 h and remained higher than those in control subjects 168 h after transplantation but did not correlate with early graft outcome [86]. Recently published paper by Pianta et al. showed that clusterin and IL-18 are more useful markers in triaging of patients with DGF within 4 h of transplantation [87].

9. KIM-1 in Critically Ill Patients

Acute kidney injury is one of the most frequent problems occurring in the critically ill patients in the intensive care units. Despite novel therapeutic strategies, it remains an unresolved problem in pharmacotherapy with high mortality rate, incidence of which varies from 28% to 90% [88, 89].

Despite this, until now, just few studies have examined the importance of the application of new biomarkers in these patients. One of them evaluated its interest in critically ill patients and indicated KIM-1 as a potential marker for prediction of the need for RRT and 7-day mortality [90].

Another study described that urinary KIM-1 levels correlated with dialysis requirement and hospital mortality in 201 critically ill hospitalized patients who developed AKI. A more recent report in the pediatric literature described a 252-patient cohort study in which KIM-1 levels predict the development of AKI in the emergency department [66].

Regardless, it is true that KIM-1 has not proved particularly effective in predicting clinical outcome in critically ill patients in some other studies. For example, Endre et al. conducted prospective observational study among patients in general intensive care units in order to better understand the diagnostic and predictive performance of some urinary biomarkers of kidney injury. Comparisons were made using the area under the curve (AUC) for diagnosis or prediction of acute kidney injury (AKI), dialysis, or death. It was shown that KIM-1 was not particularly useful in prediction of dialysis and death in 7 days, although its utility was improved with stratification for duration of AKI and baseline GFR [91].

Table 1: Advantages and disadvantages of KIM-1 (adapted from [19, 20]).

Advantages	Disadvantages
Can detect AKI earlier than serum creatinine	Primarily research tools
May suggest type of acute kidney injury	Is itself enough in diagnosis and prognosis, just as a part of "panel of biomarkers"
Can be measured in tissue, urine, and serum/plasma	Can be affected by numerous confounding variables
Urinary kidney injury molecule-1 (KIM-1) is a marker of tubular damage	Needs validation in appropriate clinical settings
Good sensitivity and specificity	High cost and poor availability
High prognostic value	
ELISA commercial assay	

10. Limitation of KIM-1 as a Diagnostic and Prognostic Marker in Cardiovascular Diseases

Previous research indicates the importance of the introduction of KIM-1 as a diagnostic and prognostic marker in kidney and heart disease. Nevertheless, it should be noted that it is unlikely that a determination of the one single marker may be sufficient in many clinical entities with very complex pathogenesis and diverse etiology. For such complicated processes it is more appropriate to combine biomarkers to maximize the features and to minimize disadvantages of each one [19].

At this point it seems that KIM-1 represents a promising candidate for inclusion in the urinary "AKI Biomarker Panel" together with NGAL. One advantage of KIM-1 as a urinary biomarker is the fact that its expression seems to be limited to the injured or diseased kidney, although its value may affect number of other confounding variables [20]. KIM-1 in the kidney and urine is also induced in a variety of chronic proteinuric, inflammatory, and fibrotic disease states in humans [92].

According to Endre and Pickering, the need for biomarker panel may include the requirement for heterogeneity in timing (biomarkers have varying time courses which are usually shorter than that of creatinine), heterogeneity of etiology (biomarker levels are dependent on preexisting conditions, and some of them may influence biomarker threshold), and heterogeneity of background function (e.g., reduced baseline glomerular filtration rate also modifies the concentrations and time course of both injury and function biomarkers) [93]. The advantages and disadvantages of KIM-1 biomarker are presented in Table 1.

11. Conclusion

Numerous animal and human studies promoted KIM-1 as a promising, new biomarker for early diagnosis, monitoring of therapeutic effects, and prediction of clinical outcome in cardiovascular diseases. Also, it should be pointed out that precise assessments of validity and establishing standards for measurement of KIM-1 as a novel marker in preclinical and clinical studies are highly required. At this moment, larger trials are necessary before a strong endorsement for establishment of KIM-1 in broader clinical use.

Finally, we believe that the future studies will demonstrate the right place and the right role of each of novel biomarkers in clinical use, including KIM-1, which was evaluated in this paper.

Conflict of Interests

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

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Clinical Study

Increased Circulating Advanced Oxidation Protein Products and High-Sensitive Troponin T in Cirrhotic Patients with Chronic Hepatitis C: A Preliminary Report

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Aim. To investigate the relationship between advanced oxidation protein products (AOPPs) and myocardial injury by comparing the selected biomarker for detecting myocardial injury [high-sensitive troponin T (hs-TnT)] in patients with chronic HCV infection. *Methods and Results*. Eighty-eight patients with cirrhosis and 40 healthy control subjects were enrolled in the study. Circulating levels of AOPPs-albumin (the ratio of AOPPs to albumin content), hs-TnT, tumor necrosis factor α (TNF-α), and high-sensitivity C-reactive protein (hs-CRP) were assessed. Compared with healthy controls, the cirrhotic patients with chronic HCV infection had higher levels of AOPPs-albumin, which were associated with increased hs-TnT. When the presence of ascites was considered, the plasma levels of AOPPs-albumin were higher, as well as TNF-α. AOPPs-albumin positively correlated with hs-TnT level in all cirrhotic patients with chronic HCV infection and this correlation was stronger in decompensated cirrhotic patients. In multivariate logistic regression analysis, the independent factors associated with the presence of ascites were high AOPPs-albumin levels and elevated hs-TnT levels. *Conclusion*. The simultaneous monitoring of plasma AOPPs and hs-TnT can be helpful for the alterations in myocardial function control in cirrhotic patients with chronic HCV infection.

1. Introduction

Patients with hepatitis C virus (HCV) infection who are chronically infected may go on to develop cirrhosis and hepatocellular carcinoma. Most authors favor the opinion that HCV infection is frequently associated with myocarditis and cardiomyopathy [1, 2]. In fact, it has been reported that HCV is replicated in myocardial tissue of patients with myocarditis; thus, HCV infection may contribute to the development of this form of myocarditis [2]. The severity of myocarditis associated with HCV infection is highly variable [2, 3]. In HCV heart failure, most patients develop chronic inflammation of the myocardium and later dilated cardiomyopathy attributable to necrosis and loss of myocytes. However, because myocytes do not replicate, the proliferative stimuli induced by HCV infection may promote myocyte hypertrophy and hypertrophic cardiomyopathy [4]. The presence of left ventricular hypertrophy and diastolic dysfunction in patients with chronic hepatitis C (CHC) during the precirrhotic stage also suggests a possible role of HCV in this structural abnormality of the heart [5]. Furthermore, the functional and structural cardiac abnormalities are present in the majority of CHC patients with moderately or severely advanced failure (Child-Pugh stage B or C) [6, 7]. Generally, myocardial injury worsens with the progression of the underlying liver failure. Therefore, the use of reliable biochemical markers for the detection of myocardial damage is essential in these patients.

Troponin T (TnT) and troponin I (TnI) are present in cardiac muscles that regulate inflammatory processes and play a leading role in myocardial hypertrophy associated with heart failure and left ventricular hypertrophy. Beyond the potential to screen for structural cardiac abnormalities, recent data suggest that cardiac troponins are valuable for screening asymptomatic individuals to prevalent subclinical cardiovascular disease [8, 9]. The high prevalence of elevated serum levels of cardiac troponins in heart failure raises the question of what mechanism of cardiomyocyte damage results in troponin release in heart failure. Proposed mechanisms include inflammatory cytokines, oxidative stress, and apoptosis [10, 11]. However, relationships between apoptosis and troponin plasma concentrations remain to be shown. Because cardiovascular disease begins early in the course of liver failure, differentiation of sources of elevated hs-TnT levels in cirrhotic patients is difficult. Nevertheless, the hs-TnT elevation is an independent predictor for adverse events [12]. Everett et al. [13] speculated that the very low, but detectable hs-TnT levels, may reflect a normal biological process of myocyte turnover. Even though the regeneration of myocytes may contribute to an increase in the muscle mass of the myocardium, a gradual decrease <50% of cardiomyocytes is exchanged during a normal lifespan [14]. Thus, it is not surprising that higher hs-TnT levels predicted a worse prognosis regardless of the conditions in cardiac or noncardiac diseases.

Oxidative stress leads to formation of glycoxidation products, including advanced oxidation protein products (AOPPs) and advanced glycation end products (AGEs). AOPPs can be formed *in vitro* by the exposure of serum albumin to hypochlorous acid. *In vivo*, plasma AOPPs are mainly carried by albumin, and their concentrations are closely correlated with the levels of dityrosine. The receptor for advanced glycation end products (RAGE) is a signal transduction receptor that binds both AGEs and AOPPs. RAGE is expressed by various cell types, including endothelial cells, smooth muscle cells, renal cells, and cardiomyocytes [15, 16]. Binding of AOPPs to the RAGE results in the generation of reactive oxygen species. Thus, AOPPs, formed as a result of oxidative stress, induce ROS generation *via* NADPH oxidase and can perpetuate oxidative stress conditions. Of note, *RAGE* is also an oxidative stress-responsive gene. The *RAGE* gene promoter contains several transcription factor-binding sites, including nuclear transcription factor [kappa]B (NF-κB) [17]. In cardiomyocytes, activation of NF-κB has been generally shown to activate cell survival pathways [18]. However, AOPPs suppress cell proliferation *via* the activation of NF-κB and induce cardiomyocyte death *via* RAGE [16, 19]. Valente et al. [16] first elucidated the role of AOPPs in proapoptotic signaling in cardiomyocytes. Of note, apoptosis of cardiomyocytes is a prerequisite for myocardial hypertrophy and heart failure-related remodeling. Systemic levels of AOPPs are increased in diverse chronic oxidative conditions, including diabetes mellitus, chronic kidney, and contribute to cardiac diseases [20, 21]. Since cardiovascular diseases are the major contributors of morbidity and mortality, it is possible that increased AOPPs-mediated cardiomyocyte death might perpetuate myocardial injury in CHC patients with moderately or severely advanced liver failure.

The aims of this study were to evaluate preliminarily the plasma levels of both AOPPs and hs-TnT of patients with chronic HCV infection for comparison with cirrhotic patients and to determine what factors significantly influence the hs-TnT level of CHC patients.

2. Patients and Methods

2.1. Patients. This study was performed in 120 patients with chronic HCV infection admitted to the Clinic of Infectious Diseases, Liver Diseases and Acquired Immune Deficiency for evaluation. The patients with chronic HCV infection were divided into two groups in function of the presence (88 patients) or the absence (32 patients) of cirrhosis. A total of 88 cirrhotic patients were included in the study.

Fifty-three were male and 35 were female, and they were aged 21–74 years (median age 56 years). The control group consisted of healthy blood donors with normal aminotransferases, normal blood counts, and negative markers for virus hepatitis and HIV (23 males/17 females, median age: 55 years). Blood samples were collected in the Department of Physiology and Biochemistry, University of Physical Education in Wroclaw. Clinical and biochemical characteristics of the study group are reported in detail in Table 1.

Inclusion criteria were histological or clinical diagnosis of cirrhosis, no evidence of metabolic, toxic, or autoimmune liver disease, and at least 1 year of alcohol abstinence. Diagnosis of cirrhosis was established according histological criteria when liver biopsy was performed, or by the combination of clinical, biochemical, and ultrasound imaging data (presence of irregular margins on ultrasound, portal hypertension with laboratory evidence of chronic liver disease), consistent with such a diagnosis. Patients were grouped according to Child-Pugh classification. Three biochemical variables [serum albumin, bilirubin, and prothrombin time (international normalized ratio, INR)] in addition to the presence or absence of ascites determine the Child-Pugh score. At the time of the study, no Child-Pugh A patients showed clinical features of decompensated liver cirrhosis (ascites or hepatic encephalopathy). At enrollment, esophageal varices were detected by endoscopy in 53% of patients; ascites and hepatic encephalopathy grade I were present by physical examination in 53 (60%) and 23 (26%) patients, respectively. Presence of ascites was assessed by ultrasonography. Bacterial infection was ruled out by clinical history, physical examination, differential and total white blood cell count, analysis and culture of urine, thorax X-ray, and culture and white blood cell count of ascetic fluid in patients with ascites.

Exclusion criteria were concurrent use of antioxidant drugs, coexisting diseases like chronic kidney disease, diabetes mellitus, cardiovascular disease, cardiac decompensation, and hepatocellular carcinoma, gastrointestinal bleeding, bacterial infection, and blood transfusion within previous two weeks. Patients received no diuretic, antibiotic, vasoactive drug (nitrates, β-blockers) and lactulose or lactitol therapy during the eight days before inclusion in the study.

After 2 h of bed rest, blood pressure was determined with an automatic digital sphygmomanometer and blood samples were collected in ice-cooled, ethylenediaminetetraacetic

Table 1: Clinical and biochemical characteristics of the study subjects.

	Healthy controls	Noncirrhotic patients	Cirrhotic patients
(*n*)	40	32	88
Male : female ratio	23 : 17	7 : 25	53 : 35
Age (years)	55 (29–56)	56 (38–69)	56 (21–74)
Ascites *n* (%)	—	—	53 (73)
Esophageal varices *n* (%)	—	—	47 (53)
Albumin (g/L)	45 (36–57)	37* (29–49)	30* (16–45)
ALT (U/L)	24 (20–28)	28 (24–33)	47** (16–79)
AST (U/L)	27 (23–30)	41 (19–64)	79** (19–150)
Bilirubin (mg/dL)	0.7 (0.6–0.9)	0.92 (0.90–0.95)	1.6* (1.0–3.6)
γGT (U/L)	26 (25–28)	48 (41–56)	92** (78–106)
Creatinine (mg/dL)	0.8 (0.7–1.2)	0.96 (0.9–1.2)	1.2* (0.7–2.4)
Serum sodium (mmol/L)	140 (138–141)	136 (129–138)	130** (129–142)
Mean arterial blood pressure (mmg/Hg)	—	—	83 (76–93)
hs-TnT (ng/L)	—	5.6 (3.0–7.1)	7.9 (3.0–18.5)
AOPPs-albumin (μmol/g)	1.7 (0.8–2.7)	2.1* (0.9–3.0)	2.4* (1.3–5.2)

Continuous variables are expressed as median (interquartile range, IQR) and categorical variables as number (percentage). Statistical significance: $^{*}P < 0.05$; $^{**}P < 0.01$ *versus* healthy controls. ALT: alanine aminotransferase; AOPPs: advanced oxidation protein products; AST: aspartate aminotransferase; hs-TnT: high-sensitive troponin T; INR: international normalised ratio; γGT: gamma glutamyltransferase.

acid- (EDTA-) containing tubes for the determination of plasma renin activity, antidiuretic hormone, and plasma AOPPs or hs-TnT in tubes with no additive for routine biochemical study and aldosterone and inflammatory markers concentrations. All samples were separated immediately by centrifugation at 4°C and stored at −80°C until further analysis.

The consent of the Bioethics Committee of the Wroclaw Medical University was obtained and all patients were informed about the character of the analyses made. Studies were conducted in compliance with the ethical standards formulated in the Helsinki Declaration of 1975 (revised in 1983).

2.2. Determination of Circulating AOPPs. Determination of AOPPs was based on spectrophotometric detection according to Šebeková et al. [22]. Two hundred microliters of plasma diluted 1 : 5 in 20 mM phosphate buffer pH 7.4 containing 0.9% sodium chloride (PBS), or chloramine-T standard solutions (0 to 100 μmol/L), was placed in each well of a 96-well microtiter plate (Becton Dickinson Labware, Lincoln Park, NJ, USA), followed by 20 μL of 10% acetic acid. Ten microliters of 1.16 M potassium iodide (Sigma) were then added, followed by 20 μL of 10% acetic acid. The absorbance of the reaction mixture was immediately read at 340 nm in a microplate reader against a blank containing 200 μL of PBS, 10 μL of KI, and 20 μL of 10% acetic acid. The chloramine-T absorbance at 340 nm was linear within the range of 0 to 100 μmol/L. The ratio of AOPPs concentration to albumin level (AOPPs-albumin) was expressed in micromoles of AOPPs per gram of albumin (μmol/g). The ratio of AOPPs to albumin content allows the evaluation of whether the proportion of oxidatively modified albumin is altered. Coefficient of variation (CV) served as an indicator of precision. Intraday and interday CV values were <10%.

2.3. Laboratory Determinations. Biochemical parameters were measured using routine laboratory methods. Serum high-sensitivity C-reactive protein (hs-CRP) level was determined with a high-sensitivity nephelometric method using the Beckman IMMAGE Immunochemistry System (Beckman Instruments, Fullerton, CA), which has a minimum level of detection of 0.2 mg/L. Serum levels of TNF-α were assayed with enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions. The minimum levels of detection were 1.6 pg/mL for TNF-α. The intra- and interassay coefficients of variation for measurements of hs-CRP and TNF-α were 2.7% and 5.0%, respectively, and 3.0% and 6.9%, respectively.

High-sensitive troponin T was measured using Cobas Troponin T hs (highly sensitive) STAT (short turn-around time) (Roche Diagnostics). The assay working range is reported as 3–10 000 ng/L, with an interassay CV, according to the manufacturer, of 3.1% at 24 ng/L and 1.3% at 300 ng/L. The lower limit of quantification is 13 ng/L, the limit of detection is 5 ng/L, and the limit of blank is 3 ng/L, as listed by the manufacturer.

2.4. Statistical Analysis. Continuous variables are expressed as median [interquartile range (IQR)] and categorical variables as number (percentage). Frequency data were compared using the χ^2 test or Fischer's exact test when necessary. Because many of the variables analyzed did not have a normal distribution as determined by the Kolmogorov-Smirnov test, nonparametric tests were used for comparison of data. The Mann-Whitney *U*-test and the Kruskal-Wallis test were used to analyze differences among two or more groups, respectively. Multivariate analysis by conditional logistical regression with a forward stepwise method was performed to find independent variables associated with the presence of

Table 2: Plasma concentrations of AOPPs-albumin and hs-TnT in cirrhotic patients with chronic HCV infection.

	Healthy controls	Child-Pugh A	Child-Pugh B	Child-Pugh C
(n)	40	34	34	20
Age (years)	55 (29–56)	54 (21–74)	58 (24–71)	56 (29–69)
Albumin (g/L)	45 (36–57)	34^{*} (28–45)	30^{*} (20–40)	25^{*} (16–32)
Bilirubin (mg/dL)	0.7 (0.6–0.9)	1.01 (1.02–1.03)	1.56^{*} (1.0–2.0)	2.15^{*} (1.1–3.6)
INR (0.8–1.1)	—	0.9 (0.8–1.09)	1.2 (1.1–1.3)	2.3^{+} (1.6–2.9)
MELD score (6–8)	—	7 (5–10)	9 (8–13)	14^{++} (10–28)
TNF-α (pg/mL)	25.0 (20.5–30.2)	34.2 (31.6–45.6)	42.0^{*} (37.6–47.2)	58.7^{*++} (48.7–64.0)
hs-CRP (mg/L)	1.05 (0.58–2.5)	3.8^{*} (3.1–7.0)	5.2^{**} (4.9–7.7)	6.3^{**} (5.8–11.0)
hs-TnT (ng/L)	—	5.2 (3.0–6.6)	5.9 (3.0–8.4)	8.6^{+} (3.0–18.5)
AOPPs-albumin (μmol/g)	1.7 (0.8–2.7)	2.8^{*} (1.3–4.4)	3.2^{*} (1.9–4.5)	4.1^{*+} (2.3–5.2)

Continuous variables are expressed as median (interquartile range).
Significance between groups: $^{*}P < 0.05$; $^{**}P < 0.01$ *versus* healthy controls; $^{+}P < 0.05$; $^{++}P < 0.01$ *versus* Child-Pugh A.
AOPPs: advanced oxidation protein products; hs-TnT: high-sensitive troponin T; INR: international normalized ratio; MELD: model of end-stage liver disease.

ascites. Regression analysis to determine significant correlations among different parameters was performed using the Spearman correlation coefficient. Statistical significance was established at $P < 0.05$.

3. Results

3.1. AOPPs-Albumin and hs-TnT Plasma Levels in Patients with Liver Cirrhosis and Healthy Controls. We analyzed 88 cirrhotic patients (53 males/35 females, median age: 56 years, range: 21–74 years) with chronic HCV infection. AOPPs-albumin plasma concentrations were significantly higher in CHC patients without ascites than in healthy controls (controls median 1.7 μmol/g, IQR 0.8–2.7 μmol/g) ($P < 0.05$, Table 1). In healthy controls, the plasma AOPPs-albumin concentrations were similar to those in control groups in other studies [22]. AOPPs-albumin plasma concentration was significantly higher in cirrhotic patients ($n = 88$; median 2.4 μmol/g, IQR 1.3–5.2 μmol/g) compared to CHC patients without cirrhosis ($n = 32$; median 2.1 μmol/g, IQR 0.9–3.0 μmol/g) ($P < 0.05$, Table 1).

The distribution of the stages of liver cirrhosis as defined according to the Child-Pugh score, and measurements of AOPPs-albumin, and hs-TnT concentrations is presented in Table 2. Patients with Child-Pugh class C exhibited significantly higher plasma concentrations of AOPPs-albumin than patients with Child-Pugh class A and controls ($P < 0.05$, $P < 0.01$, resp.). There was a significant difference between Child-Pugh B cirrhotic patients and control subjects with respect to AOPPs-albumin level (Table 2). In CHC patients without cirrhosis, hs-TnT had a median value of 5.6 ng/L (IQR 3.0–7.1 ng/L) (Table 1). Plasma hs-TnT concentrations were higher in Child-Pugh A to Child-Pugh C cirrhotic patients ($n = 88$; median 7.9 ng/L, IQR 3.0–18.5 ng/L) than in patients without cirrhosis, but this difference was not statistically significant (Table 1). hs-TnT plasma concentration was significantly higher in patients with Child-Pugh class C cirrhosis compared to patients with Child-Pugh class A cirrhosis ($P < 0.05$, Table 2). There was statistically significant correlation between hs-TnT levels and the Child-Pugh score in cirrhotic patients ($r = 0.25$, $P < 0.01$, Table 3). AOPPs-albumin positively correlated with the hs-TnT, both when the whole group of cirrhotic patients was evaluated ($r = 0.28$, $P < 0.05$) and when correlation analysis was limited to patients with ascites ($r = 0.35$, $P < 0.01$).

According to an analysis relating AOPPs-albumin and hs-TnT level to the presence of complications of cirrhosis for patients as indicated by the presence of esophageal varices, hyperbilirubinemia, and prolonged INR, there were no significant differences. However, in CHC patients with cirrhosis, AOPPs-albumin correlated inversely with the serum albumin ($r = -0.38$, $P < 0.05$). Significant correlations between AOPPs-albumin and hs-TnT level and MELD scores ($r = 0.43$, $P < 0.001$; $r = 0.31$, $P < 0.001$, resp.) were observed among the cirrhotic patients belonging to all three Child-Pugh classes. In the study group, no significant correlations were also observed between AOPPs-albumin and hs-TnT level and biochemical markers of liver injury (not reported in detail).

We assessed the levels of several inflammatory markers and their association with the levels of AOPPs-albumin and hs-TnT. Serum high-sensitivity C-reactive protein (hs-CRP) levels were significantly elevated in cirrhotic patients (Table 2). Serum TNF-α levels were higher in the Child-Pugh class C cirrhosis than in the Child-Pugh class A cirrhosis ($P < 0.05$, Table 2). Moreover, TNF-α concentrations were positively correlated with Child-Pugh score in cirrhotic patients ($r = 0.31$, $P < 0.05$). There was no statistically significant correlation between AOPPs-albumin and hs-TnT level and hs-CRP or TNF-α levels in all liver cirrhotic patients (data not shown).

3.2. Clinical and Biochemical Characteristics of Patients with Liver Cirrhosis according to the Presence of Ascites. The biochemical and clinical characteristics of cirrhotic patients both with and without ascites are shown in Table 3. Distribution of sex was similar among groups. By design, creatinine, aldosterone levels, and plasma renin activity were higher in cirrhotic patients with ascites than in patients without

Table 3: Clinical and biochemical characteristics of patients with liver cirrhosis according to the presence of ascites.

	Healthy controls	No ascites	Ascites
(*n*)	40	35	53
Age (years)	55 (29–56)	51 (21–74)	58 (24–70)
Esophageal varices *n* (%)	—	17 (49)	39 (74)
Refractory ascites *n* (%)	—	—	11 (21)
Child-Pugh score	—	6 (4–7)	$9\ (8–10)^{++}$
Creatinine (mg/dL)	0.8 (0.7–1.0)	0.9 (0.7–1.4)	1.32^{***+++} (0.8–2.4)
Albumin (g/L)	45 (36–57)	34^{*} (28–45)	29^{*} (16–40)
MAP (mmHg)	—	88 (85–93)	77^{+} (73–89)
Plasma renin activity ($ng\,mL^{-1}\,h^{-1}$)	—	0.48 (0.13–1.4)	1.9^{++} (0.95–7.6)
Aldosterone (ng/dL)	—	13.2 (5.5–20.9)	33.0^{+++} (13.7–52.2)
Antidiuretic hormone (pg/mL)	—	4.6 (2.5–5.7)	4.5 (3.6–6.4)
TNF-α (pg/mL)	25.0 (25.5–30.2)	32.3 (31.6–45.6)	55.0^{***+} (37.6–64.0)
hs-CRP (mg/L)	1.05 (0.58–2.5)	3.8^{*} (3.1–7.0)	5.9^{**} (4.9–11.0)
hs-TnT (ng/L)	—	5.1 (3.0–6.6)	10.7^{++} (3.0–18.5)
AOPPs-albumin (μmol/g)	1.7 (0.8–2.7)	2.2^{*} (1.3–4.4)	3.6^{**+} (1.9–5.2)

Continuous variables are expressed as median (interquartile range: IQR) and categorical variables as number (percentage). Significance between groups $^{+}P < 0.05$; $^{++}P < 0.01$, $^{+++}P < 0.001$ *versus* cirrhosis without ascites. AOPPs: advanced oxidation protein products; hs-TnT: high-sensitive troponin T; MAP: mean arterial pressure.

ascites. However, similar values of antidiuretic hormone were detected in all patients grouped according to the presence of ascites. CHC patients with ascites had significantly lower values of mean arterial pressure (MAP) when compared with CHC patients without ascites ($P < 0.05$).

AOPPs-albumin levels were significantly different between nonascites and CHC patients with ascites ($n = 53$; median 3.6 μmol/g, IQR 1.9–5.2 μmol/g) (Table 3). The association study revealed negative correlation between AOPPs-albumin levels and MAP in all cirrhotic patients ($r = -0.27$, $P < 0.05$).

Plasma hs-TnT levels in ascites group were significantly increased compared with those in nonascites group ($P < 0.01$, Table 3). Additionally, hs-TnT and AOPPs-albumin level in refractory ascites ($n = 11$) was significantly increased compared with the nonascites group [median 12.9 ng/L (IQR 8.4–18.5 ng/L), median 4.3 μmol/g (IQR 3.3–5.2 μmol/g), resp.]; $P < 0.001$.

The median TNF-α levels were higher in CHC patients with ascites than patients without ascites (55.0 pg/mL versus 32.3 pg/mL, $P < 0.01$) (Table 3). There was also no significant difference between serum TNF-α levels in patients with the elevated levels of circulating AOPPs-albumin and levels in patients with the elevated levels of hs-TnT (data not shown).

hs-CRP plasma levels were increased in the nonascites and ascites group, with higher levels in the latter (Table 2). Serum hs-CRP levels were variable and no correlation with the hs-TnT was found ($P > 0.05$).

3.3. Multiple Regression Analysis. Based on stepwise multiple logistic regression analysis of factors (Child-Pugh classification, MELD score, bilirubin, albumin, creatinine, low serum sodium concentration, INR, MAP AOPPs-albumin, hs-TnT, and TNF-α), plasma AOPPs-albumin level [odds ratio (OR) = 0.19, 95% confidence interval (CI) = 0.21–0.44, $P < 0.001$], hs-TnT level [odds ratio (OR) = 0.11, 95% confidence interval (CI) = 0.17–0.61, $P < 0.001$], and creatinine level (OR = 0.64, 95% CI = 0.02–0.03, $P < 0.001$) were found to be independent predictors of ascites (Table 4).

Table 4: Multiple logistic regression analysis of factors associated with the presence of ascites.

	OR	95% CI	*P* value
AOPPs-albumin level (μmol/g)	0.19	0.21–0.44	0.001
hs-TnT level	0.11	0.11–0.61	0.01
Creatinine level (mg/dL)	0.64	0.02–0.03	0.001

AOPPs: advanced oxidation protein products; hs-TnT: high-sensitive troponin T.

4. Discussion

In the present study, we investigated the relationship between AOPPs-albumin and myocardial injury by comparing the selected biomarker for detecting cardiac injury (hs-TnT) in patients with chronic HCV infection showing high levels of AOPPs-albumin in the presence of high serum TNF-α levels. The main findings are as follows. (1) Compared with healthy controls, the cirrhotic patients had higher levels of AOPPs-albumin, which were associated with increased hs-TnT. (2) When the presence of ascites was considered, the plasma levels of AOPPs-albumin were higher, as well as TNF-α. (3) AOPPs-albumin positively correlated with hs-TnT level in all cirrhotic patients with chronic HCV infection and this correlation was stronger in decompensated cirrhotic patients. (4) In multivariate logistic regression analysis, the independent factors associated with the presence of ascites were high AOPPs levels and elevated hs-TnT levels.

The associations between HCV infection and cardiovascular diseases are supported by a robust body of evidence [2,

4, 23]. It is therefore possible that myocardial inflammation or virus persistence, or both, may cause an asymmetrical septal hypertrophy enabling the occurrence of hypertrophic cardiomyopathy [24]. Myocardial hypertrophy and remodeling are pathological features of many cardiac diseases, with the underlying causes including cirrhotic cardiomyopathy in patients with chronic HCV infection. This syndrome designates a cardiac dysfunction that includes the macroscopic structural changes, systolic and/or diastolic dysfunction, and electrophysiological changes [6]. In the present study, elevated plasma concentrations of hs-TnT were associated with increases in the concentrations of AOPPs-albumin and TNF-α in cirrhotic patients with chronic HCV infection. Although these biochemical substances are known to be elevated in patients with heart failure whose prognosis is poor [25–27], there have been several reports suggesting they induce cardiomyocyte apoptosis, a critical component in the pathogenesis of heart failure [28]. Elevated hs-TnT serum levels were associated with the severity of hypertrophic cardiomyopathy, indicating that the hs-TnT levels could be a reliable indicator of subclinical ongoing myocyte damage [10]. In fact, high-sensitive assay methods for cardiac troponins are nowadays under an intense development since cardiac troponins are biomarkers of cardiomyocyte apoptosis [11], remodelling processes, or increased physiological cell turnover occurring in different etiologic origins of cardiac injury [10]. Very recently, Valente et al. [16] first elucidated the role of AOPPs in proapoptotic signaling in cardiomyocytes. These studies concluded that AOPPs-modified mouse serum albumin induces cell death in both neonatal and adult mouse cardiomyocytes, and this effect is mediated *via* RAGE. AOPPs-induced Nox2/Rac1-dependent superoxide generation and increased TRAF3 interacting protein 2 (TRAF3IP2) expression and TRAF3IP2-dependent c-Jun N-terminal kinase (JNK) activation. Further, AOPPs-MSA induced mitochondrial Bax translocation and release of cytochrome c into cytoplasm. Moreover, AOPPs-MSA suppressed antiapoptotic Bcl-2 and Bcl-xL expression. Knockdown of the adapter protein TRAF3IP2 blunted AOPPs-induced apoptosis in both neonatal and adult cardiomyocytes [16]. The ability of the appropriate stimulus to drive cardiomyocytes into apoptosis indicated that these cells were primed for apoptosis and were susceptible to clinically relevant apoptotic triggers, such as AOPPs. Our preliminary results are the first to show a correlation between marker of cardiomyocyte injury (hs-TnT) and circulating AOPPs-albumin in CHC patients with cirrhosis. These results further demonstrate that the myocardial troponin T release was dependent on the AOPPs-albumin elevation because, in cirrhotic patients with normal AOPPs-albumin levels, detectable hs-TnT concentration was not significantly elevated in comparison with patients with chronic HCV infection with normal AOPPs-albumin levels (not shown). Accordingly, there are reasons to believe that the cirrhotic myocardium may be more vulnerable to the deleterious effects of elevated AOPPs.

Most cirrhotic patients remain asymptomatic until the occurrence of decompensation characterized by ascites. The changes in cardiac function during cirrhotic cardiomyopathy are more evident in decompensated cirrhosis, and TNF-α is key factor in the signaling pathways regulating myocardial dysfunction. Studies, in fact, show that TNF-α induces apoptosis in cardiomyocytes [29, 30], suppresses cardiac contractility [31, 32], and provokes myocardial hypertrophy [33, 34]. Very recently, Che et al. [35] reported the possible association between diastolic dysfunction and inflammation reflected by serum TNF-α levels in patients with HCV infection. The other study assessing the role of NF-κB in mediating systolic dysfunction in cirrhosis showed an improvement of diastolic relaxation in cardiomyocytes when its inhibitors blocked NF-κB activity, with reduction in TNF-α expression [36]. This suggests that an inflammatory milieu, with increased TNF-α levels, may also be partly responsible for the diastolic dysfunction in cirrhosis, but the exact mechanism with which TNF-α affects diastolic dysfunction has not been elucidated. Increased TNF-α level, along with hs-TnT levels in cirrhotic patients, especially higher values in patients with ascites, was observed in the present study. Since TNF-α is associated with diastolic dysfunction, it is likely to affect the progress of myocardial injury in patients with advanced liver failure. However, in the present study, there was no significant correlation between serum TNF-α levels and increased plasma levels of hs-TnT in cirrhotic patients. These data suggest that, although TNF-α might contribute to the heart failure in CHC patients with cirrhosis, especially with advanced disease, other factors acting through different pathways probably exist.

Elevated levels of both AOPPs-albumin and hs-TnT were detected in the early stages of liver dysfunction: plasma concentrations were increased in patients in Child-Pugh class A, with higher values found in those in class B or C. Furthermore, circulating levels of both AOPPs-albumin and hs-TnT were associated with disease severity by significant relations to the MELD score ($r = 0.43$, $P < 0.001$; $r = 0.31$, $P < 0.001$, resp.). This is in keeping with previously reported data [12, 37]. Our results also showed that hs-TnT levels in all cirrhotic patients were independently associated with the presence of ascites and showed significant correlations with AOPPs levels. Additionally, increased circulating AOPPs-albumin level, along with hs-TnT levels in decompensated cirrhotic patients, especially higher values in refractory ascites group, were observed in this study. While our data must be interpreted cautiously because they are of a cross-sectional nature, our findings are consistent with those of Wiese et al. showing a relation between cardiac dysfunction and development of refractory ascites [12]. Finally, the values of both AOPPs-albumin and hs-TnT in CHC patients with a low Child-Pugh score and absence of ascites suggest that AOPPs might have a role in the late stages of cirrhosis by aggravating the already initiated cardiac dysfunction.

Analysis of plasma hs-TnT levels in cirrhotic patients has to deal with the presence of impaired renal function as a possible confounder. It has been suggested that especially in subjects with impaired renal function TnT may be falsely elevated. Reduced clearance of troponin molecules or their fragments by dysfunctional kidneys may explain persistently elevated circulating concentrations of troponin in patients with chronic heart failure [38], but this hypothesis is controversial [39]. Rather, elevated troponin levels most

likely indicate myocardial injury [39–41]. It has recently been shown that renal excretion of TnT is significantly impaired in patients with alcohol-related cirrhosis, and this may at least in part explain elevated plasma levels in these patients [12]. In contrast, in the present study, elevated serum levels of creatinine were not significantly correlated with increased levels of hs-TnT (not shown). Our data agree with other studies [40, 42] showing that the clearance of cardiac troponins is not changed by renal failure. The results of the measurements of troponins renal clearance might depend on which of the epitopes that are detected by individual assays and by the degradation of troponin.

Our study has a number of limitations that merit consideration. First, our sample size is relatively small, and a larger cohort of cirrhotic patients with HCV infection will need to be examined to confirm our findings. Second, interpreting the present data is limited by the small number of the patients studied, resulting in a limited statistical power. Third, the limit of the present study was the unavailability of brain natriuretic peptide and probrain natriuretic peptide, which are also recognized to be important in the evaluation of diastolic dysfunction. Lastly, we did not perform serial measurements and only focused on baseline values. Accordingly, our cross-sectional study design does not permit any conclusions on causality.

In conclusion, results from this study show that elevated levels of both AOPPs and hs-TnT are common in cirrhotic patients with HCV infection. Moreover, the present results provide new evidence for an association between plasma levels of AOPPs and hs-TnT, a specific marker of myocardial injury, in patients with decompensated cirrhosis. Although it does not prove causal relationship, it might strengthen the hypothesis that AOPPs are associated with cardiac disease in cirrhotic patients with HCV infection. Further investigation will be needed to elucidate the mechanisms underlying the regulation of advanced oxidation protein products, hs-TnT and other cardiovascular markers in cirrhotic patients with hepatitis C virus (HCV) infection.

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

Transcriptome and Molecular Endocrinology Aspects of Epicardial Adipose Tissue in Cardiovascular Diseases: A Systematic Review and Meta-Analysis of Observational Studies

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The objective of this study was to perform a systematic review of published literature on differentially expressed genes (DEGs) in human epicardial adipose tissue (EAT) to identify molecules associated with CVDs. A systematic literature search was conducted in PubMed, SCOPUS, and ISI Web of Science literature databases for papers published before October 2014 that addressed EAT genes and cardiovascular diseases (CVDs). We included original papers that had performed gene expressions in EAT of patients undergoing open-heart surgery. The Reporting Recommendations for Tumor Marker Prognostic Studies (PRIMARK) assessment tool was also used for methodological quality assessment. From the 180 papers identified by our initial search strategy, 40 studies met the inclusion criteria and presented DEGs in EAT samples from patients with and without CVDs. The included studies reported 42 DEGs identified through comparison of EAT-specific gene expression in patients with and without CVDs. Among the 42 DEGs, genes involved in regulating apoptosis had higher enrichment scores. Notably, interleukin-6 (IL-6) and tumor protein p53 (TP53) were the main hub genes in the network. The results suggest that regulation of apoptosis in EAT is critical for CVD development. Moreover, IL-6 and TP53 as hub genes could serve as biomarkers and therapeutic targets for CVDs.

1. Introduction

Among noncommunicable diseases, cardiovascular diseases (CVDs) are a major contributor to total global mortality and will continue to rise in the future. Thus, early detection of CVDs is critical for reducing the mortality and economic burden of this disease. Moreover, improving the understanding of the etiology associated with CVDs is highly important.

Over the last few decades, the pathophysiological concept of visceral adipose tissue has become an accepted indicator for CVD risk. Visceral adipose tissue is a metabolically active tissue that is highly involved in regulating different specific metabolic processes, including lipid metabolism, glucose homeostasis, angiogenesis, hemostasis, and blood pressure as well as the modulation of inflammation responses [1–5]. Recent evidence suggests that epicardial adipose tissue (EAT) as an index of cardiac visceral adiposity plays an essential role in cardiac morphology and function [6, 7]. EAT exists in the fat layer between the myocardium and visceral pericardium. Epicardial fat deposits are situated predominantly on the right-ventricular free wall and the left-ventricular apex but can also be directly located within the myocardium or around the coronary artery adventitia [8]. Anatomically, these fat deposits are not separated from the underlying myocardium.

Studies have shown that EAT generates a variety of bioactive molecules, such as pro- and anti-inflammatory mediators and cytokines [9], which may significantly enhance paracrine effects on cardiac function or produce systemic effects that affect many physiological processes [10].

A growing body of research on EAT has focused mainly on target genes at the transcriptome level. These studies identified numerous differentially expressed genes in EAT that are associated with cardiovascular and metabolic risk factors. However, only a small number of these genes represent efficient biomarkers and therapeutic targets [10–12]. Nonetheless, molecular knowledge based on tissue-specific gene expression profiles is helpful for understanding many aspects of the pathogenic mechanisms of CVDs and cardiometabolic components as well as identifying tissue structures that may serve as potential targets for treating CVD.

In this study we conducted a systematic review of published gene expression studies on EAT that compared differentially expressed genes (DEGs) between patients with and without cardiometabolic risk factors for CVDs, especially coronary artery disease (CAD).

2. Methods

2.1. Search Strategy. Electronic searches in PubMed, Scopus, and ISI Web of Knowledge literature databases were performed by two investigators (Arash Hossein-nezhad and Zhila Maghbooli). The databases were searched for all relevant published studies published before October 18, 2014, using the search terms (TITLE-ABS-KEY ("epicardial adipose tissue") OR TITLE-ABS-KEY ("epicardial fat")) AND (TITLE-ABS-KEY (RT-qPCR) OR TITLE-ABS-KEY (real-time PCR) OR TITLE-ABS-KEY (real time PCR) OR TITLE-ABS-KEY (microarray) OR TITLE-ABS-KEY (gene expression profile) OR TITLE-ABS-KEY (gene expression) OR TITLE-ABS-KEY (transcriptome)). The first search was not restricted to human, animal, or experimental studies. Studies that analyzed EAT gene expression in humans were then selected.

2.2. Study Selection. The criteria for considering studies for inclusion were formalized in an inclusion criteria form (S1 Appendix a) (see Supplementary Material available online at http://dx.doi.org/10.1155/2015/926567). Two investigators (Arash Hossein-nezhad and Zhila Maghbooli) independently examined the titles and abstracts of the identified studies. If study eligibility was unclear from the abstract, then the full text of the paper was retrieved and independently evaluated by the assessors. Any disagreement about inclusion was resolved by discussion.

Eligible studies included in this review had the following criteria: human subjects undergoing open-heart surgery, differential gene expression, increase or decrease in differential expression or fold change in EAT, description of specific genes, and reliable definition of diseases and classification. Studies that did not meet one or more of the eligibility criteria were excluded. The studies were not limited to any language.

2.3. Data Extraction. Two investigators (Zhila Maghbooli and Arash Hossein-nezhad) independently extracted the data using a standardized form (S1 Appendix b). The form was pilot-tested on three studies to identify and reduce misinterpretations. The following topics were recorded from the included studies: author name, year of publication, study design, population (health/disease status, setting, sample size, age, and sex), phenotype (cardiovascular diseases: CAD, ischemic heart disease, and heart failure, and cardiometabolic risk factors: hypertension, insulin resistance, diabetes, and metabolic syndrome), clinical subtypes of interest, case and control definition, diagnostic criteria for CVDs and cardiometabolic risk factors, details on sampling and RNA preparation, and details of the statistical analysis used.

2.4. Quality Assessment. The methodological quality of included papers was assessed using the PRIMARK (Reporting Recommendations for Tumor Marker Prognostic Studies) assessment tool. We used 11 items included with the PRIMARK tool (S1 Appendix c). The study quality was based on the information reported in the papers and was simultaneously and independently assessed by two investigators (Zhila Maghbooli and Arash Hossein-nezhad) in the data extraction phase.

2.5. Differentially Expressed Gene (DEG) Analysis. From each selected paper, we extracted the published DEGs and selected the official gene symbol as the gene identifier. If only an alias name was given, then we used NCBI to obtain the official gene symbol provided by HUNC (HUGO Gene Nomenclature Committee). All included studies compared EAT gene expression between CVDs and/or cardiometabolic risk factors and control samples. Only those DEGs with a fold change value >1.5 and a p value < 0.05 were selected. For multiple testing corrections, we used the false discovery rate (FDR) [13, 14]. We performed further analyses on those genes that were identified as differentially expressed with a FDR < 0.05.

2.6. Enrichment and Functional Annotation. Enrichment and functional annotation analyses were performed using DAVID (the database for annotation, visualization, and integrated discovery, http://david.abcc.ncifcrf.gov/home.jsp), which is a web-accessible program aimed at systematically extracting biological meaning from large lists of genes [15]. In the present study, overrepresented Gene Ontology (GO) and functional annotation were detected with a value of $p < 0.05$ and an enrichment score ≥ 1. We selected the top ten significantly enriched GO terms.

2.7. Protein-Protein Interaction (PPI) Network Construction and Pathway Analyses. To demonstrate potential PPI networks, DEGs were mapped to the PPI data via the STRING database v.9.1 (Search Tool for the Retrieval of Interacting Genes) (http://www.stringdb.org/) [16]. The STRING database takes a meta-analysis approach toward protein-protein association information and identifies functional links between proteins. We constructed an

extended network based on a high confidence score of 0.9, which implies that only interactions with a high level of confidence were extracted from the database and considered as valid links for the PPI network. Subcluster analysis was performed by K-means clustering. Indeed, STRING was previously used to identify significant KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways (http://www.genome.jp/kegg/pathway.html).

3. Results

The systematic search identified 180 publications. Duplicate titles ($N = 94$) were removed and the title and abstract of the remaining 86 papers were screened based on inclusion and exclusion criteria. Of these, 35 studies did not meet the inclusion criteria as described: (i) 17 studies were not conducted in humans, (ii) 12 were clearly unrelated titles, (iii) 4 did not utilize gene expression profile assays, (iv) 2 did provide data on isolated adipocytes and stromal cells in EAT deposits, and (v) 3 studies were excluded for other reasons (one duplicate published article and related abstract; two studies did not provide original data). The full text of the remaining titles was then examined in more detail. Three new studies were identified from reference lists included in the eligible studies. All selected studies ($N = 51$) determined the mRNA expression in EAT obtained from patients undergoing elective heart surgery for either coronary artery bypass grafting or valve surgery. Of these, 40 studies reported DEGs in EAT samples from patients with and without CVDs [17–56] and 11 studies had a self-control design and only compared DEGs between epicardial and paired subcutaneous/abdominal adipose tissue samples [9, 57–65]. Finally, we included 40 studies in our analysis that compared EAT gene expression between patients with and without various CVDs, especially CAD and cardiometabolic risk factors (i.e., hypertension, insulin resistance, diabetes, and metabolic syndrome) (Figure 1).

3.1. Quality Assessment. Study characteristics and gene panels as described in the original papers were applied for quality assessment (Supplemental Table S1). Most studies adequately reported an acceptable definition of CVDs and cardiometabolic risk factors, the type and location of tissue sampling, RNA storage and isolation conditions, and the expression detection methods used. Most samples were stored in liquid nitrogen or at −80°C, and most studies used either RNeasy (Qiagen) or Trizol for RNA isolation. The majority of studies used a case-control design with an appropriate control group and adequately reported the characteristics of the case and control (e.g., age, sex, BMI, CAD severity, taking drugs, and cardiometabolic risk factors). Two studies did not report the results of DEGs despite collecting EAT from CAD and non-CAD patients and using a case-control study design [19, 44]. Several studies determined protein expression levels in EAT tissue in addition to the mRNA expression levels [17, 29, 32, 34, 39, 40, 46, 53], while some others investigated the correlation of EAT mRNA expression with CAD markers such as C-reactive protein (CRP) [32, 33]. The major obstacle in the quality assessment was the diversity in statistical procedures used to analyze the DEGs (normalization and analysis methods).

3.2. Identification of DEGs in EAT: Level of Association Evidence. All included studies used a candidate gene approach, except for three studies that used array-based gene expression methods and focused on only one or a few genes [25–27]. The included papers reported 112 genes as corresponding to cardiovascular dysfunction or metabolic syndrome (S2 Table). Among 112 genes, 32 were identified in at least two studies. The DEGs in patients with and without CVDs and/or cardiometabolic risk factors were identified. A total of 53 genes were selected as DEGs in EAT samples from patients with and without CVDs with a fold change >1.5 and p value < 0.05. All DEGs showed a consistent direction of expression change, except for PR Domain Containing 16 (PRDM16) and adrenomedullin (ADM) RNA expression levels, which had inconsistent changes in expression direction. Next, the resulting p values were corrected for multiple hypothesis testing by calculating the false discovery rate (FDR) with a cut-off of 0.05. There were forty-two genes that had a FDR < 0.05.

3.3. Gene Enrichment and Functional Annotation Analysis. To investigate the functional role of DEGs in EAT, the DEGs were mapped with DAVID. Among annotation clusters, *regulation of apoptosis, regulation of transcription factor activity*, and *regulation of systemic arterial blood pressure mediated by a chemical signal* had higher enrichment scores (enrichment score: 8.35, 6.51, 5.80, and 5.11, resp.). The top ten annotation clusters are shown in Table 1.

3.4. Protein-Protein Interaction Network and Pathway Analysis. To identify hub genes, a protein-protein interaction (PPI) network was constructed using the STRING database. In the network, each edge is examined by a score as the edge weight to quantify the interaction confidence. We projected DEGs with a FDR < 0.05 (42 genes) as inputs into the search tool (STRING) to determine the molecular network of interacting genes and obtain correlations with a high probability confidence score of ≥0.9 with a genome background. The results were significantly enriched in a network ($p = 1.8 * 10^{-6}$) with 52 interactions. Notably, interleukin-6 (IL-6) and tumor protein p53 (TP53) were the main nodes in the network (Figure 2). In addition, the network was further clustered using K-means clustering. Subsequent clustering identified at least three different functional clusters (Figure 3).

To gain insight into the biological processes of DEGs in EAT we used the STRING dataset. The first three biological processes were *response to external stimulus* (FDR = 1.51×10^{-14}), *aging* (FDR = 1.85×10^{-12}), and *response to activity* (FDR = 2.48×10^{-10}) (Table 2).

To construct the PPI network, PPI data were obtained from the STRING database and significant enrichment of the DEGs in multiple KEGG terms was detected. The most significantly enriched pathways of DEGs were *HIF-1 signaling pathway* (FDR = 1.82×10^{-7}), *TNF signaling pathway*

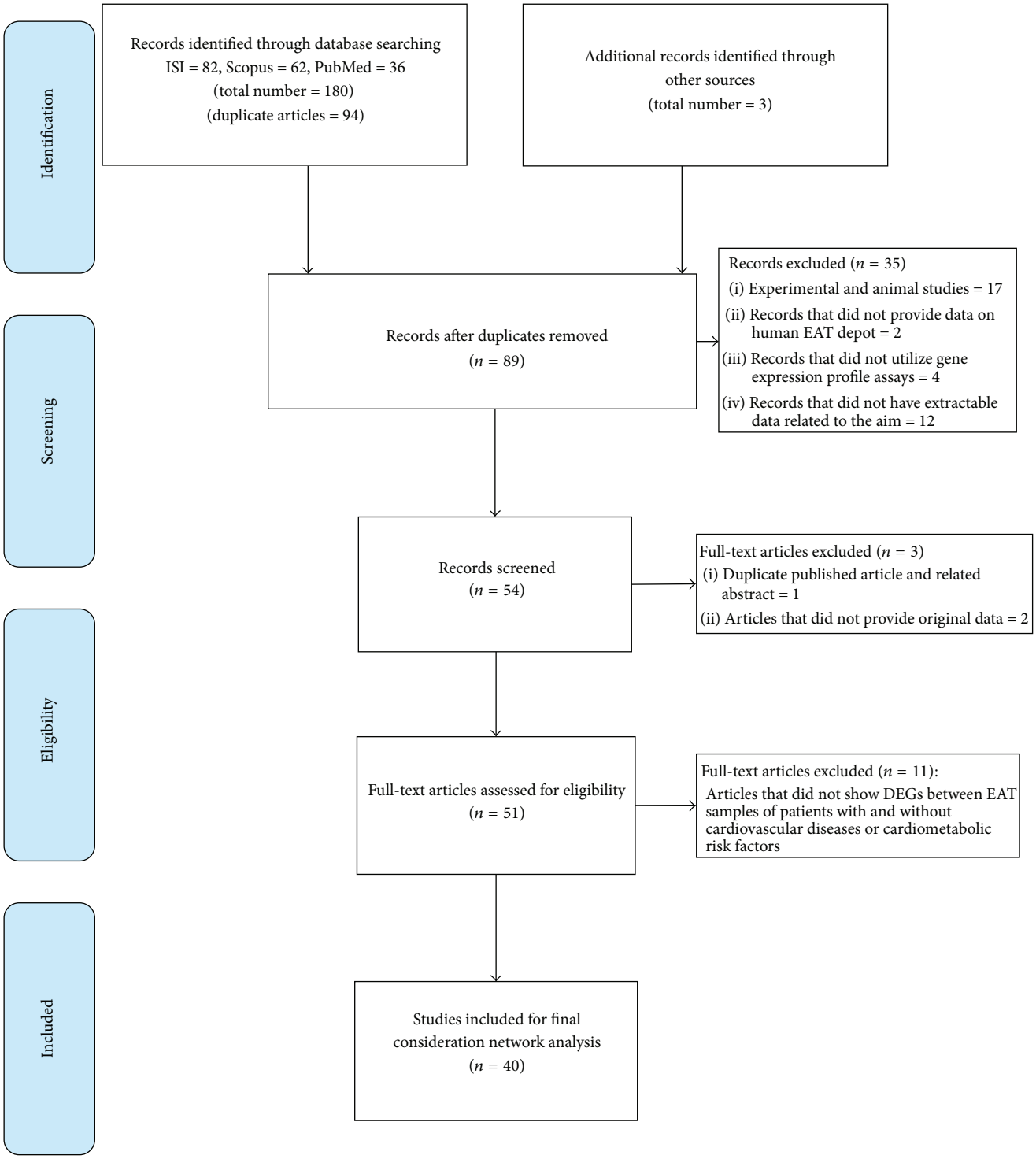

Figure 1: Flowchart of study selection process.

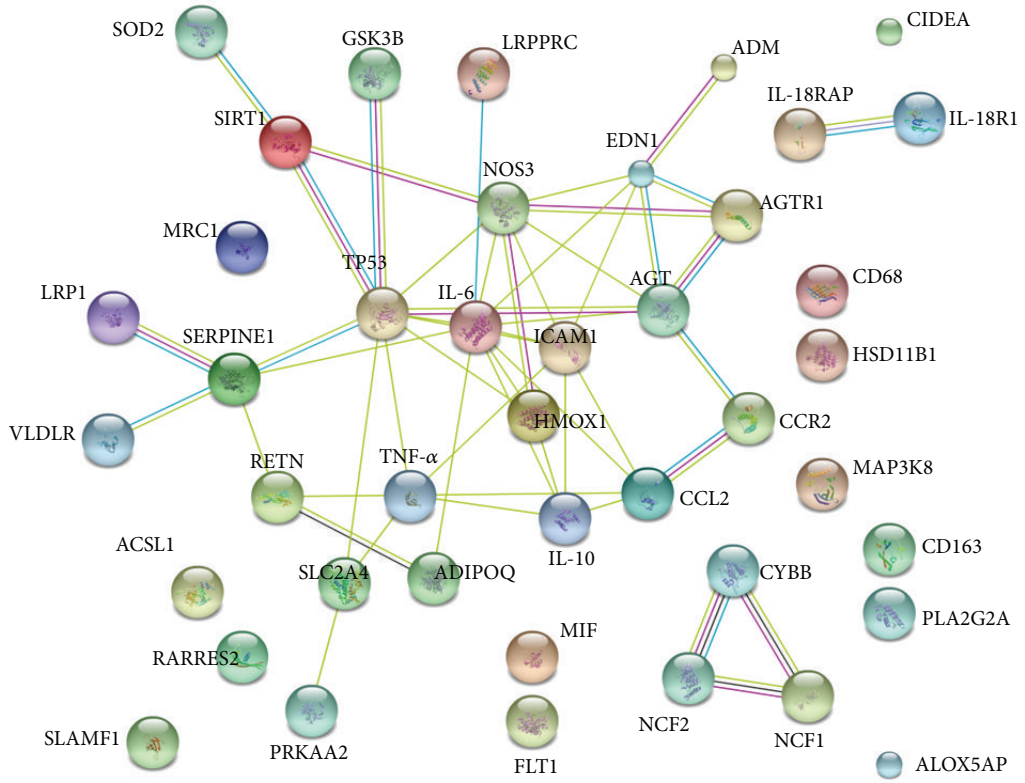

Figure 2: Protein-protein interaction (PPI) network constructed of differentially expressed genes (DEGs) identified in EAT samples. Forty-two DEGs were analyzed using the STRING database. IL-6 and TP53 were found to be the main hub genes.

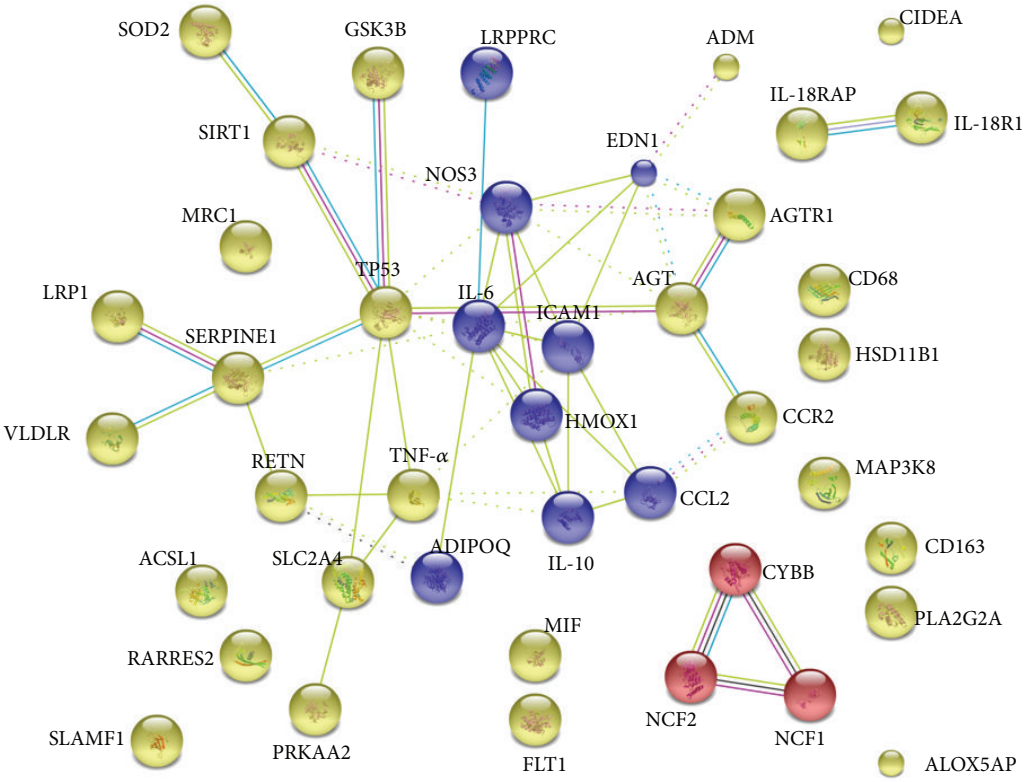

Figure 3: Subnetwork clusters identified from the PPI network. The resulting networks were clustered using K-means and confirmed IL-6 and TP53 as hub genes. Different line colors represent the types of evidence for the association.

Table 1: Gene enrichment and functional annotation analysis (top ten significantly enriched GO terms with a high count of DEGs in the EAT samples).

GO_id	Functional term	Enrichment score	p value	FDR p value
0043066	Negative regulation of apoptosis	8.356348318786127	3.95×10^{-9}	6.51×10^{-6}
0042981	Regulation of apoptosis	6.511095210354866	2.82×10^{-7}	4.64×10^{-4}
0051090	Regulation of transcription factor activity	5.808458303615781	5.23×10^{-7}	8.62×10^{-4}
0003044	Regulation of systemic arterial blood pressure mediated by a chemical signal	5.116925647742479	1.09×10^{-6}	0.001802
0006873	Cellular ion homeostasis	4.704792812343122	1.12×10^{-5}	0.018473
0051223	Regulation of protein transport	4.519156948056949	2.02×10^{-5}	0.033305
0051091	Positive regulation of transcription factor activity	4.309175470478401	2.84×10^{-5}	0.046671
0030335	Positive regulation of cell migration	3.766691351047639	1.33×10^{-4}	0.219528
0030334	Regulation of cell migration	3.7019715102579265	1.32×10^{-4}	0.21785
0016477	Cell migration	3.40004510443413	1.46×10^{-4}	0.240623

Table 2: The GO biological processes enriched for the proteins present in the STRING protein network.

GO_id	Term	Genes in test set	Number of genes	p value	FDR p value
GO:0009605	Response to external stimulus	TP53; NCF2; SOD2; ADIPOQ; ICAM1; PRKAA2; NCF1; HMOX1; FLT1; MIF; AGT; ADM; RETN; ACSL1; RARRES2; SERPINE1; GSK3B; AGTR1; NOS3; CYBB; EDN1; IL-10; LRP1; MRC1; TNF-α; PLA2G2A	26	1.12×10^{-18}	1.51×10^{-14}
GO:0007568	Aging	IL-10; AGT; ADM; LRP1; IL-6; ICAM1; RETN; SIRT1; NCF2; SERPINE1; MIF; CCL2; EDN1;	13	2.75×10^{-16}	1.85×10^{-12}
GO:0014823	Response to activity	IL-10; NCF2; AGT; SOD2; ADIPOQ; TNF-α; EDN1; CCL2	8	5.53×10^{-14}	2.48×10^{-10}
GO:0071216	Cellular response to biotic stimulus	IL-10; SERPINE1; TNF-α; GSK3B; IL-6; ICAM1; CCL2; TP53; MRC1; NOS3	10	1.09×10^{-13}	3.68×10^{-10}
GO:0032496	Response to lipopolysaccharide	IL-10; ADM; ICAM1; MRC1; NOS3; NCF2; SERPINE1; TNF-α; CCL2; SOD2; EDN1	11	1.43×10^{-12}	3.85×10^{-9}
GO:0002237	Response to molecule of bacterial origin	IL-10; ADM; ICAM1; MRC1; NOS3; SERPINE1; NCF2; TNF-α; CCL2; SOD2; EDN1	11	2.33×10^{-12}	5.23×10^{-9}
GO:0070482	Response to oxygen levels	ADM; ICAM1; TP53; NCF2; SIRT1; TNF-α; CCL2; SOD2; SLC2A4; ADIPOQ; EDN1	11	3.42×10^{-12}	6.57×10^{-09}
GO:0023057	Negative regulation of signaling	TP53; NOS3; CIDEA; SOD2; ADIPOQ; EDN1; IL-10; LRP1; ICAM1; PRKAA2; TNF-α; HMOX1; MIF; AGT; ADM; IL-6; SERPINE1	17	4.07×10^{-12}	6.79×10^{-9}
GO:0010648	Negative regulation of cell communication	TP53; NOS3; CIDEA; SOD2; ADIPOQ; EDN1; IL-10; LRP1; ICAM1; PRKAA2; TNF-α; HMOX1; MIF; AGT; ADM; IL-6; SERPINE1	17	4.54×10^{-12}	6.79×10^{-9}
GO:0045428	Regulation of nitric oxide biosynthetic process	IL-10; AGT; SOD2; TNF-α; EDN1; IL-6; ICAM1	7	6.29×10^{-12}	8.45×10^{-9}

(FDR = 1.82×10^{-7}), and *cytokine-cytokine receptor interaction* (FDR = 3.56×10^{-6}) (Table 3).

4. Discussion

Despite recent interventions to reduce cardiovascular risks, CVDs, especially CAD, remain the primary cause of death worldwide [66]. Emerging early detection biomarkers or nontraditional risk factors may have a potential role in providing new approaches to develop therapeutic targets for CAD and related risk factors. Epicardial adipose tissue (EAT) is an index of cardiac visceral adiposity and displays high metabolic activity [67]. EAT is known to secrete various bioactive proteins that contribute to local function of coronary vessels and the myocardium and/or have systemic endocrine effects on vascular tissue [68]. Both clinical and epidemiological studies have found an association between EAT and cardiometabolic risk factors and the progression of atherogenesis [48, 69], while EAT volume can act as

Table 3: Top ten enriched KEGG pathway of DEGs in EAT samples from patients with and without cardiovascular diseases.

GO id	Functional description KEGG ID	Genes in test set	Number of genes	p value	FDR p value
4066	HIF-1 signaling pathway	SERPINE1, HMOX1, IL-6, FLT1, CYBB, EDN1, NOS3	7	1.56×10^{-9}	1.82×10^{-7}
4668	TNF signaling pathway	MAP3K8, IL-18R1, TNF-α, IL-6, ICAM1, CCL2, EDN1	7	1.91×10^{-9}	1.82×10^{-7}
4060	Cytokine-cytokine receptor interaction	IL-18RAP, IL-10, IL-6, CCR2, IL-18R1, TNF-α, FLT1, CCL2	8	4.97×10^{-8}	3.56×10^{-6}
4068	FoxO signaling pathway	IL-10, SIRT1, IL-6, PRKAA2, SOD2, SLC2A4	6	1.59×10^{-7}	8.1×10^{-6}
5321	Inflammatory bowel disease (IBD)	IL-18RAP, IL-10, IL-18R1, TNF-α, IL-6	5	1.69×10^{-7}	8.1×10^{-6}
4920	Adipocytokine signaling pathway	ACSL1, SLC2A4, ADIPOQ, TNF-α, PRKAA2	5	2.51×10^{-7}	1.03×10^{-5}
5143	African trypanosomiasis	IL-6, IL-10, ICAM1, TNF-α	4	6.01×10^{-7}	2.16×10^{-5}
5323	Rheumatoid arthritis	TNF-α, IL-6, ICAM1, FLT1, CCL2	5	8.29×10^{-7}	2.64×10^{-5}
5142	Chagas disease (American trypanosomiasis)	IL-10, SERPINE1, TNF-α, IL-6, CCL2	5	1.52×10^{-6}	4.36×10^{-5}
5140	Leishmaniasis	IL-10, NCF2, TNF-α, NCF1	4	1.2×10^{-5}	2.94×10^{-4}

a significant CAD predictor [48]. Therefore, identification of genes that are differentially expressed in EAT is critically important to understand the molecular mediators of CVD and to develop effective disease management strategies. Given that different gene expression studies used different candidate genes in EAT and different processing methods (statistical procedures), there is a lack of comprehensive information on the biological role of EAT in cardiovascular disease risk.

Our systematic review of studies focused on EAT and provides an overview of EAT DEGs in CVDs, especially CAD, and/or cardiometabolic risk factors. Here we extracted information from all of the included studies on DEGs in EAT samples and performed a meta-analysis based on gene-networks consisting of upregulated and downregulated genes in EAT to identify genes that may be involved in disease pathogenesis. When all 40 studies that generated data on mRNA expression in EAT were included, we identified 112 genes that could be related to CVDs and/or cardiometabolic risk factors. Our findings from the included studies showed that only 32 genes were identified in at least two studies, and of these, only seven genes were identified by more than three studies. The remainder of the genes appeared only once in the included studies. Furthermore, groups of genes that appeared once in a study might represent regulatory programs that are specific to disease mechanisms, such as pathways that are over- or underactive in CVD. Consequently, we included all DEGs in our network meta-analysis. Multiple test corrections showed that 42 genes were significantly differentially expressed in EAT from patients with and without CVDs and/or cardiometabolic risk factors. After removal of insignificantly expressed genes, functional annotation and enrichment analysis showed that the most important functions related to DEGs were *regulation of apoptosis* and *regulation of systemic arterial blood pressure*.

Apoptosis is a biological process of programmed cell death that is tightly regulated. Genes involved in regulating apoptotic processes that were identified in our clustering analysis included IL-6, CCL2, TNF-α, TP53, CIDEA, ADIPOQ, SIRT1, IL10, MIF, SOD2, HMOX1, AGT, GSK3B, and NOS3. All of these genes were previously reported to be apoptosis biomarkers. Dysregulated apoptosis signalling pathways have been shown to play an important role in the pathogenesis of CVDs [70]. Recent studies on the biochemical hallmarks of myocardial apoptosis and heart dysfunction suggest that excessive amounts of reactive oxygen species (ROS) in EAT can modulate apoptosis [17, 40, 71, 72]. Moreover, in CVD patients EAT produces higher levels of reactive oxygen species (ROS) relative to subcutaneous adipose tissue (SAT) [40]. Subsequent studies showed higher protein levels of IL-6, CCL2, and TNF-α in EAT compared to SAT in CAD patients [9], who were also found to have higher IL-6 and TNF-α and lower ADIPOQ in EAT compared with non-CAD control subjects [9, 20, 73]. Reactive oxygen species (ROS) have been proposed to be potential contributors to inflammatory pathways [41]. Thus, an imbalance between inflammatory and anti-inflammatory cytokines secreted by EAT may be strongly involved in the development and progression of CAD [48].

Dysregulation of processes involved in *systemic arterial blood pressure* has also been implicated in affecting heart function and the risk of developing CVDs [74, 75]. AGTR1, AGT (functional category: renin-angiotensin system), EDN1, NOS3 (functional category: endothelial integrity), and SOD2 (functional category: oxidation-reduction state), which participate in systemic arterial blood pressure regulation, are also EAT markers involved in the pathogenesis of hypertension and CVDs [20, 40, 41, 62, 76]. By mapping DEGs to the STRING database, we were able to construct a PPI network that identified IL-6 and TP53 as hub nodes.

Interleukin-6 is a cytokine that has both pro- and anti-inflammatory actions [77]. IL-6 is produced by several cell types in the cardiovascular system, including fibroblasts, monocytes, endothelial cells, and adipocytes, and has important roles in activating immune responses and metabolic balance as well as in maintaining cardiovascular homeostasis [78]. Visceral adipose tissue has been shown to release more IL-6 than SAT. As mentioned above, increased mRNA and protein expression levels of IL-6 were observed in EAT compared to paired SAT from patients with CAD [9, 58].

EAT IL-6 synthesis is thought to increase in response to hypoxia and subsequently alter local and/or systemic vascular inflammation that in turn increases the risk of CVDs. In fact, the proximity of EAT to coronary arteries and the absence of muscle fascia between the adipocytes and myocardial layer can cause upregulated IL-6 mRNA and protein levels to disturb ventricular function and increase the risk of CAD [9, 58, 78].

The implication of IL-6 in CVDs by the analyzed studies is consistent with conclusions drawn in a study by Eiras et al. [29], which observed that EAT IL-6 mRNA levels were significantly higher in CAD relative to non-CAD patients and that these elevated levels were positively correlated with the severity of CAD as well as an increased predicted risk of CAD. In addition, Eiras et al. showed that IL-6 was the only independently significant risk factor for CAD [29]. Taken together, these results suggest that IL-6 expression in EAT may have an important local effect on the extension of CAD.

In accordance with the present findings, Nair et al. [79] also showed that IL-6 occupies the center of a backbone network in a patient with CAD. Together these results suggest that IL-6 could be considered as a super-hub gene involved in heart dysfunction. Our findings confirm the hypothesis that IL-6 may locally affect heart function and thus could act as a biomarker of CVDs and as a predictor for disease onset.

TP53 is a protein that is well known for its association with cancer and is often described as "the guardian of the genome." As a tumor suppressor and regulator of hundreds of target genes, TP53 can regulate numerous cellular processes, including cell cycle progression, apoptosis, cellular senescence, and DNA repair [80]. More recently, TP53 was implicated as a regulator of aging and thus could contribute to many aspects of aging and age-related diseases, such as cardiovascular and metabolic disorders [81, 82]. The actions of aging proteins like TP53 on CVD have been well studied [83] and suggest that TP53 and its cellular pathways contribute to disease pathogenesis.

In a recent follow-up study, Agra et al. showed higher TP53 mRNA expression levels in EAT than in paired SAT from patients undergoing cardiac surgery [17]. In addition, the authors demonstrated that EAT samples obtained from heart failure patients showed higher TP53 mRNA expression levels than those without heart failure and that TP53 expression was not associated with plasma adipokine levels. Moreover, patients who died during the follow-up period expressed lower levels of EAT TP53 relative to surviving patients [17]. These findings suggest that TP53 expression could be related to the inflammatory state present in heart failure patient EAT. The fact that TP53 expression was not associated with plasma adipokine levels also suggests the presence of local rather than systemic effects and regulation. Under normal conditions, TP53 induces expression of reactive oxygen scavenging genes that in turn provide protection to adipocytes from ROS. Conversely, during hypoxic, lipotoxic, or inflammatory situations, TP53 interacts with several downstream genes to induce apoptosis [38, 82].

In our meta-analysis of signaling pathways, *HIF-1 signaling pathway* and *TNF signaling pathway* headed the list of genes that are dysregulated in EAT from CVD patients. Indeed, the relationship of the HIF-1 and TNF signaling pathways to cardiometabolic risks has been extensively documented. Hypoxia inducible factor 1 (HIF-1) is a key regulator of oxygen homeostasis and mediates genomic responses to hypoxia. The activated HIF complex upregulates hypoxia inducible genes involved in cell proliferation, angiogenesis, glycolytic energy metabolism, and apoptosis [84]. The potential role of the HIF-1 transcriptional complex at a molecular and cellular level as well as functional responses in the heart to oxygen supply impairment has been broadly studied in the context of CVDs [85]. Under hypoxic conditions, transcriptional responses, such as TNF-α, IL-6, and TP53, are mediated by the HIF signaling pathway to promote angiogenesis and increase the oxygen supply to the heart [86, 87].

In recent years, it has emerged that TNF signaling plays a role in CAD pathogenesis, the development of atherosclerosis, heart failure, and the progression of myocardial disease [88]. Since TNF-α can induce apoptosis, its pathogenic effect on heart function may at least in part be due to its ability to induce cell death [89]. In contrast, there is also evidence to support a prosurvival role of TNF-α in the heart whereby TNF-α regulates adaptive responses to biomechanical stress [90]. Meanwhile, Shibasaki et al. demonstrated that higher expression of TNF-α and IL-6 in CAD patient EAT did not reflect the plasma levels of these markers [47], which suggests that the TNF signaling pathway in EAT may act locally via paracrine effects rather than circulating factors.

Our findings identified 40 DEGs and found that among these studies, there was a good overall agreement on the direction in which DEGs changed. An exception to this pattern was the data for PRDM16 and ADM RNA expression, which showed inconsistent directions of expression change. For ADM, Iacobellis et al. reported that ADM mRNA levels in EAT were significantly lower in patients with CAD than in those without CAD [34], while the mRNA expression levels of ADM in the EAT tissue were significantly higher in the CAD group than in the non-CAD group [47]. For PRDM16, Sacks et al. determined that PRDM16 expression in EAT was significantly lower in diabetes patients DM and higher in patients with metabolic syndrome (MetS) than control subjects [43]. However, all patients with DM and MetS had evidence of critical CAD. In another study performed by these authors, EAT PRDM16 expression was upregulated 1.84-fold in CAD patients compared to non-CAD patients [41].

There are limitations in our study. First, this systematic review focused on the role of mRNA expression in EAT samples rather than protein expression in the pathogenesis of CVD. However, a few studies determined the tissue protein expressions as well as their mRNA expression levels in EAT. Next, the statistical procedures used to analyze the DEGs of EAT, including normalization, are still unclear. Finally, due to diversity in statistical methods used for detecting differentially expressed genes, variation in patient classifications, and the lack of an appropriate control group in some studies, the evidence of that association remains weak.

5. Conclusion

In conclusion, we have shown complementary approaches that identified EAT transcriptomic information for patients with and without CVDs. We used network analysis and found that IL-6 and TP53 were the most important key genes related to cardiovascular risk which were expressed in EAT. These data suggest that IL-6 and TP53 in EAT could act to modulate heart function through HIF-1 and TNF signaling pathways. Confirmation of this link requires additional studies that will enhance our understanding of the pathogenesis role of EAT in cardiovascular diseases.

Conflict of Interests

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

Authors' Contribution

Zhila Maghbooli and Arash Hossein-nezhad contributed equally to this work.

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Research Article

Endogenous Ouabain: An Old Cardiotonic Steroid as a New Biomarker of Heart Failure and a Predictor of Mortality after Cardiac Surgery

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Cardiovascular diseases remain the main cause of mortality and morbidity worldwide; primary prevention is a priority for physicians. Biomarkers are useful tools able to identify high-risk individuals, guide treatments, and determine prognosis. Our aim is to investigate Endogenous Ouabain (EO), an adrenal stress hormone with hemodynamic effects, as a valuable biomarker of heart failure. In a population of 845 patients undergoing elective cardiac surgery, we have investigated the relationships between EO and echocardiography parameters/plasmatic biomarker of cardiac function. EO was found to be correlated negatively with left ventricular EF ($p = 0.001$), positively with Cardiac End-Diastolic Diameter ($p = 0.047$), and positively with plasmatic NT-proBNP level ($p = 0.02$). Moreover, a different plasmatic EO level (both preoperative and postoperative) was found according to NYHA class ($p = 0.013$). All these results have been replicated on an independent cohort of patients (147 subjects from US). Finally, a higher EO level in the immediate postoperative time was indicative of a more severe cardiological condition and it was associated with increased perioperative mortality risk ($p = 0.023$ for 30-day morality). Our data suggest that preoperative and postoperative plasmatic EO level identifies patients with a more severe cardiovascular presentation at baseline. These patients have a higher risk of morbidity and mortality after cardiac surgery.

1. Introduction

Cardiovascular diseases are the leading cause of mortality and morbidity in the world [1]. Their primary prevention and secondary prevention are a priority for the health system and require multiple approaches to increase effectiveness [2]. Biomarkers are useful tools used to identify with greater accuracy high-risk individuals, establish a faster diagnosis, guide treatment, and determine prognosis [3].

Our research group deals with the role of Endogenous Ouabain (EO) as biomarker of clinical and subclinical cardiovascular disease.

Endogenous Ouabain (EO) is cardiac glycoside acting as an adrenal stress hormone with cardiological, hemodynamic, and renal effects. This hormone increases to picomolar (10^{-12}) range in the plasma of hypertensive humans [4], after acute physical exercise [5], and in pregnancy [6]. EO is also known to be higher in patients with kidney failure [7], myocardial infarction [8], and congestive heart failure [9]. In addition to its hypertensive effects, EO even modifies cardiac function and modulates cellular proliferation and differentiation in heart [10], kidney [11, 12], and vascular smooth muscle [13]. Finally, it is also able to increase myogenic tone and reduce renal blood flow [7]. The primary site of Ouabain

action is generally assumed to be the α-subunit of Na^+K^+-ATPase. Ouabain inhibits Na^+K^+-ATPase whit high affinity binding mainly to the $\alpha 2$ and $\alpha 3$ isoforms in vascular and brain tissues, respectively. This inhibition increases the Na^+ concentration in the cytoplasm, which reduces the activity of the Na^+/Ca^{2+} exchanger (NCX) and, consequently, increases the amount of Ca^{2+} available to activate contraction in tissues such as the heart. This produces a positive inotropic effect [14, 15]. A large number of experiments support the hypothesis that Na^+K^+-ATPase inhibition is not necessary for the inotropic effect of cardiac glycoside in the myocardium [16–20]. Recent studies have shown that the binding of Ouabain to Na^+K^+-ATPase elicits numerous additional changes in cell function. These include activation of intracellular signal transduction, activation of cytoplasmic Ca^{2+} oscillation, stimulation of endocytosis, and inhibition of endocytosis membrane traffic, as well as cell proliferation and adhesion [21–24]. Considering the important role of cardiac glycoside in cell signaling, growth, and apoptosis, it seems clear that these molecules represent potential biomarkers for acute and chronic kidney failure, heart failure, and cardiovascular remodeling as well as potential therapeutic targets.

1.1. EO and Heart Failure. The impact of an endogenous Na^+K^+-ATPase inhibitor on individuals with congestive heart failure could be considerable. First, the myocardial inotropic state is directly dependent on the function of the sodium potassium pump. Second, inhibition of the Na^+K^+-ATPase in the renal tubule may lead to natriuresis. Third, inhibition of the pump in the vasculature might maintain or increase blood pressure by causing vasoconstriction either directly [25] or by effects on sympathetic innervation [26]. These possible consequences of the actions of Na^+K^+-ATPase inhibitor explain the efficacy of cardiac glycosides in some patients and suggest that deficiency of EO might exacerbate congestive heart failure. On the other hand, the decreased cardiac output, fluid overload, and hypotension associated with congestive heart failure might increase adrenal release of EO. The relationship between Cardiotonic Steroids, cardiac geometry, and central hemodynamic parameters has been analyzed in several studies. Gottlieb et al. [27] found that although plasma EO did not exhibit graded increases with the progression of cardiac failure, EO levels were elevated in patients with severely impaired left ventricular (LV) performance (LV ejection fraction less than 21%); Manunta et al. [4] demonstrated that plasma EO positively correlated with systolic and diastolic blood pressure in a group of 110 normotensive subjects and 100 hypertensive subjects. These works demonstrated that EO levels correlated positively with LV mass index and LV end-diastolic volume only in hypertensive subjects. Later it was found that circulating EO levels in 92 hypertensive patients were positively correlated with mean BP and total peripheral resistance index, whereas LV end-diastolic volume index, stroke index, and cardiac index exhibited inverse correlations with this hormone. Plasmatic EO was found as independent predictor of total peripheral resistance index, cardiac index, and relative wall thickness. Moreover, the plasma EO was substantially higher in patients with eccentric remodeling compared with those subjects with normal LV geometry or concentric hypertrophy [28]. In another study performed in patients with LV dysfunction, the plasma EO was found to be elevated if compared with normal subjects [29]. Experimental data also indicate an association between elevated plasmatic Cardiotonic Steroids levels and cardiovascular remodeling. Moreover, sustained Ouabain infusion, which causes a 2-fold elevation of plasmatic Ouabain immunoreactivity, is sufficient to induce LV hypertrophy in normotensive rats [30].

1.2. Aim. The aim of this study is to evaluate if EO could be used as a valuable biomarker of heart failure. Moreover, we have studied the possible relationship between EO and clinical outcomes (as development of postsurgical complication and mortality rate) for those patients undergoing cardiac surgery.

2. Methods

2.1. Study Population. We enrolled more than 840 patients undergoing elective cardiac surgery (Coronary Artery Bypass Graft, valve surgery, Aortic Arch surgery, or a combination of previous interventions) at our hospital in the last five years (from December 2009 to December 2014). Cardiovascular risk factors, demographic data, clinical data (including past medical history), and medications were obtained from patient interview and chart review. We excluded patients with evidence of severe renal disease (as acute kidney injury (AKI) before surgery or End Stage Renal Disease (ESRD)), prior kidney or heart transplantation, or surgery performed in urgency. Participants with multiple surgeries were only enrolled in the study once. All participants provided written informed consent and institution research ethics board approved the study.

2.2. Validation Cohort. 147 patients were enrolled in a prospective observational validation study conducted from January to April 2012 in the State University of New York, SUNY Downstate Medical Center (New York, US). Patients were admitted to the cardiology service or in the Intensive Care Unit (ICU) of the Cardiology Department. We measured EO levels in patients with heart failure, acute coronary disease, atrial and ventricular arrhythmias, and systemic or pulmonary hypertension admitted to the cardiology service. Written consent was obtained from the participating subjects (see Supplementary Material for details, available online at http://dx.doi.org/10.1155/2015/714793).

2.3. Sample Collection. We collected urine and plasma specimens preoperatively and daily until ICU discharge. The first postoperative samples were collected 24 h after cardiac surgery was performed. In addition to routine preoperative assessments, blood samples were obtained for plasma EO determinations within 24 hours from admission to the clinic and for the first 24 hours postoperatively. Samples were stored at -80°C until analysis.

2.4. Determination of EO. EO was extracted from plasma and measured by using a specific radioimmunoassay (RIA) in accordance with those methods previously published [31, 32].

Plasma samples are preextracted with methanol, dried with speed vacuum, and reconstituted with 0.1% trifluoroacetic acid. Preconditioned C18 Bond Elut columns (Varian, Inc., Palo Alto, California, US) are used for sample extraction. Following several water washes and one wash with 2.5% acetonitrile, Endogenous Ouabain is eluted with 25% acetonitrile. These steps are critical for two main reasons: first, the recovery of EO from the column can be variable if the preconditioning is not correct, and second the 2.5% acetonitrile wash reduces the highly polar material present in plasma extracts. Rabbit polyclonal antiouabain antisera of high titer (prepared by two different Institutes) were used in the radioimmunoassay. These antiouabain antisera possessed panels of cross-reactivity very specific for EO and similar aglycones. The cross-reactivity of the two antisera with the purified human plasma Endogenous Ouabain differed slightly from one to each other, while always remaining within experimental error (Hamlyn, unpublished observation). The possibility that the antisera used in our radioimmunoassay may recognize the human placental inhibitor of Na^+-K^+-ATPase, whose suggested structure resembles that of a bufadienolide, is very unlikely because the two Ouabain antisera we used show very weak cross-reactivity with the bufadienolides (<1%). Moreover, the sample extraction process we employ precludes the presence of bufadienolides in the Endogenous Ouabain assay because they are not sufficiently polar to be eluted by 25% acetonitrile under our conditions. Another variable is the method used to separate bound from free-labeled Ouabain. For the Milan Endogenous Ouabain radioimmunoassay system, the intra-assay and interassay coefficient of variation were approximately 5 and 9%, respectively, and remain remarkably steady over the years. When all aspects of the radioimmunoassay method are respected, all tissues from rats and humans including plasma contain measurable and reproducible amounts of EO by radioimmunoassay and ATPase assay, both before and after HPLC fractionation. An investigator blinded to the plasma biomarker concentration collected data from patient chart notes and the computerized data system.

2.5. Statistical Analyses. Continuous data are expressed as means ± standard deviation. Dichotomous variables are presented as percentages. Median and interquartile ranges (IQR) are presented for nonparametric variables. Reflecting the nonnormal distribution of EO among the population [30], we used logarithmic transformation for the statistical analysis or nonparametric tests when appropriate. ANOVA or Kruskal-Wallis and median tests were used to compare continuous variables among NYHA or left ventricular EF class, whereas Chi-square analysis or Fisher's test was used to compare discrete variables. The Mann-Whitney test was used to compare EO between different groups. Logistic Regression was used to study the effect of different variables on mortality rate. ROC was used to examine the predicting power of different variables on mortality rate; AUC (i.e., C-index) was calculated from the ROC curve. A statistically derived value, based on the Youden index [33], maximizing the sum of the sensitivity and specificity, was used to define the optimal cut-off value. A two-sided p value of <0.05 was considered to indicate statistical significance. All analyses were performed with SPSS 22.0 software (IBM, Inc., Armonk, NY, USA).

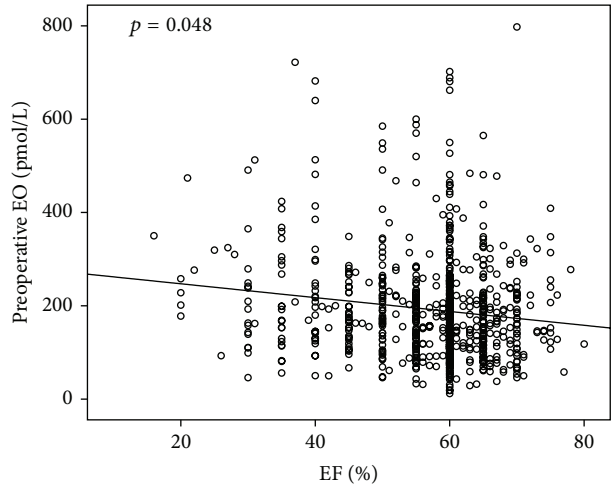

Figure 1: Correlation between baseline (preoperative) EO level and cardiac left ventricular ejection fraction (LVEF). Patients with higher Endogenous Ouabain baseline levels are those with lower LVEF (Pearson Correlation with logarithmic EO $r = 0.135$; $p = 0.001$ (0.048 after correction for sex, age, BMI, preoperative GFR, and clinical presentation expressed as EuroSCORE)).

3. Results

The study population was composed of 845 patients (34.4% females and 65.6% males; details in Table 1). Postoperative AKI (according to AKIN criteria [34]) was observed in 197 patients (23.3%). Total in-hospital mortality was 1.7% (14 patients) for cardiovascular complications after surgery; 30-day mortality was 1.3% (11 patients). All deceased patients developed AKI before exitus.

3.1. EO as Biomarker of Cardiac Failure. We have focused our attention on the relationships among preoperative clinical status of the patient, specific echocardiographic parameters of cardiac function, and plasmatic markers used in common clinical practice. In particular, as it is known from the literature [35–38], in our population a significant negative correlation between left ventricular ejection fraction (LVEF) with Cardiac End-Diastolic Diameter ($p < 0.001$, $r = 0.487$, Pearson Correlation) and with preoperative value of NT-proBNP (Pearson Correlation $r = 0.569$; $p < 0.001$) was confirmed.

We found a correlation between baseline (preoperative) EO level and cardiac ejection fraction. Patients with a higher baseline Endogenous Ouabain are those with lower left ventricular ejection fraction (Pearson Correlation with logarithmic EO $r = 0.135$; $p = 0.001$ (0.048 after correction for covariates); Figure 1). Results remain significant even

Table 1: Characteristics of population.

Population characteristics (845 subjects)	
Anthropometric and preoperative parameters	
Gender (f/m, %)	34.4/65.6
Age (years)	62.40 ± 13.14
BMI (kg/m^2)	25.28 ± 4.11
Plasma creatinine (mg/dL)	0.90 ± 0.23
eGFR (mL/m 1.73 m^2)	80.25 ± 20.47
Hypertension (%)	55.4
Diabetes (%)	14.5
Peripheral vascular disease (%)	17.7
Chronic obstructive pulmonary disease (%)	11.5
EuroSCORE	4.03 ± 4.54
CLIN-RISK[#]	9.38 ± 4.08
Plasma EO (pmol/L)[*]	174 [118–241] 204.62 ± 145.96
NT-ProBNP (pg/mL)	680.72 ± 910.53
Cardiological characteristics	
Left ventricular ejection fraction (%)	56.98 ± 10.27
Class LVEF (%)	
<30%	3.3
30–50%	19.9
>50%	76.8
Interventricular septum (mm)	11.31 ± 2.12
Cardiac End-Diastolic Diameter (mm)	53.86 ± 7.95
NYHA classification (%)	
I	22.1
II	55.9
III	21.0
IV	1.1
Surgical characteristics	
Surgery type (%)	
Valve repair (VR)	49.5
Isolated coronary bypass (CABG)	16.3
CABG + VR	18.5
Aortic Arch surgery	13.3
Other	2.2
Reoperation cardiac surgery (%)	10.7
Combined surgery (%)	18.6
Cardiopulmonary bypass used (%)	88.9
Cardiopulmonary bypass duration (min)	68.85 ± 30.04
Postoperative time	
Plasma creatinine (mg/dL)	1.20 ± 0.63
Plasma EO (pmol/L)[*]	267 [200–357] 311.55 ± 200.70
ΔPlasma EO (pmol/L)	91.51 ± 195.52
Outcomes	
AKI (%)	
AKIN Stage I	23.3
AKIN Stage II	9.3
AKIN Stage II	2.3
In-hospital mortality (n, %)	14 (1.7%)

Dichotomy variables: expressed as % (positive).
Parametric variables: expressed as mean ± s.d.
Nonparametric variables (∗): expressed as median (25–75 percentile) and mean ± s.d.
[#]Clinical risk model for postoperative severe AKI (based on gender, age, LVEF, hypertension, diabetes, renal function, reintervention, and surgery type (see [41, 42])).

after LVEF was recorded according to EuroSCORE [39] classification (three classes: EF < 30%; EF 30–50%; EF > 50%; Kruskal-Wallis $p = 0.001$ (0.013 after correction for covariates); Figure 2). Moreover a positive correlation between preoperative level of Endogenous Ouabain and Cardiac End-Diastolic Diameter was also observed (Pearson Correlation with logarithmic EO: $r = 0.147$; $p = 0.047$ (0.05 after correction for covariates)). Furthermore, a positive correlation was observed also between the plasmatic values of EO and NT-proBNP (Pearson Correlation with logarithmic EO: $r = 0.321$; $p = 0.02$ (0.021 after correction for covariates)). Statistical adjustment was made for sex, age, BMI, preoperative GFR, and clinical presentation (summarized by EuroSCORE preoperative value).

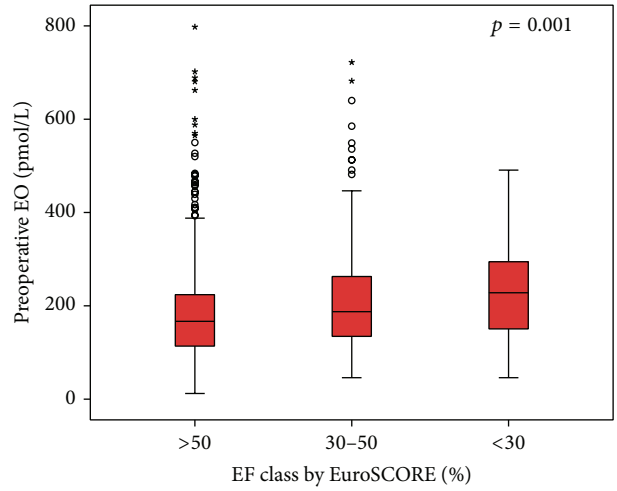

Figure 2: EO preoperative level according to EuroSCORE EF classification (three classes: EF < 30%; EF 30–50%; EF > 50%; Kruskal-Wallis $p = 0.001$).

Finally, those patients with a more severe heart failure index, expressed as NYHA class, have a higher baseline EO plasmatic level ($p = 0.047$). According to each NYHA class, mean (±SD) EO preoperative level (expressed in pmol/L) was as follows: 179.84 ± 107.58 for class I; 192.07 ± 107.45 for class II; 209.08 ± 125.67 for class III; and 247.98 ± 133.52 for class IV. More interesting, we observed the same trend, but with a stronger evidence, for postoperative EO level (mean ± SD, resp.: 272.81 ± 127.59 versus 276.89 ± 126.50 versus 333.62 ± 164.78 versus 427.79 ± 246.65 pmol/L) according to NYHA class (Kruskal-Wallis $p = 0.0001$; Figure 3). Correlation between postoperative EO and NYHA class was corrected for clinical variables (see above) and also for preoperative level of EO ($p = 0.013$).

As replication we performed the same analysis on a different population of 147 subjects from SUNY Downstate Medical Center (New York, US). Despite the small sample size, we confirmed the main results observed on the Italian cohort. In particular, we observed a negative correlation between EO preoperative level and LVEF ($p = 0.008$) and a positive correlation EO-BNP ($p = 0.05$). See supplementary data for details.

3.2. EO as Biomarker of Cardiac Stress. In order to support the hypothesis that EO was secreted during hemodynamics

Table 2: EO levels according to different surgical intervention.

Time		Number of patients	Mean (pmol/L)	SE	ANOVA p value[#]	Multiple comparison p value[#]
Preoperative	VR	401	183.74	5.57	0.01	
	CABG	136	206.17	8.52		0.005 versus VR
	Complex	280	201.01	7.002		0.07 versus VR
Postoperative	VR		270.84	7.91	0.009	
	CABG		278.93	9.64		
	Complex		313.34	10.91		0.003 versus VR
ΔEO	VR		79.54	7.62	0.033	
	CABG		56.08	11.10		
	Complex		96.98	9.94		0.01 versus CABG

VR: valve repair; CABG: Coronary Artery Bypass Graft; complex: combined surgery + Aortic Arch surgery.
[#] p value for ANOVA (with EO transformed as logarithmic).
ΔEO: postoperative − preoperative.

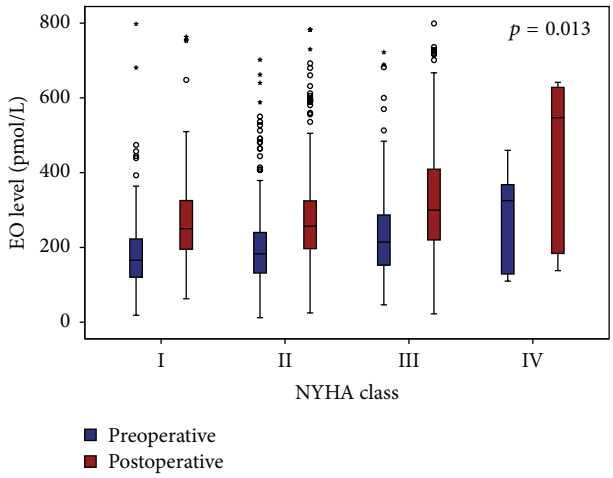

Figure 3: EO levels (blue = preoperative; red = postoperative) according to each NYHA class ($p = 0.013$ after correction for sex, age, BMI, preoperative GFR, EuroSCORE, and baseline level of EO).

stress [40], we investigated the change in EO plasmatic level according to different kind of cardiac pathology or surgical technique.

A higher circulating preoperative level of EO was found in patients undergoing Coronary Artery Bypass Graft (CABG) intervention. This difference is most evident considering the values of patients with simple valvular disease eligible for surgical repair (ANOVA $p = 0.01$). In the same way, those patients undergoing a more complex surgical procedure (e.g., patient with surgery on Aorta Arch or undergoing combined intervention) reached a higher level of postoperative EO plasmatic concentration ($p = 0.009$). See Table 2 for details.

Moreover, when we looked to surgical technique, a greater plasmatic EO levels change was observed (meant as postoperative/preoperative EO variation (ΔEO)), in those patients undergoing cardiopulmonary bypass (CPB). ΔEO was, respectively, 43.73 ± 134.65 pmol/L (NO-CPB) versus 87.62 ± 131.56 pmol/L (CBP), ANOVA $p = 0.007$. This result was even more significant when we have restricted the analysis only to those patients undergoing CABG as surgical intervention (NO-CBP ($n = 55$): 26.89 ± 124.05 versus CBP ($n = 37$): 97.90 ± 80.87 pmol/L; ANOVA $p = 0.003$).

3.3. EO as Predictor of Postoperative Morbidity and Mortality. In our previous studies we have already demonstrated that baseline values of Endogenous Ouabain correlate with the development of AKI after cardiac surgery [41, 42]. Also in this subset of patients we confirmed the same predictive power. Indeed, patients with higher EO baseline values have higher chance to develop mild to severe AKI after cardiac surgery (Logistic Regression for AKI $p < 0.0001$). All data were corrected for clinical risk model for AKI [42]. This result was greatly expected because the population studied in this work is largely superimposed on those previously published and represents an expansion of the same population. In this work we tried to find any correlation between postoperative EO levels and the development of postoperative renal failure. Unfortunately, a weaker correlation with AKI of postoperative EO if compared with preoperative EO was observed (Logistic Regression for AKI $p = 0.017$); moreover, in a risk model including clinical variables and both EO levels (pre- and postoperative), only basal EO was related with AKI development.

In our sample the mortality was approximately 1.4%; this data was concordant with those reported in the literature [43, 44]. In particular, deaths by cardiovascular events were 14 (indeed patients who died from septic events or surgical complications were excluded from this work).

In our population mortality rate has been associated with baseline eGFR, previous cardiac surgery, and preoperative NYHA class (see Table 3). Moreover, all deceased patients developed AKI before "exitus" occurred (probably as a consequence of the development of heart failure). These data are perfectly in agreement with the literature [45]. All these variables are summarized by EuroSCORE (European System for Cardiac Operative Risk Evaluation) value [39]. EuroSCORE is able to predict perioperative (30 days) mortality [46] for patients undergoing cardiac surgery. This score is very well codified and widely accepted: indeed the use of this score to

Table 3: Logistic Regression with mortality.

	Variables	p value[#]	p value[*]	Exp(B)[*]	CI (95%)[*]
Reference	EuroSCORE	0.001	—	1.10	1.03–1.18
Clinical characteristics	Age	0.004	0.039	1.08	1.00–1.16
	LVEF	n.s.	n.s.		
	NYHA	0.027	n.s.		
	Basal creatinine	0.006	n.s.		
	Hypertension	n.s.	n.s.		
	Diabetes	n.s.	n.s.		
Surgery type	REDO	0.001	0.011	5.02	1.45–17.33
	Complex	0.023	0.04	2.17	1.04–4.56
	EEC	n.s.	n.s.		
Complications	AKI	<0.001	<0.001	53.24	10.99–257.87
EO	Preoperative EO	n.s.	n.s.		
	Postoperative EO	0.025	0.046	1.04	1.00–1.07
	Postoperative EO > 363 pmol/L	0.04	0.049	3.58	1.03–12.78

[#] p value for Logistic Regression (not correct); [*] p value and Exp(B) for Logistic Regression after correction for EuroSCORE.
n.s: not significant; Exp(B): expected beta for Logistic Regression; LVEF: left ventricular ejection fraction; REDO: reintervention; complex: combined surgery or Aortic Arch surgery; EEC: extracorporeal circulation; AKI: acute kidney injury (by AKIN criteria; see [34]).

predict mortality risk is codified by International Guidelines [44].

We confirmed the excellent existing correlation between EuroSCORE and mortality rate. A greater EuroSCORE preoperative value was observed in those patients who died after surgery if compared to those who have survived (resp., 3.89 ± 4.04 versus 11.36 ± 14.68; ANOVA $p < 0.0001$). Moreover, Logistic Regression for total in-hospital mortality was strictly significant (Logistic Regression $p = 0.003$; Table 3).

Any significant relationship between preoperative value of EO and mortality rate was not observed. Furthermore, also the change in EO plasmatic levels was not correlated with total in-hospital mortality after correction for EuroSCORE.

We found that only the postoperative value of Endogenous Ouabain was strongly related with mortality rate after correction for baseline EuroSCORE value: in particular, those patients with a higher level of postoperative EO have a higher mortality risk after cardiac surgery (Logistic Regression $p = 0.046$). To better understand the impact of postoperative EO on mortality risk we performed Receiver Operating Characteristic (ROC) curve (AUC 0.68 ± 0.08; $p = 0.035$) to allow Youden's index calculation [33]: we identified 363 pmol/L as critical EO level. Those patients with plasmatic EO concentration after cardiac surgery > 363 pmol/L had a mortality risk 3.5 times higher if compared to other patients.

It is also well know that NT-proBNP is a good predictor of postoperative mortality [47–49]. Even in the studied population pre- and postoperative levels of NT-proBNP were higher in deceased patients if compared to those who have survived (Kruskal-Wallis $p = 0.036$ and $p = 0.039$ for preoperative and postoperative NT-proBNP values, resp.). However, the predictive power of this biomarker of heart failure (expressed as Logistic Regression for mortality) was found to be of borderline significance and, surprisingly, less effective if compared to EO (Logistic Regression for NT-proBNP: $p = 0.085$ and $p = 0.052$ for preoperative and postoperative NT-proBNP values, resp.). After correction for EuroSCORE no association between NT-proBNP and mortality rate was observed. In our opinion this unexpected result could be explained by the small number of recorded events that could mask the real "potential" of NT-proBNP.

Finally, because EuroSCORE was codified for 30-day mortality prediction [46], we performed a survival analysis at 30 days after cardiac surgery. In this case mortality was 1.3% (11 patients). Also in this analysis postoperative EO was confirmed to be associated with 30-day mortality (Cox regression $p = 0.023$ after correction for EuroSCORE; Figure 4).

4. Conclusion

This work tried to investigate the relationship among EO and several clinical and biological heart-related variables in order to assess EO as a new and valuable biomarker for heart failure. Moreover, we investigated the role of EO pre- and postoperative levels on cardiovascular mortality after cardiac surgery.

The main results were the evidence of correlations between EO and several echocardiographic parameters of cardiac function. EO correlates negatively with LVEF ($p = 0.001$) and positively with Cardiac End-Diastolic Diameter ($p = 0.047$). Moreover, an increase in plasma EO concentration is associated with an analogue increase of plasmatic NT-proBNP ($p = 0.02$), a well-known and well-accepted biomarker of cardiac failure. All these results are of special interest because they are clinically related one to each other: a patient with heart failure will show reduced EF, increased BNP, and a concomitant rise in EO level. The evidence of different plasmatic EO level according to NYHA class is not a simple consequence of the previous results; it is another demonstration of the close relationship between high levels of EO and impaired cardiac function. Furthermore, all these results have been replicated on an independent control cohort of patients (147 subjects from Cardiology Unit of SUNY

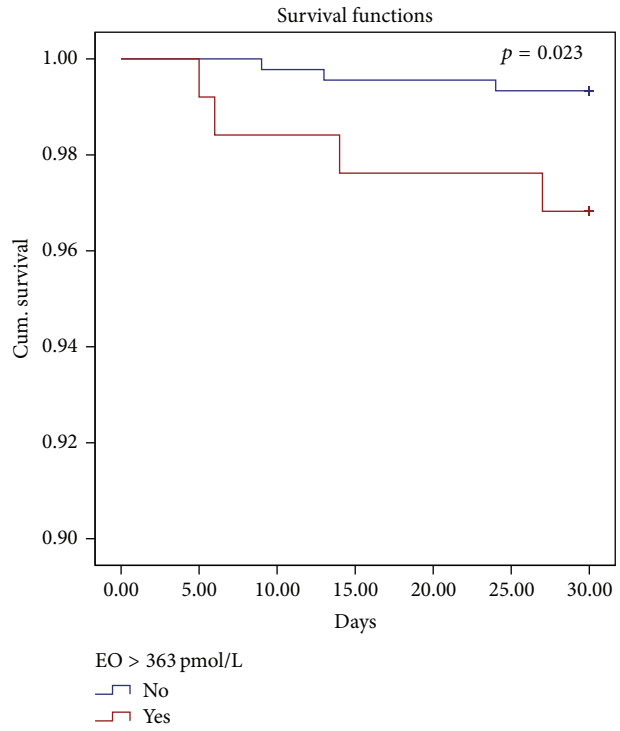

Figure 4: 30-day survival rate for patients with high (>363 pmol/L, red line) postoperative EO level (Cox regression corrected for EuroSCORE $p = 0.023$).

Downstate Medical Center). This makes such results even more significant.

The second evidence of this paper is the further demonstration that EO acts as an acute stress hormone. Patients undergoing a more complex surgical procedure (as a combined intervention or a procedure that involves Aortic Arch) reached a higher level of postoperative EO. Moreover patients undergoing CPB (a surgical technique in which cardiorenal system is exposed to an intense hemodynamic stress with a significant release of proinflammatory cytokines [50]) have a greater increase in postoperative EO plasmatic level ($p = 0.009$).

Finally, we have shown that high levels of EO in the immediate postoperative time are indicative of a more severe heart condition and how they are associated with increased perioperative mortality ($p = 0.023$ for 30-day morality).

All these data, taken together, bring to reconsider EO no longer as a simple natriuretic hormone. These evidences raise awareness that EO is a real cardiac stress hormone and that could be an indicator of the presence of subclinical cardiovascular damage. It is yet to be investigated whether this subclinical damage is mainly important for the cardiac, vascular, or kidney district and what is the first organ to be involved. Furthermore, our data suggest that those patients with a more severe heart failure (and, as consequence, with a reduction in blood pressure (BP) levels) may have an increase in EO plasmatic levels in response to the hypotensive stimulus. This could occur in attempt to restore BP values to physiological levels. These findings are in agreement with other previous observations on the effects of extracorporeal circulation (ECC) on EO values. Indeed, Bignami et al. [40] have shown how EO markedly and rapidly increases after significant reduction of BP values induced by the anesthesia.

Our data indicate that EO acts as a biomarker of individual cardiovascular condition. Presence of higher preoperative and postoperative plasmatic EO level identifies patients with a higher risk of morbidity and mortality after surgery. Those patients may benefit from inhibition of EO action. Indeed an inhibitor of EO (rostafuroxin) has been recently developed [51] and might help to minimize perioperative mortality. Moreover, the theoretical possibility of a pharmacological intervention able to change EO levels makes the study of this hormone of particular interest. Actually, in contrast to traditional biomarkers of heart failure (such as NT-proBNP), which are used only for diagnostic, EO could be used with a double purpose: diagnostic (preoperative) and therapeutic (postoperative). But to reach this futuristic goal we need further investigations to understand the real impact of EO on the development/progression of cardiovascular damage and its role of "link" among HF, AKI, and mortality.

5. Limitation

There are several limitations in this study that will require further investigation.

The most important one seems to be the small number of events (death) on which we have conducted the analysis. Actually the mortality rate of our population is comparable with the literature. In addition, our hospital is considered a "Center of Excellence" in Italy for the cardiac surgery, especially for the great experience of our surgical team; this further reduces the number of recorded events. Nonetheless, we believe that the results presented should be considered truthful for three reasons. First, all surgical operations were conducted by the same surgical team. Moreover, all preoperative echocardiographic studies were conducted by the same team of two people, minimizing the intraoperator variability. These peculiarities make this population highly homogeneous and reduce confounding variables. This allows us to study in detail the pathophysiological mechanisms underlying the development/worsening of cardiovascular diseases and to obtain interesting results even with a small number of reported events. Second, to further reduce confounding factors, we have chosen to include in the study only surgical interventions performed electively, excluding emergencies and patients with too severe comorbidities (such as ESRD). Third, in order to be sure of investigating the effect of EO on cardiovascular mortality, we excluded "a priori" from the analysis all patients who died from other causes (such as septic complications or issues related to surgical procedure).

Another point of discussion could be the choice not to correct "EO-mortality" association for presence of postoperative AKI. In fact, as is well known, there is a very strong association between postoperative mortality and the onset of renal damage. This is also evident in our population (see Section 3 and Table 3). However, considering that all the deceased patients developed kidney damage, AKI has

a statistical power so strong to be able to cover all other variables under examination (including EuroSCORE, EF, and other clinical characteristics well known in the literature as being associated with perioperative mortality). For this reason, we have chosen excluding AKI from the analysis. In addition, in our opinion, kidney damage is secondary to the development of heart failure (triggering factor of reported mortality events) and the final aim of this study is to investigate the relationship between EO and heart failure (also because the relationship between EO and AKI has already been thoroughly demonstrated [41, 42, 52]).

Finally, for the statistical analysis was used EuroSCORE instead of EuroSCORE II (a recent evolution EuroSCORE). This was decided for two reasons. First, patient enrollment was started before the publication of this new score (2009 versus 2011) and not for all patients could the new EuroSCORE II be calculated (especially for older ones). So we chose to maintain the old score in order to preserve sample size. Second, in the most recent guidelines [44] EuroSCORE is still the reference model for calculation of perioperative mortality.

Abbreviations

Δ:	Variation from baseline
AKI:	Acute kidney injury
BNP:	Brain natriuretic peptide
CABG:	Coronary Artery Bypass Graft
CPB:	Cardiopulmonary bypass
eGFR:	Estimated glomerular filtration rate
EO:	Endogenous Ouabain
EF:	Ejection fraction
ECC:	Extracorporeal circuit circulation
EDD:	Cardiac End-Diastolic Diameter
ESRD:	End Stage Renal Disease
HF:	Heart Failure
LV:	Left ventricular
NT-proBNP:	N-terminal prohormone of brain natriuretic peptide.

Conflict of Interests

No conflict of interests was declared.

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Research Article

Pentraxin-3 Predicts Long-Term Cardiac Events in Patients with Chronic Heart Failure

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The aim of this study was to investigate the long-term prognostic value of pentraxin-3 (PTX3) in patients with chronic heart failure (CHF). 377 patients were prospectively followed up for 3 years to determine cardiac events including cardiac death or rehospitalization for worsening heart failure. The plasma PTX3 levels were significantly higher in CHF patients than in healthy subjects ($p < 0.001$), and they increased with advancing New York Heart Association (NYHA) Functional Classification ($p < 0.001$). Plasma PTX3 levels in CHF patients with cardiac events were significantly higher than in event-free patients ($p < 0.001$). We determined the normal upper limit of plasma PTX3 levels from the mean + 2 SD value of 64 control subjects (3.64 ng/mL). A Kaplan-Meier analysis revealed that patients with increased PTX3 (≥3.64 ng/mL) were at a higher risk for cardiac events than those without increased PTX3 ($p < 0.01$). A multifactorial Cox proportional hazards model showed that increased PTX3 (≥3.64 ngImL) was an independent risk factor for cardiac events in CHF patients (hazard ratio (HR) = 4.224, $p < 0.01$; 95% CI: 1.130–15.783). Plasma PTX3 levels are a long-term independent predictor of prognosis in patients with CHF.

1. Introduction

Chronic heart failure (CHF) is the end stage of many types of cardiovascular diseases, and the prognosis of patients with CHF is very poor. CHF is a leading cause of hospital admissions and death in developed countries [1], and CHF has also become a major cause of morbidity and mortality in developing countries. Thus, there is an urgent need for improvements in the risk stratification and prognosis of patients with CHF.

In recent years, studies have shown that inflammation isz related to CHF and that plasma C-reactive protein (CRP) levels are associated with the prognosis of CHF patients [2–4]. Pentraxin-3 (PTX3) and CRP are members of the pentraxin family. CRP is a short pentraxin synthesized in the liver and may be an indicator of a systemic response to local inflammation [5, 6], while PTX3 is a long pentraxin produced mainly by dendritic cells, monocytes, fibroblasts, and vascular endothelial cells in response to primary inflammatory stimuli [7, 8], and this factor is highly expressed in the cardiovascular system [9–11]. PTX3 may reflect local inflammatory status in the cardiovascular system and thus may be a new biomarker of inflammation; however, this possibility will require more research.

We recently demonstrated that plasma levels of PTX3 were significantly higher in patients with stable coronary artery disease (CAD) after drug-eluting stent (DES) implantation and that these elevated levels of PTX3 were significantly and independently associated with the prevalence of major cardiovascular events (MACE) after DES implantation [12]. Moreover, a few recent studies also indicated that PTX3 levels were significantly higher in CHF patients and could potentially function as a predictor of adverse clinical outcomes in CHF patients [13–16].

All of these data were obtained in developed countries such as Japan and various European countries, and the causes

of CHF in these countries differ from the causes of CHF in developing countries. To our knowledge, no data have been published about the association between PTX3 and CHF in developing countries. China is the largest developing country in the world; thus, data from China are likely to be valuable.

This study was designed to assess the efficacy of plasma PTX3 levels for the prediction of long-term cardiac events in CHF patients in China.

2. Materials and Methods

2.1. Patients. Between June 2010 and December 2011, we prospectively enrolled 406 patients with CHF who were admitted to Ningbo Yinzhou People's Hospital, Medical School of Ningbo University. Sixty-four age- and gender-matched healthy subjects from our medical examination center served as the control group for determining normal plasma levels of PTX3. These subjects were diagnosed as normal by physical examinations, chest X-ray, electrocardiogram, and echocardiography.

Two cardiovascular specialists confirmed all recruited CHF patients. The diagnostic criteria for patients with CHF included the following: (1) underlying heart disease; (2) dyspnea and edema of the lower limbs as well as other symptoms of heart failure; (3) abnormalities in at least one of the following objective indicators: etiological factors, the anatomy of the heart, and the cardiac function index (as assessed by chest X-ray or echocardiography). The inclusion criteria included the following: (1) impaired heart function (NYHA classes II to IV) on admission; (2) left ventricular ejection fraction (LVEF) ≤45%; and (3) impaired heart function (NYHA classes I and II) before discharge. Exclusion criteria included patients with renal dysfunction (serum creatinine >1.5 mg/dL), severe hepatic or lung disease, chronic or acute inflammation, and malignant disease. Patients who experienced myocardial infarction (MI) during the month prior to enrollment were also excluded.

This study was approved by the Ethics Committees of Ningbo Yinzhou People's Hospital, and all patients provided written informed consent.

In this study, demographic and clinical data, including age, gender, heart rate, diabetes mellitus, hypertension, hyperlipidemia, smoking, a previous history of myocardial infarction, NYHA class, and LVEF on admission, were collected from in-hospital medical records and patient interviews.

2.2. Venous Blood Samples and Laboratory Analyses. Venous blood samples were collected from patients on the second morning after admission under fasting conditions to measure levels of serum hsCRP and plasma PTX3. Whole blood was immediately collected into a tube containing ethylenediaminetetraacetate (EDTA) and then centrifuged at 2000 ×g for 15 min at room temperature; the plasma was kept frozen at −80°C until analysis. Plasma PTX3 concentrations were measured by enzyme-linked immunosorbent assay (ELISA; Perseus Proteomics Inc., Tokyo, Japan) as reported previously [17]. This assay can measure the plasma PTX3 concentration linearly between 0.1 and 20 ng/mL.

Normal PTX3 levels were determined based on the upper limit of plasma PTX3 levels from the mean + 2 SD value in control subjects.

2.3. Follow-Up and Endpoints. A total of 377 patients underwent clinical follow-up (follow-up rate, 92.9%) for three years after enrollment. The primary endpoint was cardiac events, which were defined as cardiac death or rehospitalization for worsening heart failure. A review of medical records and follow-up telephone interviews were conducted to survey for cardiac events among the enrolled patients. Reviews of the follow-up case notes were performed by two of the authors (Wang Chunming and Chen Guozhong) who were blinded to the PTX3 levels. All deaths were considered to be from cardiac causes unless an unequivocal noncardiac cause could be established.

2.4. Statistical Analysis. All data analyses were performed using SPSS, version 18.0 (SPSS, Chicago, IL, USA). Continuous data are expressed as the mean ± SD, and skewed variables are presented as the median value. Continuous variables were analyzed using unpaired Student's t-test or linear regression analysis; categorical variables were compared using the χ^2 test. If data were not normally distributed, the Mann Whitney U-test was used. Cox proportional hazard regression analysis was used to determine which variables were significantly related to cardiac events. Only variables with p values less than 0.05 in the univariate Cox regression analysis were entered into the multivariate Cox regression analysis. The hazard ratio (HR) and 95% confidence intervals (CIs) are presented. The log-rank test was performed to obtain the Kaplan-Meier probability estimates. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Plasma PTX3 Levels in Control Subjects and CHF Patients. The plasma level of PTX3 in control subjects (37 males, 27 females, mean age: 76 ± 9 years) was 2.58 ± 0.53 ng/mL, but the corresponding value was 3.42 ± 0.88 ng/mL in the CHF patients (220 males, 157 females, mean age: 77 ± 9 years). As shown in Figure 1, the PTX3 levels were significantly higher in CHF patients than in control subjects ($p < 0.01$) and increased significantly with advancing NYHA functional class ($p < 0.01$).

3.2. Clinical Characteristics of CHF Patients with and without Cardiac Events. During the 3 years of follow-up, there were 152 (40.3%) cardiac events, including 54 (14.3%) cardiac deaths and 98 (26.0%) rehospitalization cases for worsening heart failure. Patients with a cardiac event had higher concentrations of PTX3, high-sensitivity C-reactive protein (hsCRP), and cardiac troponin (cTnI) ($p < 0.001$) compared with those without a cardiac event. Patients with a cardiac event had a higher prevalence of NYHA class >II and a higher heart rate and age ($p < 0.001$), but they showed decreased use of angiotensin converting enzyme inhibitor (ACE-I) or angiotensin receptor blocker (ARB) ($p < 0.05$) (Table 1).

Table 1: Clinical characteristics of patients with and without a cardiac event.

	All patients (n = 377)	Event-free (n = 225)	Cardiac event (n = 152)	p value
Age (years)	77.1 ± 9.1	75.0 ± 8.9	79.6 ± 9.3	<0.001
Males, n (%)	220 (58.5)	131 (58.2)	89 (58.6)	0.5175
Heart rate, beats/min	76 ± 18	74 ± 15	80 ± 18	<0.001
NYHA class >II, n (%)	266 (70.7)	134 (59.6)	129 (84.9)	<0.001
CHD, n (%)	196 (52.1)	114 (50.7)	82 (53.8)	0.599
Diabetes mellitus, n (%)	52 (13.8)	29 (12.9)	23 (15.1)	0.546
Hypertension, n (%)	143 (38.0)	89 (39.6)	54 (35.5)	0.450
Hyperlipidemia, n (%)	94 (25.0)	54 (24.0)	40 (26.3)	0.629
Current smoking, n (%)	115 (30.6)	68 (30.2)	47 (30.9)	0.910
PTX3 (ng/mL)	3.42 ± 0.88	3.088 ± 0.99	3.911 ± 0.83	<0.001
cTnI (ng/mL)	0.49 ± 0.24	0.24 ± 0.14	0.84 ± 0.45	<0.001
hsCRP (ng/mL)	6.72 ± 4.67	6.44 ± 4.35	7.22 ± 4.37	<0.001
Medical therapy				
Aspirin, n (%)	349 (92.8)	204 (90.6)	145 (95.4)	0.211
β-blockers, n (%)	254 (67.6)	160 (71.1)	94 (61.8)	0.073
ACE-I/ARB, n (%)	266 (70.7)	169 (75.1)	97 (63.8)	0.021
Diuretics, n (%)	344 (91.5)	204 (90.7)	140 (92.1)	0.712

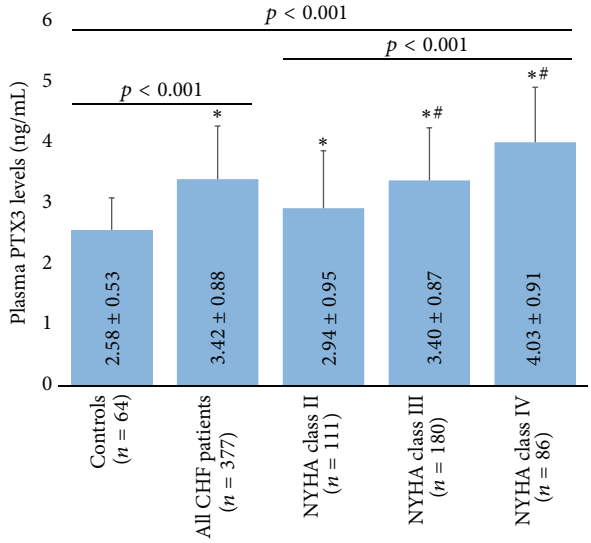

Figure 1: Plasma PTX3 levels in control subjects and CHF patients. $^{*}p < 0.01$ versus control, $^{\#}p < 0.01$ versus NYHA class II.

3.3. Clinical Characteristics according to the Upper Limit Normal Plasma PTX3 Levels. The normal PTX3 levels were determined by the upper limit of plasma PTX3 levels (3.64 ng/mL) from the mean + 2 SD value in 64 control subjects (2.58 ± 0.53 ng/mL). Patients with increased PTX3 (≥3.64 ng/mL) had a higher prevalence of NYHA class >II ($p = 0.039$) as well as higher concentrations of hsCRP and cTnI ($p < 0.001$) compared to patients without increased PTX3 (Table 2).

3.4. Cardiac Events in CHF Patients. As shown in Table 3, the CHF patients with increased PTX3 levels had a higher prevalence of cardiac death ($p < 0.05$), rehospitalization for worsening HF ($p < 0.001$), and cardiac events ($p < 0.001$). Kaplan-Meier analysis also showed that the probability of cardiac events, cardiac death, and rehospitalization was significantly higher in the high-PTX3 group than in the low-PTX3 group ($p < 0.001$ or = 0.001, log-rank test) (Figure 2).

3.5. Univariate and Multivariate Cox Regression Analysis of Cardiac Events. PTX3, age, cTnI, NYHA class >II ($p < 0.001$), heart rate ($p = 0.003$), and hsCRP ($p = 0.016$) were significantly associated with cardiac events over the three-year period based on univariate Cox regression analysis (Table 4). A stepwise multivariate Cox regression analysis was performed that included age, heart rate, and prevalence of NYHA class >II, PTX3, cTnI, and hsCRP. PTX3 (hazard ratio (HR) 4.154, $p = 0.005$; 95% CI, 1.130–15.783), cTnI (HR 1.808, $p = 0.008$; 95% CI, 1.208–2.686), NYHA class >II (HR 3.018, $p < 0.001$; 95% CI, 1.818–4.998), and age (HR 2.518, $p = 0.020$; 95% CI, 1.030–6.158) were independently associated with cardiac events over the three-year follow-up period. hsCRP was not independently associated with cardiac events over that same time period (Table 4).

4. Discussion

In this study, we found that plasma PTX3 levels were significantly higher in CHF patients than in healthy subjects, that these levels increased with advancing NYHA functional classification, and that the plasma levels of PTX3 can significantly

Table 2: Clinical characteristics according to the upper limit normal plasma PTX3 levels* in CHF patients.

	PTX3 <3.64 ng/mL (n = 205)	PTX3 ≥3.64 ng/mL (n = 172)	p value
Age (years)	76.8 ± 9.5	77.5 ± 9.7	0.412
Males, n (%)	116 (56.6)	104 (60.5)	0.464
Heart rate, beats/min	75 ± 17	77 ± 20	0.346
NYHA class >II, n (%)	123 (60)	143 (83.1)	<0.001
CHD, n (%)	103 (50.2)	93 (54.1)	0.471
Diabetes mellitus n (%)	22 (10.7)	30 (17.4)	0.072
Hypertension, n (%)	83 (40.6)	60 (34.9)	0.287
Hyperlipidemia, n (%)	50 (24.4)	44 (25.6)	0.812
Current smoking, n (%)	60 (29.3)	55 (32.0)	0.577
cTnI (ng/mL)	0.34 ± 0.16	0.64 ± 0.28	<0.001
hsCRP (ng/mL)	5.27 ± 4.45	8.17 ± 5.43	<0.001
Medical therapy			
Aspirin, n (%)	189 (92.2)	160 (93.0)	0.845
β-blockers, n (%)	142 (69.3)	112 (65.1)	0.440
ACE-I/ARB, n (%)	151 (73.7)	115 (66.9)	0.174
Diuretics, n (%)	185 (90.2)	159 (92.4)	0.471

*Normal PTX3 levels are determined based on the upper limit of plasma PTX3 levels from the mean ± 2 SD value in 64 control subjects (2.58 ± 0.53 ng/mL).

Table 3: Risk stratification of CHF patients based on increased PTX3 (above the upper limit normal plasma PTX3 level of 3.64 ng/mL).

	PTX3 <3.64 ng/mL (n = 205)	PTX3 ≥3.64 ng/mL (n = 172)	p value
Cardiac events	43	109	<0.001
Cardiac death	22	32	0.038
Rehospitalization for worsening HF	21	77	<0.001

predict future cardiac events in CHF patients. Moreover, in a stepwise multivariate Cox regression analysis, which included well-known clinical and biochemical risk factors for CHF, the plasma levels of PTX3 remained an independent predictor of cardiac events in CHF patients. These findings suggest that PTX3 may be a reliable predictor for risk stratification in CHF patients, and measuring PTX3 may substantially improve the risk stratification of CHF patients.

In recent years, studies on the relationship between plasma PTX3 levels and CHF have made little progress. Suzuki et al. [16] found that PTX3 levels increased significantly in 196 CHF patients compared to 60 healthy controls and that they increased with advancing NYHA functional class. These authors also demonstrated that the plasma PTX3 level could be a prognostic risk factor in CHF patients. Similarly, Kotooka et al. [18] indicated that the plasma PTX3 levels were higher in 37 CHF patients with dilated cardiomyopathy than in healthy subjects. PTX3 levels might be a potentially useful biomarker for predicting prognosis as well as detecting inflammatory status in CHF patients. Recently, the CORONA and GISSI-HF trials showed that an elevated PTX3 concentration was associated with age and advanced NYHA class, and this marker independently predicted fatal outcomes in CHF patients over a 3-month period [14]. Further studies showed that high plasma PTX3 levels were correlated with future cardiovascular events in CHF patients with a normal ejection fraction [13, 15].

However, all of these data were obtained in developed countries, such as Japan and various European countries. To the best of our knowledge, we are the first to report an independent association between plasma levels of PTX3 and adverse cardiac events among CHF patients in a developing country. The risk stratification of CHF patients may be different between developed and developing countries because the causes of CHF and the extent of inflammatory activation are not the same.

In the present study, the normal plasma level of PTX3 from 64 healthy subjects was 2.58 ± 0.53 ng/mL, which is higher than that obtained in a large sample of healthy Japanese subjects [19]. This result might have occurred because our subjects were significantly older (76 ± 9 versus 60 ± 11 years), as plasma levels of PTX3 are known to increase with age [19]. However, the plasma PTX3 level in our CHF population was 3.42 ± 0.88 ng/mL, which is slightly lower than that reported by other studies in developed countries. Although the blood samples were drawn at a similar time, the average age in our study was older than that in the other studies [13, 14, 16]. This indicates that the increased inflammatory activation of CHF patients in China was not as significant as that observed in developed countries. There are

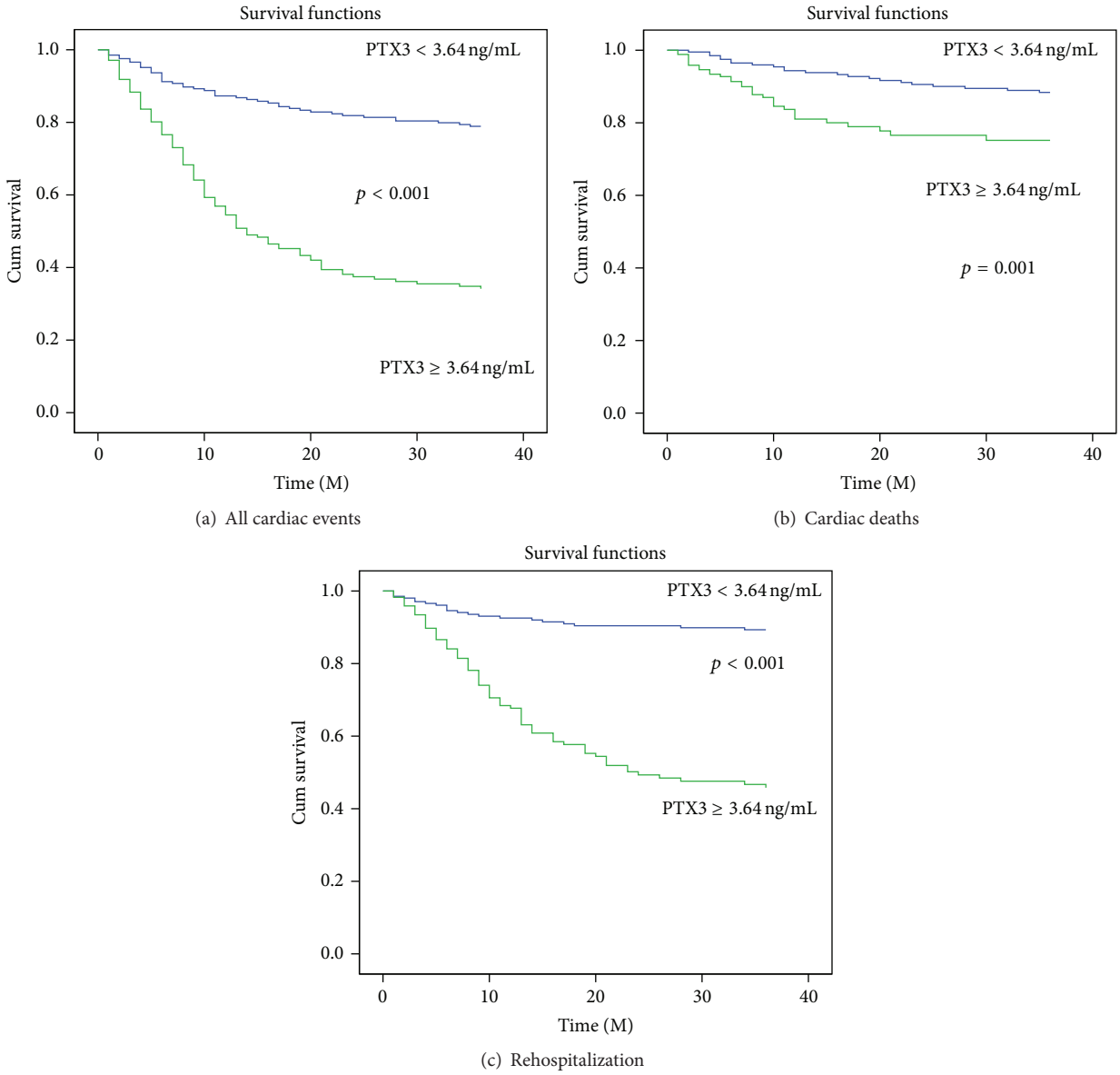

Figure 2: Kaplan-Meier analysis for all cardiac events (a), cardiac deaths (b), and rehospitalization (c) according to the upper limit normal plasma PTX3 levels (3.64 ng/mL). The p values were calculated using the log-rank test.

no prior data on whether plasma PTX3 levels are associated with adverse cardiac events in China.

Our study demonstrated that the plasma level of PTX3 was not only risk factor but also an independent predictor of cardiac events in CHF patients. These results suggest that PTX3 could be considered as a universal risk factor in CHF patients.

In the present study, we indicated that both PTX3 and hsCRP were associated with cardiac events in CHF patients based on univariate Cox regression analysis; however, in the stepwise multivariate Cox regression analysis, only PTX3 was an independent predictor of adverse cardiac events in CHF patients. Our results are consistent with those of previous studies [13, 18], which suggest that PTX3 is a more reliable inflammatory predictor than CRP in CHF patients. Recent studies indicated that PTX3 was significantly related to hsCRP in CHF patients by correlation analysis [16, 18]. This association and the higher specificity of PTX3 for localized inflammation in the cardiovascular system [10, 11] might be responsible for the superior prognostic value of PTX3. In addition, our findings that age, NYHA class >II, and cTnI were also independently associated with cardiac events in CHF patients are consistent with the data in previous studies [20–22].

This study had several limitations. First, although this was a long-term prospective study, our data were derived from a relatively small number of patients in a single center in China; therefore, more studies must be performed in developing countries to confirm that PTX3 could be considered as a universal risk factor in CHF patients. Second, we only

Table 4: Univariate and multivariate Cox regression analysis of major adverse cardiovascular events.

	HR	95% CI	p value
Univariate analysis			
Age	2.609	1.371–4.992	<0.001
Males	0.760	0.534–1.082	0.128
Heart rate	1.189	1.058–1.328	0.003
NYHA class >II	3.493	2.089–5.848	<0.001
PTX3	4.399	3.081–6.281	<0.001
cTnI	1.591	1.296–1.954	<0.001
hsCRP	1.248	1.043–1.492	0.016
CHD	0.778	0.548–1.218	0.278
Diabetes mellitus	1.013	0.978–1.048	0.477
Hypertension	0.923	0.521–1.622	0.773
Hyperlipidemia	1.022	0.989–1.055	0.193
Current smoking	1.018	0.948–1.091	0.568
Aspirin	1.091	0.793–1.501	0.594
β-blockers	0.780	0.314–1.864	0.526
ACE-I/ARB	0.962	0.710–1.385	0.962
Diuretics	0.990	0.975–1.006	0.226
Multivariate analysis			
Age	2.518	1.030–6.158	0.020
PTX3	4.154	1.130–15.783	0.005
cTnI	1.808	1.208–2.686	0.008
NYHA class >II	3.018	1.818–4.998	<0.001

assessed the plasma PTX3 levels on the second morning after admission under fasting conditions. Serial measurements of PTX3 might be more useful for evaluating changes in inflammatory status in CHF patients and estimating risk during the follow-up period. Finally, our multifactorial Cox proportional hazards model did not include brain natriuretic peptide (BNP) or LVEF, which are strong predictors of CHF prognosis, due to insufficient data. Therefore, a multicenter trial with a large study population derived from different developing countries that includes BNP and LVEF data is necessary to validate the clinical importance of PTX3 in the future.

5. Conclusions

In conclusion, PTX3 could be considered as a universal risk factor and was more reliable than CRP in CHF patients. Measuring plasma PTX3 levels may substantially improve the risk stratification of CHF patients.

Conflict of Interests

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

Authors' Contribution

Liu Haibo and Guo Xiaofang contributed equally to this work as co-first authors. Yu Wanjun and Ge Junbo contributed equally to this work as co-last authors.

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Research Article

Kinetics of Highly Sensitive Troponin T after Cardiac Surgery

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Perioperative myocardial infarction (PMI) confers a considerable risk in cardiac surgery settings; finding the ideal biomarker seems to be an ideal goal. Our aim was to assess the diagnostic accuracy of highly sensitive troponin T (hsTnT) in cardiac surgery settings and to define a diagnostic level for PMI diagnosis. This was a single-center prospective observational study analyzing data from all patients who underwent cardiac surgeries. The primary outcome was the diagnosis of PMI through a specific level. The secondary outcome measures were the lengths of mechanical ventilation (LOV), stay in the intensive care unit (LOSICU), and hospitalization. Based on the third universal definition of PMI, patients were divided into two groups: no PMI (Group I) and PMI (Group II). Data from 413 patients were analyzed. Nine patients fulfilled the diagnostic criteria of PMI, while 41 patients were identified with a 5-fold increase in their CK-MB (≥120 U/L). Using ROC analysis, a hsTnT level of 3,466 ng/L or above showed 90% sensitivity and 90% specificity for the diagnosis of PMI. Secondary outcome measures in patients with PMI were significantly prolonged. In conclusion, the hsTnT levels detected here paralleled those of CK-MB and a cut-off level of 3466 ng/L could be diagnostic of PMI.

1. Introduction

Biomarkers are important diagnostic tools for addressing clinical problems. Recent changes in laboratory diagnostic power have resulted in these markers being incorporated into international guidelines and into the updated definition of myocardial infarction [1]. An ideal biomarker for myocardial infarction diagnosis should possess high sensitivity and specificity, also rapidly released and slowly eliminated, for early and late diagnoses. In addition, such a biomarker should be cost-effective and simple to use, without affecting patients' outcome or impacting their therapy [1]. Three subunits (C, I, and T) located on the myofibrillar thin (actin) filament of striated (skeletal and cardiac) muscle shape the backbone of the troponin complex. Cardiac muscle expresses the troponin T and I isoforms, so cardiac troponin T (cTnT) and I (cTnI) are more specific than creatine kinase (CK) values for myocardial injury and, owing to their high sensitivity, may rise when creatine kinase MB (CK-MB) concentrations do not [2].

Many contributors may raise cardiac enzyme levels after cardiac surgery, such as acute coronary syndrome (ACS) related to recent myocardial infarction (AMI) before the surgery [3], or directly related to cardiac surgery in perioperative myocardial infarction (PMI), such as inadequate cardiac protection, reperfusion injury, and direct surgical trauma [4]. Such elevations during cardiac surgery may not be ACS-related, as these enzymes may be already elevated in patients with end-stage renal disease [5], acute pericarditis, acute heart failure (AHF) [6], sepsis [7], or rhabdomyolysis [8].

The PMI could not be solely attributed to coronary surgery (whether on- or off-pump), as it might also be associated with isolated valvular surgeries, although this association is not common [9, 10]; McGregor et al. reported an incidence of 4% with valvular surgeries [10].

Highly sensitive troponin T (hsTnT) is a reliable biomarker with high sensitivity and negative predictive values compared with conventional troponin (cTn) [11]. For the diagnosis of acute myocardial infarction (AMI), hsTnT offers excellent diagnostic performance even with early presentation to the emergency department [12] and some evidence exists for a better diagnostic accuracy than cTn [13].

Open heart surgeries carry a well-established risk of PMI [14]; 2–5% incidence had been reported with intense mortality and morbidity [15]. Since the prognosis of patients after cardiac surgery must also be addressed, a marker with predictive power in both short- and long-term mortality should be optimum. In a meta-analysis by Lurati Buse et al., the authors studied the prognostic value of cTn in cardiac surgery settings, where postoperative cTn release was found to be associated with mid- and short-term all-cause mortality (12 mo and 30 d, resp.) [16]. The universal diagnosis of PMI is based on an increase in the CK-MB by more than 5 times the 99th percentile upper reference level plus either new pathological Q-waves or left bundle branch block (LBBB) on 12 lead electrocardiogram (ECG), image evidence of new viable myocardium loss, or angiographic findings of native coronary occlusion or new graft occlusion [17]. The role of hsTnT in the diagnosis of PMI has not yet been defined.

2. Aim of the Study

The aim of the study is to define the role of hsTnT in diagnosing PMI in the cardiac surgery setting.

3. Methods

3.1. Study Design. This is a single-center prospective observational study conducted over a 2-year period (October 2011 to September 2013) in a 12-bed cardiothoracic ICU of a Qatari heart hospital (Hamad Medical Corporation, Doha, Qatar). The ethical committee gave the approval to conduct the study (reference number 13223/13), with a waiver of informed consent, as no specific intervention was performed and no extra blood sampling was required. Patients with chronic renal impairment, sepsis, or a preexisting high level of hsTnT were excluded (unless postoperative difference is significant by more than a 50% increment). Patients with marked intraoperative hypotension (mean arterial blood pressure less than 80 for more than 5 min) were also excluded (32 patients).

We enrolled 413 patients who underwent cardiac surgeries. Based on the association of PMI, patients were divided into two groups (Tables 3 and 4). According to their hsTnT levels (corresponding to a 5-fold increase in CK-MB), patients were further divided into 2 different groups (Tables 6 and 7).

3.2. Study Assessments. The following datasets were recorded for all patients: age, sex, existing diabetes or hypertension, type of surgery, anesthesia time, cardiopulmonary bypass (CPB) time, aortic cross clamp (ACC) time, use of inotropes and vasopressors, EuroSCORE, and statin therapy. Length of mechanical ventilation (LOV), stay in the ICU, and stay in the hospital were also recorded. Routine renal and liver functions were recorded. Outcome variables including acute kidney injury (AKI), postoperative atrial fibrillation (POAF), infection, stroke, wound infection, and death were noted for each patient. Data were retrieved using Dendrite Clinical Systems (London, UK). Blood samples for hsTnT and CK-MB looking for myocardial injury were collected in the first 24 h after surgery at 6 h intervals and then as per clinical indications. The hsTnT was measured using COBAS Troponin T hs (highly sensitive) STAT (short turn-around time) (Roche Diagnostics). ECG was performed routinely before and immediately after the surgery and then every 12 h. Transthoracic echocardiography was requested when indicated to trace new regional wall motion abnormalities in patients with high levels of cardiac enzymes and patients requiring high doses of inotropes.

3.3. Outcome Definitions. The primary outcome was the diagnosis of PMI. This was defined as a 5-fold increase in CK-MB plus one of the confirmatory criteria, including ECG, echocardiographic, or angiographic evidence. The secondary outcome measures were the length of mechanical ventilation (LOV), length of stay in the intensive care unit (LOSICU), and length of hospitalization. Based on the third universal definition of PMI (a fivefold increase in the CK-MB plus one confirmatory criterion), patients [17] were divided into two groups: no PMI (Group I) and PMI (Group II). Group II accounted for 2.17% of the study population.

3.4. Statistical Analysis. Normally distributed continuous variables were expressed as mean ± standard deviation (SD). Skewed variables were presented as median (interquartile range (IQR)). Patients were divided into two groups based on the existence of PMI. The groups were compared through parametric and nonparametric tests or by chi-square tests, as appropriate. Significant association was defined by a probability (P) value ≤ 0.05. Correlations of log hsTnT were first examined by single variable linear or logistic regression and presented as nonadjusted coefficient (NAC) and 95% confidence interval (95% CI). Analysis was done with and without adjustment of age, gender, and BMI. Factors with a P value ≤ 0.05 by single variable regression analyses were included in a multivariable linear regression model, presented as adjusted coefficient (AC) (95% CI) [18]. Receiver operating curve (ROC) was used to test the validity of hsTnT as a marker of PMI and to assess sensitivity and specificity. Statistical analyses were performed using the SPSS software (version 19, Chicago, IL, USA).

4. Results

Of the 445 patients screened, 32 were excluded; hence 413 were enrolled. The study population had a mean age of 54.9 ± 10.9 years and was predominantly male 349 (86.9%). In addition, 48.9% of the patients were diabetics and 45.6% were hypertensive. The majority of patients underwent CABG surgery (84%) (Tables 1 and 2). When patients fulfilled the diagnoses of PMI [16], ROC (Figure 1) was used to draw a corresponding level of hsTnT; a level of 3466 ng/L or above

Table 1: Description of the studied group.

Variable	Number	Minimum	Maximum	Mean ± SD
Age	413	19	85	54.9 ± 10.9
BMI (kg/m^2)	412	14.5	44.8	27.4 ± 5.1
Creatinine (micromole/L)	413	43	145	92.4 ± 53.1
EF%	413	20	65	48.6 ± 10
Additive EuroSCORE	411	0	17	3.6 ± 2.9
CPB time (minutes)	290	0	342	121.1 ± 48.1
ACC time (minutes)	286	0	215	77.2 ± 35.1
CK-MB (U/L)	413	3	737	76.9 ± 44.4
hsTnT (ng/L)	413	24	66299	1812.2 ± 111.5
Anesthesia time (minutes)	413	180	378	333.3 ± 97
LOV (minutes)	409	181	12980	566 ± 444
LOS_{ICU} (hours)	410	15	491	147 ± 68
LOS_{hosp} (days)	410	4	49	31.7 ± 29.9

BMI: body mass index; EF: ejection fraction; HgA1C: glycated hemoglobin; CPB: cardiopulmonary bypass; ACC: aortic cross clamp; hsTnT: high sensitive troponin T; LOV: length of mechanical ventilation; LOS_{ICU}: length of stay in ICU; LOS_{hosp}: hospital length of stay.

Table 2: Intergroup statistics.

Variable	Number (%)
Gender	
Male	359 (86.9)
Female	54 (13.9)
Hypertension	183 (45.6)
Diabetes	194 (48.9)
Operative urgency	
Elective	222 (53.1)
Urgent	107 (25.6)
Emergent	13 (3.1)
Surgery type	
CABG	347 (84)
Valvular surgery	60 (14.5)
Aortic dissection	6 (1.45)
Outcome	
Readmission to ICU	11 (2.6)
Reexploration	35 (14.5)
POAF	19 (4.6)
AKI	117 (28.3)
Mortality	12 (2.9)

CABG: coronary artery bypass graft; POAF: postoperative atrial fibrillation; AKI: acute kidney injury.

Table 3: Clinical and laboratory variables in both groups.

Variable	Group I $N = 404$ (%)	Group II ($N = 9$)	P value
Age	54.9 ± 10.8	55 ± 12.8	0.56
Sex, male	352 (87.1)	7 (86.4)	0.1
Hypertension	178 (44)	5 (55.5)	0.15
Diabetes	188 (46.5)	6 (66.6)	0.01
BMI	28.2 ± 5.8	32 ± 10.7	0.6
EuroSCORE	3.65 ± .5	4 ± 0.3	0.87
Basal creatinine (micromole/L)	90.5 ± 44.2	86 ± 20.4	0.21
EF%	44.7 ± 7.6	42.7 ± .1	0.4
Surgery (elective)	97 (66.4)	57 (70.4)	0.35
Inotrops			
Dopamine	30 (7.4)	7 (77.7)	0.01
Adrenaline	23 (5.7)	3 (22.2)	0.03
Noradrenline	55 (13.6)	8 (88.8)	0.01
Dobutamine	3 (0.7)	2 (33.3)	0.01
Surgery			
CABG	336 (83.3)	8 (88.8)	0.4
Valvular	65 (16.1)	1 (11.1)	
Aortic disssection	5 (1.2)	1 (11.1)	
Highest CKMB	1938.14 ± 543.1	8169.11 ± 4690.1	0.000
Highest hsTnT	54.62 ± 14.1	167.56 ± 68.387	0.000

IDDM: insulin dependent diabetes mellitus; NIDDM: non-insulin-dependent diabetes mellitus; BMI: body mass index; HbA1C: glycated hemoglobin; EF: ejection fraction; CABG: coronary artery bypass graft.

showed 90% sensitivity and 90% specificity for diagnosis of PMI with C-statistics of 0.90 (0.75–1.0). Patients with PMI had worse outcome and more complications and constituted 2.17% of the study population (Tables 3 and 4).

We found that a hsTnT level of 2309 ng/L corresponds to the CK-MB level that is diagnostic of PMI without the additional criteria mentioned by Lurati Buse et al. [16], C-statistics of 0.87 (0.80–0.94), which showed 80% sensitivity and 86% specificity for the diagnosis of possible myocardial injury (Figure 2); therefore we conventionally considered that level as an indicator of myocardial injury and patients who were compared based on this level (Tables 5 and 6) included Groups II and IV. The two groups were well matched for

Table 4: Clinical outcome in both groups.

Variable	Group I ($N = 404$)	Group II ($N = 9$)	P value
Intraoperative parameters			
CPB time (minutes)	120.7 ± 37	134.8 ± 52.8	0.47
ACC time (minutes)	77.1 ± 34.8	77.2 ± 50.7	0.1
Anesthesia time (minutes)	6.8 ± 1.5	7 ± 1.9	0.9
Postoperative parameters			
LOV median (range) (minutes)	422 ± 211 (181–1440)	1567 ± 597 (260–129800)	0.000
LOS_{ICU} median (range) (hours)	61.6 ± 9.8 (15–320)	408.4 ± 70.5 (46–491)	0.05
LOS_{hosp} median (range) (days)	12.18 ± 2.5 (3.7–25)	18.78 ± 7.6 (5.3–49)	0.000
Postoperative complication			
POAF	14 (3.4)	5 (55.6)	0.01
AKI	120 (29)	7 (77.8)	0.04
VAP	4 (1)	2 (22.2)	
Readmision ICU	9 (2.2)	2 (22.2)	0.01
Reexploration	30 (7.4)	5 (55.6)	0.001
In-hospital mortality	9 (2.2)	3 (33.3)	0.01

CPB: cardiopulmonary bypass; ACC: aortic cross clamp; LOV: length of mechanical ventilation; LOS_{ICU}: ICU length of stay; LOS_{hosp}: hospital length of stay; POAF: postoperative atrial fibrillation; AKI: acute kidney injury; VAP: ventilator associated pneumonia.

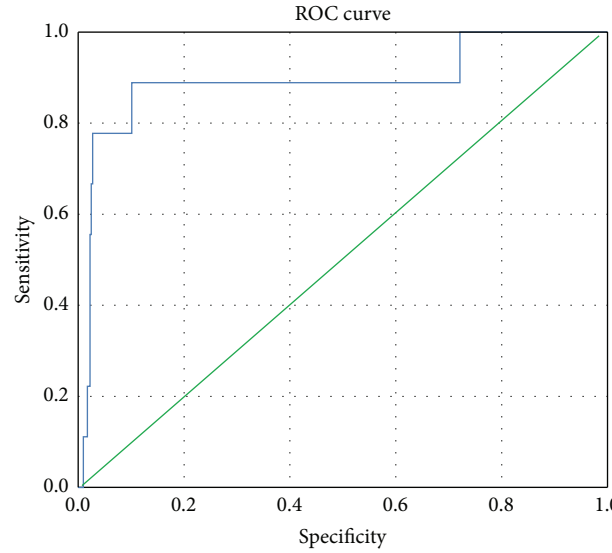

Figure 1: ROC for hsTnT associated definitive PMI. Receiver operating characteristic (ROC) curve for diagnostic level of highly sensitive troponin T (hsTnT) suggestive of perioperative myocardial infraction. ROC was used to discriminate hsTnT level based on CKMB cut points (above and below 120 for CKMB); 87% accuracy was detected with 95% confidence interval. Total number = 413; number of patients with definitive myocardial infarction = 9. Area under the curve (AUC) for *hsTnT* is 0.87 with $P = 0.001$.

age, gender, BMI, basal creatinine, association with diabetes, hypertension, and EuroSCORE. Patients with hsTnT levels of 2309 ng/L or below had a better outcome in terms of inotropes needed, length of ventilation, ICU, and hospital stay, as well as postoperative complications. We performed a multivariate analysis for significant results within the given cutoff of hsTnT (of 2309 ng/L) and found significant relations of the given level with operative emergency ($P = 0.001$); this level is a predictor for longer duration of mechanical ventilation ($P = 0.01$) and POAF ($P = 0.003$) (Table 7). This was repeated after adjustment for age, gender, and BMI, giving the same significance level.

5. Discussion

The salient findings of this study were the identification of similar PMI incidence as reported in other studies (2.2%) and identification of hsTnT levels that corresponds to CKMB levels indicative of myocardial injury and PMI where additional criteria were included. Finally, both levels of hsTtnT were associated with poor outcome.

In cardiac surgery settings, whether coronary artery bypass grafting or valvular surgeries, variable incidences of PMI exist, 4% in the former and up to 5% in the latter [10, 15]. In our study, the incidence of PMI for CABG and valvular surgeries were 2.3 and 1.7%, respectively. Whatever the incidence, PMI is a serious condition with a high mortality and morbidity. Thus, proper diagnostic and treating tools are needed to manage the expected high risk.

In managing patients after cardiac surgeries, early diagnosis of PMI and prediction of related morbidity are the elements that carry the greatest impact on clinical course (i.e., treatment and survival). In this context, looking for distinctive markers seems to be an ideal goal. An ideal marker should possess early diagnostic and prognostic properties. In our study, we used hsTnT, which is confirmed as offering higher sensitivity and specificity than conventional troponin (cTnI) [11].

5.1. Diagnostic Cutoff. We aimed to identify a diagnostic cutoff that carries high sensitivity and specificity for hsTnT, as compared to CK-MB, in the cardiac surgery setting. Thygesen and colleagues first proposed diagnostic criteria for PMI with CK-MB [17]. Lim et al. subsequently reported that the cardiac troponin I (cTnI) test at 1 h after CABG could potentially differentiate patients with significant revascularization injury; a cutoff of cTnI exceeding 5 μg/L at 1 h had 67% sensitivity and 79% specificity for detecting new late gadolinium enhancement in cardiac magnetic resonance image as confirmatory [19]. We hypothesize that quantitative evaluation of hsTnT cutoff could represent a better diagnostic tool. We found that when patients fulfill diagnoses of PMI [17], ROC analysis reported that the hsTnT level of 3466 ng/L or above is associated with 90% sensitivity and 90% specificity for diagnosis of PMI (Figure 1), where the level of 2309 ng/L is associated with 80% sensitivity and 86% specificity for an equivalent level of CKMB (Figure 2) (5-fold higher than

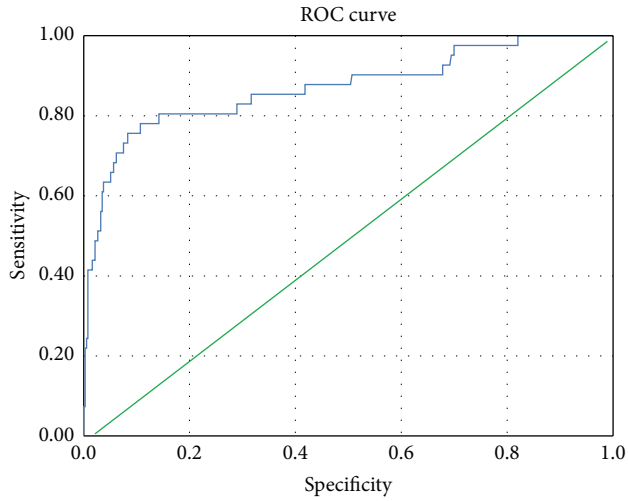

Figure 2: ROC for hsTnT associated high normal value of CK-MB. Receiver operating characteristic (ROC) curve for level of highly sensitive troponin T (hsTnT) suggestive of myocardial injury. ROC was used to discriminate hsTnT level based on CKMB cut points (above and below 120 for CKMB); 89% accuracy was detected with 95% confidence interval. Total number = 413; number of patients with suspicion of myocardial injury = 41. Area under the curve (AUC) for *hsTnT* is 0.89 with $P = 0.0001$. Diagonal segments are produced by ties.

the normal level), which would be suspicious of myocardial injury (Tables 5 and 6). No previous studies have addressed a diagnostic cutoff for hsTnT.

5.2. Prediction of Mortality and Morbidity. Prediction of outcome in terms of short-term mortality could be achieved in noncardiac surgery through cardiac troponin I [20]. Contemporary assays should supply an appropriate diagnostic performance, where high sensitivity is a basic need. Patients' prognostication in terms of risk and possible adverse events through monitoring technique provide a great value for clinicians, allowing adjustments of preventative as well as therapeutic interventions; hsTnT is thus a marker that could help in modern clinical laboratories [21]. In our study, we hypothesize that hsTnT could provide better diagnostic and prognostic properties than conventional CKMB. This was consistent with Freund et al., who stated that higher sensitivity is attributed to hsTnT when compared with conventional cTnI in patients with low to moderate MI probability [11].

Gillies et al. pointed to the high incidence of myocardial injury after major noncardiac surgeries, where hsTnT concentration could exceed the 99th percentile in 45% of patients. The authors did not find an association between the peak postoperative troponin level and outcome [22]. The latter was contrary to Nagele et al., who found a significant association between postoperative elevation of hsTnT with MI and long-term mortality after noncardiac surgery [23]. James et al. found that base troponin provides information related to the 30-day mortality in ACS and, when it is combined with C-reactive protein, could provide a better risk identification [24].

Table 5: Clinical and laboratory variables for CKMB discrimination.

Variable	Group III CKMB < 120 $N = 372$ (%)	Group IV CKMB ≥ 120 ($N = 41$)	P value
Age	55.2 ± 10.6	51.7 ± 12.8	0.4
Sex, male	324 (78.8)	35 (85.3)	0.5
Hypertension	164 (44)	19 (55.5)	0.4
Diabetes	172 (44.3)	22 (66.6)	0.01
BMI	28.4 ± 5.8	28.1 ± 7.2	0.23
EuroSCORE	3.8 ± 0.4	4.1 ± 0.36	0.7
Basal creatinine (micromole/L)	90.2 ± 44.4	91.9 ± 38.4	0.6
EF%	48.8 ± 10	47.1 ± 9.1	0.8
Surgery (elective)	194 (66.4)	16 (70.4)	0.08
Inotrops			
Dopamine	27 (7.2)	10 (24.3)	0.03
Adrenaline	18 (4.8)	8 (19.5)	0.04
Noradrenline	48 (12.9)	15 (36.5)	0.01
Dobutamine	2 (0.5)	3 (7.3)	0.01
Surgery			
CABG	314 (84.4)	33 (80.4)	0.3
Valvular	53 (14.2)	7 (17)	0.3
Aortic disssection	5 (1.3)	1 (2.4)	
Highest CKMB	36.7 ± 22	242 ± 93	0.000
Highest hsTnT	1434.14 ± 150	7884 ± 190	0.000

IDDM: insulin dependent diabetes mellitus; NIDDM: non-insulin-dependent diabetes mellitus; BMI: body mass index; HbA1C: glycated hemoglobin; EF: ejection fraction; CABG: coronary artery bypass graft.

In heart surgery, variable events could be the cause of an elevation of hsTnT, including direct surgical trauma and incomplete cardiac protection [4], end-stage renal disease [5], acute pericarditis, acute heart failure (AHF) [6], sepsis [7], and rhabdomyolysis [8]. In our study, we excluded patients with ESRD, sepsis, and preexisting high level of hsTnT.

In our study, we used equivalent levels of HsTnT to high/normal levels of CKMB to define groups. Lehrke et al., 48 h after cardiac surgery, utilized a cTnT concentration of >0.46 ng/mL to predict mortality [25]. Both groups in our work (III and IV) were matched regarding the age, sex, existence of diabetes or hypertension, BMI, EuroSCORE, basal creatinine, and EF%. The emergency of surgery did not show statistical differences, nor did the type of surgery (whether CABG or valvular). Patients with a higher level of hsTnT required more inotropes (Table 5). Similarly, Mohammed et al. found a highly significant correlation between the need for inotropic support and troponin elevation in cardiac surgery [26].

The length of the surgery did not differ between the groups; however, the lengths of CPB and ACC were significantly higher in patients with higher levels of hsTnT (Table 6).

Table 6: Clinical outcome for CKMB discrimination.

Variable	Group III CKMB < 120 $N = 372$ (%)	Group IV CKMB ≥ 120 ($N = 41$)	P value
Intraoperative parameters			
CPB time (minutes)	119 ± 43	135 ± 69.6	0.06
ACC time (minutes)	77.1 ± 33	77.4 ± 48.5	0.09
Anesthesia time (minutes)	6.8 ± 1.4	6.7 ± 2	0.9
Postoperative parameters			
LOV median (range) (minutes)	402 ± 45.1 (181–1227)	767 ± 130 (198–129800)	0.001
LOS_{ICU} median (range) (hours)	57.4 ± 8.9 (15–320)	172.4 ± 37.5 (46–491)	0.000
LOS_{hosp} median (range) (days)	11 ± 3.2 (3.7–21)	15.8 ± 2 (3.3–49)	0.000
Postoperative complication			
POAF	12 (3.2)	7 (17)	0.04
AKI	102 (27.4)	15 (36.5)	0.03
VAP	4 (1)	2 (4.9)	
Readmission ICU	9 (2.4)	2 (4.9)	
Reexploration	24 (6.5)	11 (26.8)	0.05
In-hospital mortality	7 (1.9)	5 (12.1)	0.009

CPB: cardiopulmonary bypass; ACC: aortic cross clamp; LOV: length of mechanical ventilation; LOS_{ICU}: ICU length of stay; LOS_{hosp}: hospital length of stay; POAF: postoperative atrial fibrillation; AKI: acute kidney injury; VAP: ventilator associated pneumonia.

Table 7: Multivariate logistic regression analysis for hsTnT cutoff (2309 ng/L).

Variable	Adjusted OR	95% CI	P value
Operation emergency	10.2	2.5–41.3	0.001
LOV	1.01	1.002–1.02	0.01
AKI	0.84	0.32–2.20	0.72
POAF	4.79	1.7–13.53	0.003
Mortality	3.7	0.42–32.9	0.24

CI: confidence interval; LOV: length of ventilation; AKI: acute kidney injury; POAF: postoperative atrial fibrillation.

This was consistent with Järvinen et al., who performed a multivariate logistic regression analysis that concluded that long CPB is an independent predictor for PMI [27]. Length of mechanical ventilation and lengths of stay in the ICU and hospital were all significantly higher in patients with a higher level of hsTnT. Gamble et al. expressed the prognostic value in the settings of MI [28]. The incidence of complications was significantly higher in patients with a higher level of hsTnT in terms of POAF, reexploration, and mortality, where ventilator associated pneumonia (VAP) and ICU readmission were also higher but without statistical significance (Table 6). Jaffe argued that patients with high levels of hsTnT are more likely to have a troublesome course than those without similar elevation [29]. We excluded patient with preexisting high levels of hsTnT, except when the postoperative level difference was significantly high. Patients with a marked intraoperative hypotension were also excluded due to previous reports of hsTnT elevation being associated with preoperative levels [30] and intraoperative hypotension [29, 31].

Weber et al. used the additive value of the hsTnT, revised cardiac index, and N terminal probrain natriuretic peptide (NT-proBNP) to predict adverse cardiac events in major noncardiac surgeries. The authors found that hsTnT was the strongest independent risk predictor. Furthermore, the authors found that high perioperative levels of both cardiac markers were associated with the length of hospital stay and the necessity of intensive care treatment [32].

We performed multivariate analyses for the significant results within the given cutoff of hsTnT (of 2309 ng/L) and found a significant association of the given level with procedures in the emergency settings ($P = 0.001$); the level is predictor for longer duration of mechanical ventilation ($P = 0.01$) and POAF ($P = 0.003$) (Table 7). This was consistent with Beckman, who stated that prediction of all-cause mortality, as well as cardiovascular morbidity, could be achieved through troponin elevation [33]. Hernández-romero et al. similarly found that presurgical hsTnT elevation was associated with the development of POAF events, unlike the N terminal probrain natriuretic peptide (NT-proBNP) [34], whereas Laine argued that cardiac troponin may not carry prognostic information in asymptomatic patients who lack electrocardiography changes [35]. However, the study population consisted of noncardiac surgery patients.

Our results found that 15 patients (36.5% with higher level of hsTnT) (Table 6) had AKI. Evidence suggested that cardiac troponin could increase in patients with chronic renal failure even in the absence of myocardial ischemia and may add to the complexity of diagnosing ACS in this group of patients [36]. The latter authors suggested referring to a preset level of troponin when attempting to evaluate myocardial injury. Dubin et al. studied hsTnT in 81 subjects with renal failure, finding similar increased levels. However, in our study, we excluded patients with chronic renal failure [37]. Whether the rise of hsTnT in our study was a result or a cause of AKI could not be determined with certainty; however, Aviles et al. reported that, regardless of the creatinine clearance levels in patients with ACS, short-term prognosis could be predicted with cardiac troponin T levels [38].

5.3. Perioperative Myocardial Infarction. We found the diagnostic level of hsTnT in PMI to be 3466 ng/L or above, which is associated with 90% sensitivity and 90% specificity when including one of the confirmatory criteria, as in CK-MB [17] (Tables 3 and 4). Patients with PMI had a poorer outcome and more complications. Excellent diagnostic performance of hsTnT assays was reported, which substantially improved diagnosis of AMI in the early phase [12]. Nagele et al. highlighted the power of increased levels of hsTnT in a study including 625 patients that had undergone major noncardiac surgery, which serves for risk stratification and could be used as a tool to quantify myocardial injury in patients with cardiovascular risk factors [39]. Higher sensitivity as well as

specificity due to CTnI was described when compared with CK-MB for diagnosing PMI after cardiac surgery [40], which is in line with our findings using hsTnT.

5.4. Strengths and Limitations. Our study sheds new light on the utilization of hsTnT as a prognostic tool, where a set level could differentiate PMI and another level was associated with morbidity and mortality after cardiac surgery. This study has the limitations of being performed at a single center, lacking a supportive measure to detect the extent of myocardial loss of viability and relates it to the used cutoff without PMI. The significant association of AKI in the high HsTNT group should be further investigated. The study population was predominantly male because the Qatari population consists of only ~20% natives, with the remaining 80% being expatriate. The majority of the latter are male.

Further studies are needed, either with large number of patients or multicenter study, to confirm our cutoff levels. Long-term follow-up studies are also needed.

6. Conclusion

The hsTnT levels detected here paralleled those of CK-MB and a cutoff level of 3466 ng/L could be diagnostic of PMI. Further studies are required to validate this finding. Secondary outcome measures in patients with PMI (i.e., LOV and LOS_{ICU}) were significantly prolonged.

Recommendations and Future Directions

Recommendations and future directions are as follows:

(1) Utilization of hsTnT cutoff to diagnose PMI in association with other diagnostic tools.

(2) Utilization of high level cutoff to prognosticate outcome after cardiac surgeries.

(3) High incidence of PMI after cardiac surgeries.

(4) Considering AKI when interpreting hsTnT in a justified study.

Key Messages

Key messages are as follows:

(1) Power of hsTnT to predict the outcome after cardiac surgeries.

(2) Ability to have hsTnT cutoff to diagnose PMI.

(3) Association of poor outcome and more complication in patients with hsTnT even without PMI.

(4) Value of frequent hsTnT monitoring after cardiac surgery.

(5) Value of assay of hsTnT from a reference in patients with preoperative high level.

Abbreviations

ACC: Aortic cross clamp
ACS: Acute coronary syndrome
AKI: Acute kidney injury
BG: Blood glucose
CABG: Coronary artery bypass graft
CAD: Coronary artery disease
CK: Creatine kinase
CK-MB: Creatine kinase MB
cTnI: Cardiac troponin I
cTnT: Cardiac troponin T
CPB: Cardiopulmonary bypass
HbA1c: Glycated hemoglobin
hsTnT: Highly sensitive troponin T
ICU: Intensive care unit
LOS: Length of stay
LOV: Length of ventilation
POAF: Postoperative atrial fibrillation
PMI: Perioperative myocardial infarction
ROC: Receiver operating curve
TnT: Cardiac troponin T.

Consent

The ethics review panel waived informed consent for all patients enrolled in the study. However, all study data were maintained anonymously.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Amr S. Omar carried out study design and contributed to concept, writing the paper, and revising the final form. Suraj Sudarsanan was responsible for data collection, interpretation of data, and revising the paper. Samy Hanoura was responsible for data collection and paper revision. Hany Osman was responsible for data collection. Praveen C. Sivadasan was responsible for data management and paper revision. Yasser Shouman was responsible for revision of the paper. Alejandro Kohn Tuli was responsible for data interpretation. Rajvir Singh conducted statistical analysis. Abdulaziz Al Khulaifi suggested the idea and, as a chair of the department, provided general support and substantial contribution to concept and design and acquisition of data. All authors read and approved the final paper.

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Research Article

Serum Caveolin-1 as a Novel Biomarker in Idiopathic Pulmonary Artery Hypertension

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Pulmonary arterial hypertension (PAH) is a rare disease but with significant morbidity and high mortality. There is no specific way to diagnose PAH. Thus, an easy used with good sensitivity and specificity biomarker of PAH is highly desirable to aid in the screening, diagnosis, and follow-up. Caveolin-1 (Cav1) is the structural protein of caveolae and is highly expressed in type I pneumocytes. Lungs tissues from idiopathic PAH (IPAH) patients showed decreased expression of Cav1 in vascular endothelial cells. Therefore, we developed a direct sandwich immunoassay for the determination of Cav1 in IAPH patient's serum. The result disclosed serum Cav1 level was significantly lower in IPAH than control groups. Using serum Cav1, 17.17 pg/mL as a cutoff value, the sensitivity was 0.59 and the specificity was 1.0. There were two major findings in our results. First, serum Cav1 might be a novel biomarker in the diagnosis of IPAH with fare sensitivity and good specificity. Second, Cav1 might be used to make differential diagnosis between COPD-PH and IPAH group.

1. Introduction

Pulmonary arterial hypertension (PAH) is a rare disease but with significant morbidity and high mortality. Annual incidence is 1-2 cases per million people in the USA and it is 2–4 times as common in women as in men [1, 2]. In untreated patients, the median survival rate is only 2.8 years, and the 5-year survival rate is 34% [3]. There is no specific way to diagnose PAH. According to the American and European clinical practice guidelines [4–6], the diagnosis involves sequences of steps and requires several invasive and noninvasive examinations. Well experienced specialists are needed to interpret the results and manage these patients [7, 8]. Thus, sensitive and specific biomarkers of PAH are highly desirable to aid in the screening, diagnosis, and follow-up.

Previous studies have suggested that atrial natriuretic peptide (ANP), N-terminal probrain natriuretic peptide (NT-proBNP), troponin, and uric acid are potential biomarkers for PAH [9–12]. However, these are not specific biomarkers of the pathology changed of the pulmonary artery hypertension. Endothelial cell dysfunction, proliferation without apoptosis, and vasoconstriction may play important roles in PAH; therefore vascular bed may be a good source of new biomarkers [13–15].

Caveolae are 50–100 nm vesicular invaginations of the cell plasma membrane and caveolin-1 (Cav1) is the structural protein of caveolae and is highly expressed in adipocytes, endothelial cells, and type I pneumocytes. $Cav1^{-/-}$ mice exhibit pulmonary hypertension and right ventricle hypertrophy [16–18]. In monocrotaline-induced PH rat models, Cav1 deficiency is seen in lung tissue [19]. Lungs tissues from idiopathic PAH (IPAH) patients decreased expression of Cav1 in vascular endothelial cells and also decreased in the total lung lysate [20, 21]. Furthermore, Cav1 can be secreted into serum and be detected [22]. These results suggested that Cav1 may play an important role in the pathogenesis of PAH and serum Cav1 level may be a good biomarker for diagnosis [23, 24].

2. Materials and Methods

In the study, age matched patients with normal left ventricle function were divided into 3 groups. In Group (1) IPAH patients ($n = 21$), definite diagnosis was made according to European Society of Cardiology (ECS) [4] and American Heart Association (AHA/ACC) [5] guideline, inclusion criteria including mean pulmonary artery pressure (mPAP) $\geq$ 25 mmHg, pulmonary wedge pressure less or equal to 15 mmHg, and pulmonary vascular resistance over 3 Wood units measured by right heart catheterization. In Group (2) chronic obstructive pulmonary disease with pulmonary hypertension (COPD-PH) patients ($n = 22$), COPD were diagnosed by pulmonologist and estimated mean PAP $\geq$ 25 mmHg by echocardiography. Group (3) (non-PAH group) includes healthy volunteers ($N = 26$) with mPAP less than 15 mmHg measured by echocardiography.

Demographic data and clinical features of patients included in this study were summarized in Table 1. According to Tahir et al. reports [23, 24], we developed a direct sandwich immunoassay for the determination serum Cav1 level from participants. Serum hsCRP, NT-proBNP, and BMPR2 levels were also measured by commercial ELISA kits. This study was approved by Local Ethical Committee in Taichung Veterans General Hospital, Taichung, Taiwan (number CE12022). Written informed consent was provided to all participants.

2.1. Protocol for Serum Cav1 Assay. Two commercial affinity purified monoclonal mouse Cav1 antibodies and polyclonal rabbit Cav1 antibodies were chosen for a direct sandwich ELISA. The capture Cav1 antibody used was generated from human recombinant Cav1 (R&D systems), and the detection antibody was HRP-conjugated rabbit polyclonal antibody raised against a peptide mapping at the NH_2 terminus of human Cav1 (Santa Cruz Biotechnology). Costar microplate wells were coated with 100 μL of Cav1 antibody (2.5 mg/L) in PBS (pH 7.4) and incubated overnight at 4°C. The wells were then blocked with 300 μL of PBS containing 0.5% BSA and 0.05% v/v Tween 20 for 2 hours at room temperature and were washed three times with PBS containing 0.5% v/v Tween 20 (PBST). Serum samples, calibrators, and controls were added (100 μL) to the wells and incubated overnight at 4°C. The wells were washed three times with 400 μL of PBST and 100 μL of HRP-conjugated Cav1 (Santa Cruz Biotechnology) antibody diluted 1 : 2000 in PBST. After incubation for 2 hr at room temperature, the wells were washed three times with PBST, and 100 μL of 3,3′,5,5′-tetramethylbenzidine substrate solution (Clinical Science Products, Inc.) was added and incubated for 30 min at room temperature. The reaction was stopped by adding 100 μL of 1 N H_3PO_4, and the absorbance was read at 450 nm with a microplate reader (TECAN, Grödig, Austria). Serum Cav1 levels were measured using lab-made recombinant pQE30-Cav 1 (a 101-amino acid region of Cav1 gene from GenBank accession number NM001753) as a standard. A linear standard curve was constructed using a concentration range (12.19–780 pg/mL) of recombinant Cav1 in a parallel ELISA. The levels of Cav1 in sera of 21 IPAH patients, 22 COPD-PH patients, and 26 healthy controls by the in-house ELISA were then measured. The limit of detection of the sandwich ELISA was 12.19 pg/mL. Any value below the detective limitation of the assay referred to zero. In Figure 1, Western blot data using capture and detection antibodies that react to recombinant Cav1 proteins was showed.

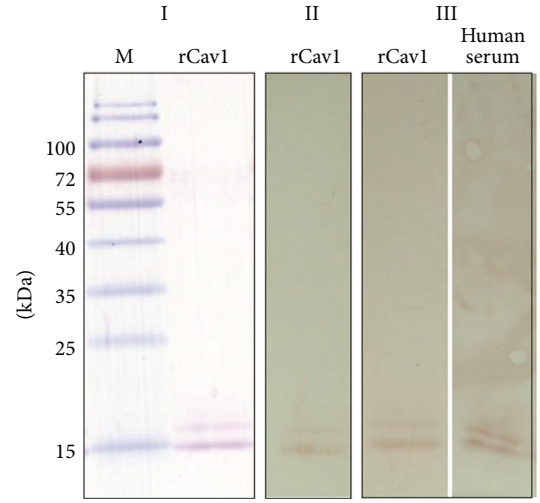

Figure 1: Specificity of capture and detection antibodies to rCav-1 and native Cav1 of human serum. Recombinant Cav1 protein was purified using affinity chromatography and analyzed by SDS-PAGE (I) and immunoblotting (II and III). In panel I, the rCav-1 protein migrated as a single band and displayed >95% purity by Coomassie blue staining. The binding specificity of the capture and detection antibodies to rCav-1 and human serum was demonstrated and showed in panel II and panel III, respectively. Numbers on the left indicate sizes of protein markers (lane M).

2.2. Enzyme-Linked Immunosorbent Assay for HsCRP, NT-proBNP, and BMPR2 Detection. The serum levels of high-sensitivity C-reactive protein (hsCRP), NT-proBNP, and bone morphogenetic protein type II receptor (BMPR2) were measured with enzyme-linked immunosorbent assay (ELISA) kits, hsCRP (Cell Biolabs, Inc., San Diego, CA), NT-proBNP (Invitrogen Corporation, Camarillo, CA), and BMPR2 (MyBioSource, San Diego, CA) according to the manual.

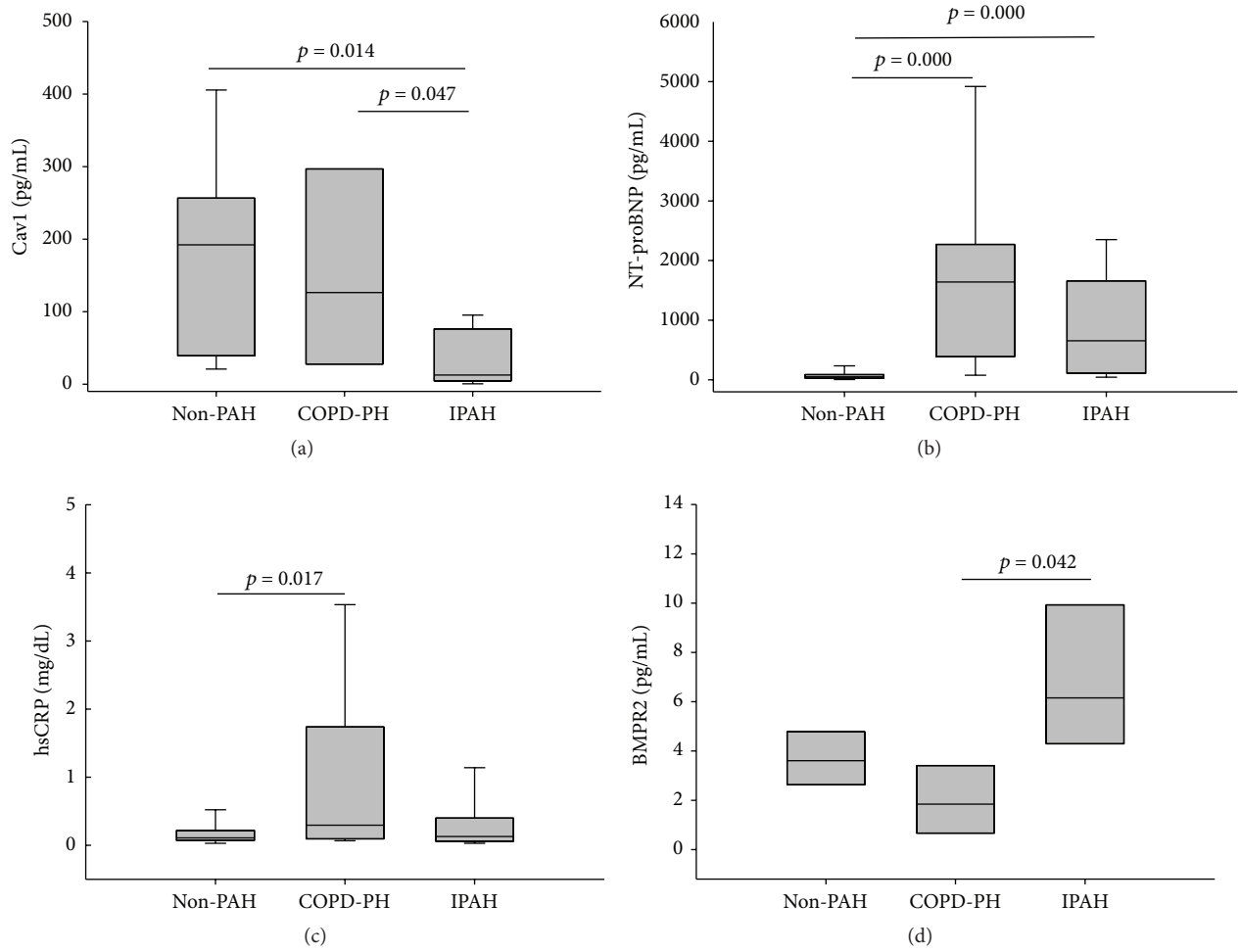

Figure 2: Serum biomarker levels in PAH patients and control subjects. PAH: pulmonary artery hypertension, COPD-PH: chronic obstructive pulmonary disease with pulmonary hypertension, and IPAH: idiopathic PAH.

2.3. Statistical Analysis. All data were expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) was used to compare continuous variables among different groups and Mann-Whitney U test was used to compare variables between groups. Optimal thresholds for survival analysis were identified using Receiver-Operated Characteristics (ROC) analysis. Statistical analysis was performed using SPSS 18 (SPSS; Chicago, IL, USA).

3. Results

In Table 1, patients in IPAH group were younger than non-PAH volunteer and COPD-PH group, but not significant ($p = 0.16$). There were more female patients ($n = 14$) than male patients ($n = 6$) in IPAH group. The systolic blood pressure was significantly lower than IPAH groups, which may result from right heart failure ($p = 0.002$). The PA pressure was significantly different between IPAH, COPD-PH, and non-PAH group ($p < 0.005$).

In Figure 2(a), serum Cav1 level was significantly low in IPAH compared to non-PAH and COPD-PH group (76.45 ± 32.41 versus 140.75 ± 59.72 pg/mL and 173.57 ± 42.75 pg/mL; $p = 0.014$ and $p = 0.047$). But there was no significant difference between non-PAH and COPD-PH. NT-proBNP (Figure 2(b)) was significantly higher in IPAH and COPD-PH than normal group (933.59 ± 210.09 and 1806.38 ± 474.07 versus 83.436 ± 22.33 pg/mL, both $p < 0.05$), but there is no difference between IPAH and COPD-PH groups. hsCRP (Figure 2(c)) was significantly higher in COPD-PH group than non-PAH group (1.02 ± 0.32 versus 0.20 ± 0.04 mg/mL, $p = 0.017$), but there is no difference between COPD-PH and IPAH group (1.02 ± 0.32 versus 0.37 ± 0.15 mg/mL, $p > 0.5$). BMPR2 (Figure 2(d)) was higher in IPAH group than COPD-PH group (22.35 ± 15.60 versus 2.57 ± 0.99 pg/mL, $p = 0.019$), but there is no significant difference between COPD-PH and non-PAH group (2.57 ± 0.99 versus 6.41 ± 3.27 pg/mL, $p > 0.5$).

In IPAH patients, using serum Cav1, 17.17 pg/mL as a cutoff value (Table 2), the sensitivity was 0.59, the specificity was 1.0, and area under ROC curve was 0.816 (Figure 3(a)). Using NT-proBNP, 89.25 pg/mL as a cutoff value, the sensitivity was 0.889, the specificity was 0.778, and area under

Table 1: Demographic data patients with pulmonary artery hypertension and healthy controls.

	Non-PAH ($n = 27$)	COPD-PH ($n = 20$)	IPAH ($n = 20$)	p value
Age, yrs	51.30 ± 11.71 (64–38)	58.9 ± 12.96 (75–39)	45.4 ± 16.16 (78–18)	0.16
Sex (male/female)	22/5	16/4	6/14	0.000
Height, cm	161.94 ± 6.84 (182–152)	160.63 ± 7.22 (169.5–144)	158.93 ± 7.53 (176–147)	0.366
Weight, kg	66.65 ± 12.31 (86.5–49.5)	67.28 ± 12.50 (94–46)	63.51 ± 13.43 (98–46)	0.578
BMI, kg/m^2	25.45 ± 3.91 (33.76–18.29)	16.16 ± 5.10 (38.58–17.10)	25.15 ± 5.64 (42.7–18.36)	0.791
History of DM	5 (18.5%)	5 (25.0%)	0 (0)	0.68
History of HTN	8 (29.6%)	10 (45.5%)	4 (18.2%)	0.117
PAP peak (mmHg)	17.69 ± 4.48 (8–25.8)	46.76 ± 12.74 (35–72.6)	96.37 ± 30.76 (47.30–169)	0.000
PAP mean (mmHg)	12.36 ± 2.89 (6.3–18.80)	31.14 ± 7.93 (23.4–48.4)	57.79 ± 14.87 (29.60–84.5)	0.000
SBP, mmHg	133.35 ± 16.01 (170–104)	132.80 ± 22.24 (176–96)	113.20 ± 23.10 (175–86)	0.002
DBP, mmHg	79.77 ± 9.60 (101–60)	81.80 ± 16.87 (119–62)	77.80 ± 16.87 (128–54)	0.683
TC, mg/dL	176.88 ± 35.35 (236–92)	183.35 ± 44.46 (281–105)	146.25 ± 33.39 (188–103)	0.078
HDL_C, mg/dL	41.6 ± 9.31 (59–29)	48.8 ± 38.5 (166–6)	52.4 ± 14.84 (66–33)	0.753
TG, mg/dL	142.88 ± 69.20 (373–53)	127.50 ± 94.72 (458–23)	79.88 ± 33.28 (148–38)	0.139
Creatinine, mg/dL	1.08 ± 0.27 (2–0.7)	1.44 ± 0.75 (4.4–0.8)	0.88 ± 1.6 (1.2–0.6)	0.515
AC_sugar, mg/dL	108.23 ± 26.36 (177–79)	110.50 ± 42.04 (235–49)	108.75 ± 34.78 (189–83)	0.980
Caveolin-1 pg/mL	173.57 ± 135.18 (47.22–409.44)	163.04 ± 146.59 (56.61–425.54)	33.81 ± 36.3 (18–235)	0.029
hsCRP mg/dL	0.18 ± 0.23 (0.1–0.95)	1.02 ± 1.30 (0.13–4.38)	0.37 ± 0.62 (0.03–2.75)	0.007
NT-proBNP pg/mL	59.83 ± 64.84 (4.0–336)	1426 ± 1231 (140–2790)	933.6 ± 891.3 (107–2120)	0.004

PAH: pulmonary artery hypertension, COPD-PH: chronic obstructive pulmonary disease with pulmonary hypertension, IPAH: idiopathic PAH, PAP: pulmonary artery pressure, SBP: systolic blood pressure, DBP: diastolic blood pressure, hsCRP: high-sensitivity C-reactive protein, and NT-proBNP: N-terminal of the prohormone brain natriuretic peptide.

Table 2: Sensitivity and specificity data for cutoff point of Cav1 and other biomarkers in IPAH patients.

Biomarker	Cutoff value	Sensitivity	Specificity
Cav1	17.17 pg/mL	0.588	1
NT-proBNP	89.25 pg/mL	0.889	0.778
hsCRP	0.27 mg/dL	0.389	0.852
BMPR2	3.71 pg/mL	1	0.429

ROC curve was 0.89 (Figure 3(b)). Using hsCRP, 0.27 mg/dL as a cutoff value, the sensitivity was 0.39, the specificity was 0.85, and area under ROC curve was 0.89 (Figure 3(c)). Using BMPR2, 3.71 pg/mL as a cutoff value, the sensitivity was 1.00, the specificity was 0.43, and area under ROC curve was 0.78 (Figure 3(d)). Linear regression analysis between Cav1 and 6 min walk test, PAP, and PVR was done but did not show good correlation. Data were not shown here.

4. Discussion

There were two major findings in our results. First, serum Cav1 might be a novel biomarker in the diagnosis of IPAH with fare sensitivity and good specificity. Second, Cav1 might be used to make differential diagnosis between COPD-PH and IPAH group.

Cav1 was highly expressed in vascular endothelial cells but less in smooth muscle cells. The expression of Cav1 was decreased in the plexiform lesion from IAPH patients' lung tissue [20]. The expression in the smooth muscle cell was increased and immunoblotting from whole lung prepared revealed decreased expression of Cav1 [25, 26]. In this study, we further demonstrate that the serum Cav1 level in IPAH patients was also decreased (Figure 2(a)), and the difference was significant between IPAH, COPD-PH, and normal subjects. By using serum Cav1 level 17.17 pg/mL as cutoff value in the diagnosis of IPAH, there were fare sensitivity (0.6) and good specificity (1.0) (Figure 3(a)).

In a small number of COPD with PH patients (mean PAP: 29.5 ± 5.1 mmHg), the intimal expression of Cav1 was decreased as compared with COPD patients without PAH (mean PAP: 16.7 ± 2.7 mmHg) [27]. Our data did not show significant difference between COPD-PH and normal subjects, but there was significant difference between COPD-PH and IPAH patients (Figure 2(a), $p = 0.047$). Although smooth muscle proliferation with increasing Cav1 expression was noted in both COPD-PH and IPAH patients, our results suggested the serum Cav1 level correlated with its expression in endothelial cells but not the smooth muscle cells. Our data suggest that Cav1 may be potential biomarkers for elevated PA pressure and could be used for differential diagnosis of COPD-PH and IPAH.

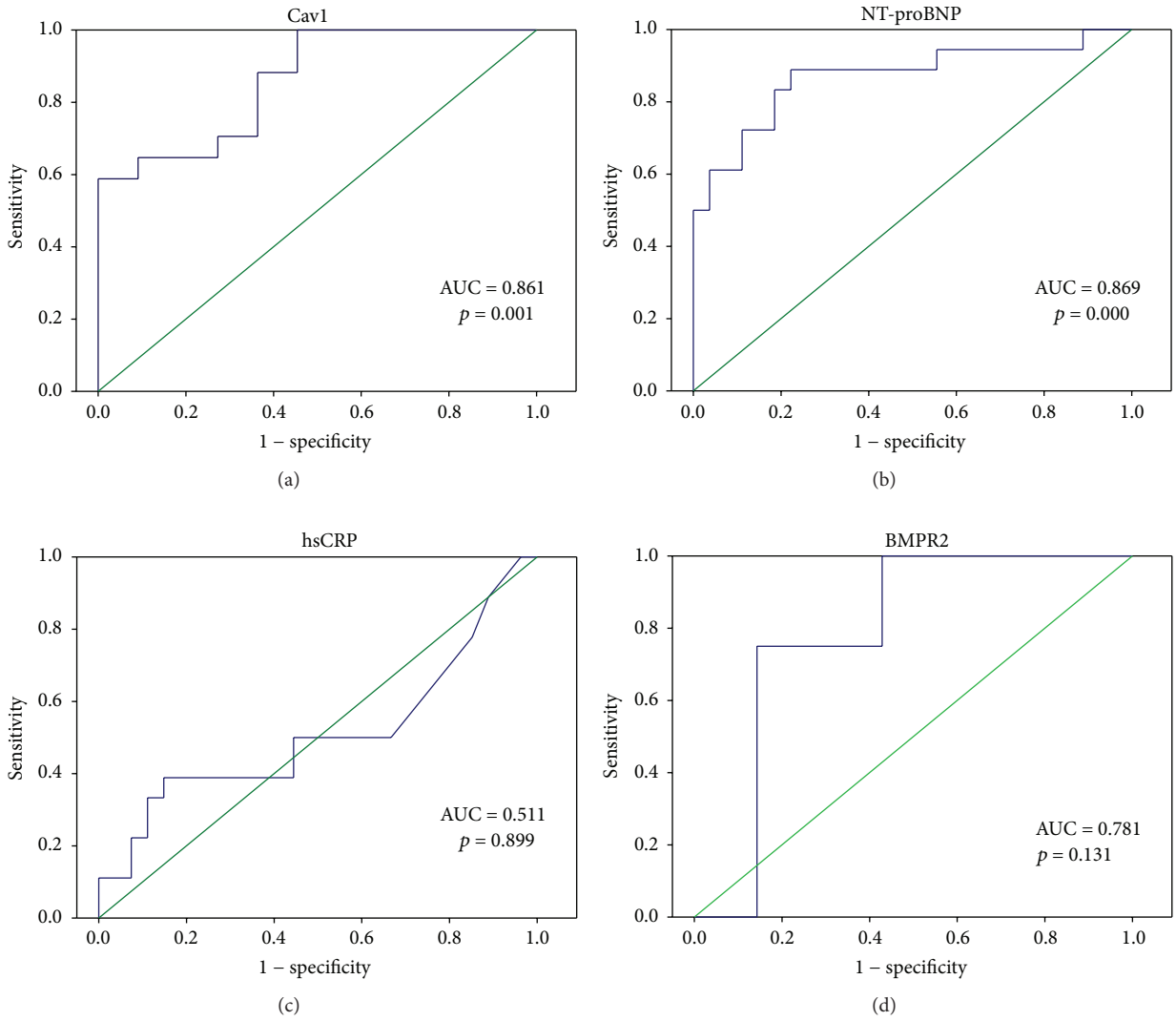

Figure 3: Receiver operator curve analysis of Cav1 and other biomarkers in idiopathic pulmonary artery hypertension (IPAH) patients.

NT-proBNP is secreted by the ventricles of the heart in response to excessive stretching of cardiomyocytes. Serum NT-proBNP elevated in both left and right ventricle dysfunction [28, 29]. In COPD patients with PAH and right heart failure, the NT-proBNP was also elevated [30]. In our results (Figure 2(b)), NT-proBNP levels were significantly higher in both COPD-PAH and IPAH groups than normal subjects, but there was no significant difference between the disease subjects. hsCRP, a nonspecific biomarker in response to different pathogenesis of inflammation, was higher in COPD patients due to chronic lung inflammation (Figure 2(c), $p = 0.017$) than normal group. But there was no difference between COPD-PH and IPAH groups. Mutations in the BMPR2 gene resulted in the development of familial primary pulmonary hypertension, but the role BMPR2 mutations play in the development of PH has not been clarified. Cav1 and BMPR2 were colocalized in both endothelial and smooth muscle cell membrane [31, 32] and Cav1 was suggested to regulate BMPR2 downstream signaling. In this study (Figure 2(d)), serum BMPR2 level was not significantly different between normal subjects versus IPHA patients and normal subjects versus COPD-PAH patients. But the differences between IPAH and COPD with PAH were significantly different ($p = 0.019$). Further study may be indicated to elucidate the relation between Cav1 and BMPR2 in IPAH patients.

Taken together, our results demonstrated that reduced serum Cav1 level may be a potential biomarker in IPAH diagnosis and could be used for differential diagnosis of pulmonary artery hypertension patients between idiopathic pulmonary hypertension and COPD.

5. Study Limitation

This is a small number cross-sectional study. COPD-PH is more frequent in males; IPAH is more frequent in females. It is difficult to correct the match number of patients in gender.

The IPAH patients included were at different treatment status, including newly diagnosed IPAH without medication to double or even triple drugs combined therapy. The functional status of heart failure may also influence Cav1 serum level. Therefore, we find only poor correlation between Cav1 level and pulmonary artery pressure, pulmonary vascular resistance, and 6-minute wall test. However, our results suggested serum Cav1 level might be used as an easy and convenient way for IAPH initial diagnosis. Future studies are necessary to include more patients at different stages of disease, to evaluate Cav1 level in response to different treatment, to predict the IPAH progression and long-term prognosis.

Conflict of Interests

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

Acknowledgments

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Research Article

Amelioration of Isoproterenol-Induced Oxidative Damage in Rat Myocardium by *Withania somnifera* Leaf Extract

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We investigated the protective role of *Withania somnifera* leaf extract (WSLEt) on isoproterenol- (ISO-) induced myocardial infarction (MI) in rats. Subcutaneous injection of ISO (85 mg/kg body weight (b.w.)) administered to rats for two consecutive days caused a significant increase in cardiac troponin I (cTnI) levels and serum lipid profiles, as well as the activities of some marker enzymes. In addition to these diagnostic markers, there were increased levels of lipid peroxidation (LPO) and decreased activities of enzymatic antioxidants (superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GRx), and glutathione-S-transferase (GST)) in the myocardium. However, oral pretreatment (100 mg/kg b.w.) with WSLEt for 4 weeks elicited a significant cardioprotective activity by lowering the levels of cTnI, lipid profiles, and marker enzymes. The levels of LPO products were also significantly decreased. Elevated activities of antioxidant enzymes were also observed in rats pretreated with WSLEt. As further confirmed histopathologically, our findings strongly suggest that the cardioprotective effect of WSLEt on myocardium experiencing ISO-induced oxidative damage may be due to an augmentation of the endogenous antioxidant system and an inhibition of LPO in the myocardial membrane. We conclude that WSLEt confers some protection against oxidative damage in ISO-induced MI in rats.

1. Introduction

Withania somnifera (Solanaceae), also known as "ashwagandha" or "winter cherry," is one of the most valuable herbs in the traditional Indian systems of medicine [1]. The plant is utilized in more than 100 formulations in Ayurveda, Unani, and Siddha [2]. It is described as an herbal tonic and health food in the famous book of Vedas and is considered akin to an "Indian Ginseng" in the traditional Indian system of healing [3]. The ethnopharmacological properties of the plant include adaptogenic, antisedative, and anticonvulsive activities. The plant is used to treat various neurological disorders, geriatric debilities, arthritis, stress, and behavior-related problems [4]. *W. somnifera* contains a variety of nutrients and phytochemicals and is therefore also used as a dietary supplement. It has been reported that all of the major parts of *W. somnifera*, such as the roots, fruits, and leaves, provide potential benefits for human health because of their high polyphenol contents and antioxidant activities [5].

Myocardial infarction (MI) is a common presentation of ischemic heart disease (IHD). MI remains the major cause of death in the developed world and is a major pathological issue worldwide despite rapid advancements made in the treatment of coronary artery diseases (CAD) [6]. It occurs as a result of increased myocardial metabolic demand and decreased supply of oxygen and nutrients via the coronary circulation to the myocardium, leading to cell injury; it is one of the most lethal manifestations of cardiovascular diseases (CVD) [7]. MI continues to be a major public health problem, not only in western countries but also increasingly more in developing countries, where it contributes significantly to mortality [8]. According to the World Health Organization, MI is predicted

to be the major cause of death in the world by the year of 2020 [9].

Isoproterenol [1–(3, 4–dihydroxyphenyl)–2–isopropyl-aminoethanol hydrochloride (ISO)] is a synthetic catecholamine and β-adrenergic agonist that is an important regulator of myocardial contractility and metabolism, thus serving as the key element of a standard model for the study of potentially beneficial effects of numerous drugs on cardiac function [7]. ISO induces cardiac necrosis by several mechanisms, including increased oxygen consumption, poor oxygen utilization, increased calcium overload and accumulation, altered myocardial cell metabolism, increased myocardial cAMP levels, deranged electrolyte milieu, altered membrane permeability, intracellular acidosis, and increased levels of lipid peroxides [10]. The pathophysiological and morphological aberrations produced in the heart of the myocardial necrotic rat model are comparable with those taking place in human MI. The various mechanisms proposed to explain ISO-induced cardiotoxicity include the generation of highly cytotoxic free radicals through the autooxidation of catecholamines, which has been implicated as one of the important causative factors [11].

Oxidation of catecholamine forms quinoid compounds giving rise to the production of superoxide anions and, subsequently, hydrogen peroxide, which, in the presence of iron, forms highly reactive hydroxyl radicals and causes protein, lipid, and DNA damage and increased MI size [12]. In addition, excessive formation of free radicals may result in the loss of function and integrity of myocardial membranes [13]. These free radicals may attack polyunsaturated fatty acids (PUFAs) within the membranes, forming peroxyl radicals. These radicals can then attack adjacent fatty acids, causing a chain reaction of lipid peroxidation (LPO). The lipid hydroperoxide end products are also harmful and may contribute to further tissue and organ damage [14].

In recent years, long-term prevention of CVD is associated with the consumption of fresh fruits, vegetables, or plants rich in natural antioxidants. As a result, there has been considerable interest in research on natural bioactive compounds, with a generally accepted view that natural products are superior in terms of efficacy and safety when compared to their synthetic analogs [15]. Medicinal plants constitute an important source of active natural products that differ widely in terms of structure and biological properties and play an important role in the protection against various human diseases including CVD [16].

A previous study reported that *W. somnifera*, particularly its leaves, has remarkable antioxidant properties [1]. *W. somnifera* leaves have been reported to contain higher amounts of polyphenols and flavonoids when compared to the roots and fruits [5]. To date, many epidemiological studies have demonstrated the effectiveness of phenolics and flavonoids as antitumor, anti-inflammatory agent or in reducing the risk of cardiovascular diseases [17]. Most importantly, *W. somnifera* leaves contain higher levels of catechin, which belongs to the flavonoid family, when compared to other parts of the plant [5]. Some previous studies have strongly suggested that catechin reduces the risk of IHD [18, 19]. To date, there is no or little available data on the potential medicinal properties of *W. somnifera* leaves, since most studies tend to focus on *W. somnifera* roots [7, 20]. Considering the more robust antioxidant potential and higher catechin content of *W. somnifera* leaves, we were interested in investigating the effect of *W. somnifera* leaves on oxidative stress-induced cardiac injury. In this study, the cardioprotective effect of *W. somnifera* leaf extract (WSLEt) was investigated in relation to cardiac marker enzymes, lipid peroxides, and the antioxidant enzyme defense system.

2. Materials and Methods

2.1. Experimental Animals. The experiments were conducted according to ethical guidelines as approved by the Bangladesh Association for Laboratory Animal Science. Adult male Wistar Albino rats ($n = 40$) (140–160 g) were bred and reared in the animal house facility of the Department of Biochemistry and Molecular Biology, Jahangirnagar University, at a constant room temperature of $23 \pm 2^{\circ}$C, and in an environment with humidity ranging between 40% and 70%. The rats were housed in plastic cages (with hard wood chips for bedding) and received a natural 12 h day-night cycle. The rats were provided with a standard laboratory pellet diet and water *ad libitum*. The pellet diet consisted of 56.17% carbohydrate, 22.02% crude protein, 4.25% crude oil, 3.25% crude fibre, 2.46% glucose, 0.8% calcium, 0.6% phosphorus, and 1.8% vitamins.

2.2. Drugs and Chemicals. The assay kit used for the estimation of cardiac troponin I (cTnI) levels was purchased from JAJ International, Inc., USA. Other assay kits for the measurement of creatine kinase (CK-MB), lactate dehydrogenase (LDH), aspartate transaminase (AST) and alanine transaminase (ALT), TC (total cholesterol), TGs (triglycerides), and high-density lipoprotein-cholesterol (HDL-C) were all purchased from Stanbio Laboratory, USA. The assay kits for superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GRx), and glutathione-S-transferase (GST) were purchased from Abnova Corporation, Taiwan. ISO and 1,1,3,3-tetraethoxy propane were purchased from Nacalai Tesque, Inc., Kyoto, Japan. All of the chemicals and reagents used in this study were of analytical grade.

2.3. Sample Collection and Extraction. *W. somnifera* leaves were collected from the Gaibanda Samriddhi Project, HELVETAS Swiss Inter Cooperation-Bangladesh, in July, 2013, and were authenticated by a botanist (Professor M. Shah Alam, Department of Botany, Rajshahi University, Rajshahi 6205, Bangladesh). The collected leaves of the medicinal plant were cleaned and then air-dried in the shade for 7 days before being ground to a fine powder by a blender (CM/L7360065, Jaipan, Mumbai, India). The fine powder was used to prepare a 5% ethanolic extract (5 g of *W. somnifera* leaf powder added to a final volume of 100 mL of a 70% ethanol solution) in the dark so as to avoid reactions in solution that may occur in the presence of light. The solution was shaken in a shaker for 72 h at room temperature. Then, the solution was filtered and dried in a rotary evaporator (Buchi,

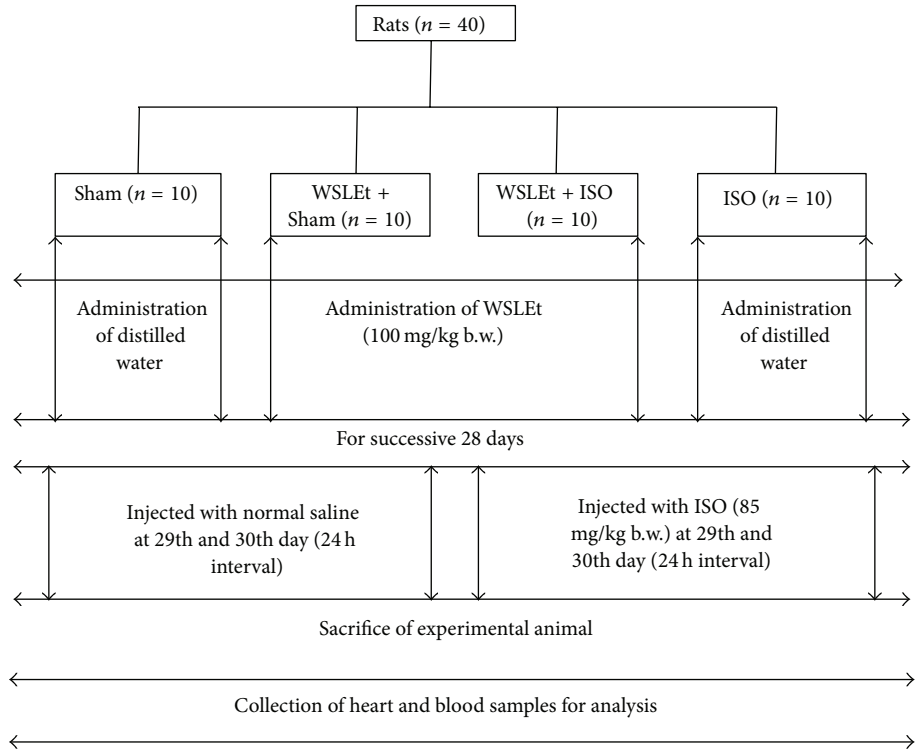

Figure 1: Schematic representation of experimental design of the study.

Tokyo, Japan) under reduced pressure (100 psi) at a controlled temperature (40°C). The dried extract was collected and then finally preserved at −20°C for subsequent *in vivo* studies. Only the required amount was withdrawn from refrigerator to ensure the stability of the extract.

2.4. Experimental Design. After a week-long acclimation period, the animals were randomly divided into 4 groups (10 rats in each group) (Figure 1).

Sham. Animals received only distilled water (2 mL/kg) for 4 weeks and were then treated by normal saline injection (1 mL) for 2 days (on the 29th and 30th days).

WSLEt + Sham. Animals were pretreated with WSLEt (100 mg/kg) for 4 weeks at 24 hr interval and then treated by normal saline injection (1 mL) for 2 days (on the 29th and 30th days).

WSLEt + ISO. Animals were pretreated with WSLEt (100 mg/kg) for 4 weeks at 24 hr interval and then treated by ISO injection (85 mg/kg) for 2 days (on the 29th and 30th days).

ISO. Animals received only distilled water (2 mL/kg) for 4 weeks and were then treated by ISO injection (85 mg/kg) for 2 days (on the 29th and 30th days).

2.5. Induction of Experimental MI. ISO (85 mg) was dissolved in normal saline (1 mL) and was subcutaneously (s.c.) injected into rats (85 mg/kg) at an interval of 24 h for 2 days to induce experimental MI. The choice of ISO dose was based on a pilot study for ISO dose fixation and on the results of a previous study [21]. The WSLEt dosage was based on previous studies [7, 22].

During the experimental period, the rats' body weights were recorded regularly and the doses were modulated accordingly. At 48 h after the first ISO injection, all animals were sacrificed by decapitation. Blood samples (3 mL) were collected and serum samples were separated by centrifugation. Immediately following blood collection, the heart samples were separated from surrounding tissues and were washed twice with ice cold phosphate-buffered saline. The samples were then homogenized in phosphate buffer (25 mM, pH 7.4) using a tissue homogenizer (F 12520121, Omni International, Kennesaw, USA) to produce an approximately 10% w/v homogenate. The homogenate was centrifuged at 1,700 rpm for 10 min, and the supernatant was collected and stored at −20°C for subsequent biochemical analyses. Some of the heart samples were stored in 10% formalin for histopathological examination.

2.6. Serum Biochemical Analysis. An enzyme immunoassay kit was employed for the determination of cTnI in serum samples using an ELISA micro-plate reader (digital and analog system RS232, Das, Italy). Standard assay kits were employed to determine the levels of CK-MB, LDH, AST, ALT, TC, TGs, and HDL-C in serum samples using a PD-303S Spectrophotometer (APEL, Japan). Serum VLDL-C levels

Table 1: Changes in the body and heart weights in different groups of rats.

Parameters	Group			
	Sham	WSLEt + Sham	WSLEt + ISO	ISO
Initial body weight (g)	143.70 ± 24.16[a]	146.71 ± 15.99[a]	141.00 ± 23.43[a]	148.63 ± 19.91[a]
Final body weight (g)	164.82 ± 19.28[a]	175.00 ± 6.36[a]	169.75 ± 16.64[a]	172.00 ± 21.59[a]
Body weight gain (g)	21.12[a]	28.29[a]	28.75[a]	23.37[a]
Absolute heart weight (g)	0.62 ± 0.03[a]	0.66 ± 0.03[a]	0.73 ± 0.02[b]	0.97 ± 0.06[c]
Relative heart weight (g/100 g)	0.39 ± 0.02[a]	0.39 ± 0.01[a]	0.49 ± 0.02[b]	0.57 ± 0.04[c]

Results are expressed as mean values ± SD; $n = 10$. [a,b,c]Values in the same row that do not share superscript letters (a, b, c) differ significantly at $p < 0.05$.

were calculated based on a formula provided by Friedewald [23]:

$$\text{VLDL-C} = \frac{\text{TG}}{5}. \quad (1)$$

2.7. Biochemical Analysis in Heart Tissue. Malondialdehyde (MDA) levels were assayed for LPO products in the heart tissues. MDA, which is also referred to as thiobarbituric acid-reactive substance (TBARS), was measured according to the method published by Ohkawa et al. [24]. Briefly, 0.2 mL of tissue homogenate, 0.2 mL of 8.1% sodium dodecyl sulphate (SDS), 1.5 mL of 20% acetic acid, and 1.5 mL of 8% TBA were mixed. The mixture was supplemented up to 4 mL with distilled water and was heated at 95°C in a water bath for 60 min. After incubation, the tubes were cooled to room temperature and the final volume was increased to 5 mL in each tube. A butanol : pyridine (15 : 1) mixture (5 mL) was added and the contents were vortexed thoroughly for 2 min. After centrifugation (3,000 rpm) for 10 min, the upper organic layer was aspirated, and its absorbance was read at 532 nm against the blank. The levels of TBARS were expressed as nmol of MDA per mg of protein.

The heart tissue homogenate was recentrifuged at 12,000 rpm for 10 min at 4°C using an Eppendorf 5415D centrifuge (Hamburg, Germany). The resulting clean supernatants of heart tissue extracts were used for further estimation of endogenous antioxidant enzymes including SOD, GPx, GRx, and GST using standard ELISA micro-plate assay kits. The levels of SOD, GPx, GRx, and GST were expressed as units/mg of protein, nmol of NADPH oxidized/min/mg of protein, nmol of NADPH oxidized/min/mg of protein, and nmol of CDNB conjugated/min/mg of protein, respectively. The total protein in the heart tissue homogenates was estimated by the method described by Lowry et al. [25]. Briefly, 0.2 mL sample (digested with 0.1 N sodium hydroxide (NaOH)) was mixed with 2 mL of working reagent (a mixture of 2% sodium carbonate, 0.1 N NaOH, 1.56% copper sulphate, and 2.37% sodium-potassium tartrate), and the reaction mixture was incubated for 10 min at room temperature. The addition of 1 N Folin-Ciocalteu's phenol reagent (0.2 mL) was followed by a 30 min incubation at room temperature. Finally, the absorbance was measured at 660 nm. Bovine serum albumin was used as the standard to calculate the protein content of samples.

2.8. Histopathological Examination. After sacrificing the animals, the hearts were rapidly dissected and immediately washed with saline before being fixed in 10% formalin. The fixed tissues were then embedded in paraffin. After that, serial sections (5 μm thickness) were cut followed by staining with hematoxylin and eosin (H & E). Microscopic observation was done using a fluorescence microscope over normal spectra (Olympus DP72, Tokyo, Japan) at 40x magnification. Photomicrographs were taken by using an attached digital camera. A dedicated pathologist who was blind to the treatment assignment of the different study groups was assigned to perform the histopathological evaluation.

2.9. Statistical Analysis. The results of all the groups are shown as mean values ± standard deviations (SD). The data was analyzed using SPSS (Statistical Packages for Social Science, version 20.0, IBM Corporation, New York, USA) and Microsoft Excel 2007 (Redmond, Washington, USA). Statistical analyses of biochemical data were performed using a one-way ANOVA followed by a Tukey *post hoc* test. A p value of <0.05 was accepted as indicating statistical significance.

3. Results

None of the rats died in any of the experimental groups over the entire 4-week treatment period. There was no significant difference in the body weights observed at the baseline time point or at the end of the experimental period between the groups (Table 1). However, the heart weights increased significantly ($p < 0.05$) in ISO-treated rats when compared with normal control rats. When compared to ISO group, rats pretreated with WSLEt had a significant ($p < 0.05$) reduction in heart weight, indicating its cardioprotective effects. No significant difference was observed in rats treated with WSLEt alone when compared to normal control rats.

Rats treated with ISO alone had a marked ($p < 0.05$) elevation in serum cTnI levels when compared to the control (Figure 2). However, oral pretreatment of WSLEt for 4 weeks significantly ($p < 0.05$) decreased serum cTnI levels in ISO-treated rats when compared with ISO group.

A marked increase in the activities of serum cardiac enzymes was observed in ISO-induced myocardial ischemic rats (Figure 3). This effect was significantly ameliorated by WSLEt.

Table 2: WSLEt ameliorates the oxidative damage caused by ISO as demonstrated by the changes in the serum lipid profiles.

Parameters	Group			
	Sham	WSLEt + Sham	WSLEt + ISO	ISO
TC (mg/dL)	50.19 ± 6.34^{a}	45.67 ± 4.93^{a}	56.37 ± 6.38^{b}	74.75 ± 12.35^{c}
TG (mg/dL)	42.69 ± 5.55^{a}	38.46 ± 8.09^{a}	67.56 ± 8.90^{b}	82.29 ± 6.12^{c}
VLDL-C (mg/dL)	8.54 ± 1.11^{a}	7.69 ± 1.39^{a}	12.33 ± 1.41^{b}	16.46 ± 1.22^{c}
HDL-C (mg/dL)	45.12 ± 2.71^{a}	49.12 ± 4.49^{b}	46.04 ± 1.15^{ab}	19.89 ± 1.21^{c}

Results are expressed as mean values ± SD; $n = 10$. ^{a,b,c}Values in the same row that do not share superscript letters (a, b, c) differ significantly at $p < 0.05$.

Table 3: WSLEt ameliorates the oxidative damage caused by ISO as demonstrated by the changes in LPO levels and the activities of SOD, GRx, GPx, and GST.

Parameters	Group			
	Sham	WSLEt + Sham	WSLEt + ISO	ISO
LPO (nmol TBARS/mg of protein)	42.77 ± 1.05^{a}	37.18 ± 1.85^{a}	40.02 ± 1.17^{a}	82.17 ± 1.35^{b}
SOD (units/mg of protein)	1.45 ± 0.02^{ab}	1.58 ± 0.16^{a}	0.33 ± 0.02^{b}	0.10 ± 0.00^{c}
GRx (nmol NADPH oxidized/min/mg of protein)	97.56 ± 2.09^{a}	90.57 ± 1.79^{a}	97.14 ± 6.05^{a}	75.59 ± 9.79^{b}
GPx (nmol NADPH oxidized/min/mg of protein)	2.15 ± 0.45^{a}	3.12 ± 0.16^{b}	1.98 ± 0.00^{ab}	0.96 ± 0.00^{c}
GST (nmol of CDNB conjugated/min/mg of protein)	2.04 ± 0.06^{a}	4.05 ± 0.19^{b}	1.99 ± 0.11^{a}	0.85 ± 0.02^{c}

Results are expressed as mean values ± SD; $n = 10$. ^{a,b,c}Values in the same row that do not share superscript letters (a, b, c) differ significantly at $p < 0.05$.

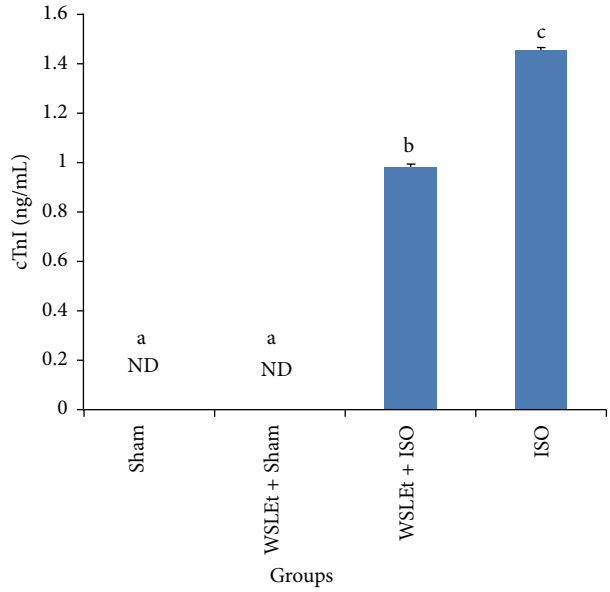

Figure 2: WSLEt ameliorates the oxidative damage caused by ISO as demonstrated by the changes in cTnI levels. Bars represent mean values ± SD ($n = 10$); bars with different letters represent significantly different mean values at $p < 0.05$. ND: not detected.

Similarly, pretreatment with WSLEt also ameliorated serum lipid profile increases (TC, TGs, VLDL-C, and HDL-C) (Table 2).

The activities of antioxidant enzymes such as SOD, GRx, GPx, GST, and LPO in the hearts of ISO-treated rats, which were significantly decreased when compared with the control, improved significantly by pretreatment with WSLEt (Table 3).

Figures 4(a)–4(d) show the effects of WSLEt on the histology of the heart in normal and ISO-induced myocardial-infarcted rats. Control rats and those treated with WSLEt (100 mg/kg) showed normal cardiac fibers (Figures 4(a) and 4(b)) with no overt damage observed. Figure 4(d) shows an ISO-treated myocardium with an area of infarction with splitting of cardiac muscle fibers, edematous intramuscular space, and inflammatory cells. Animals from WSLEt + ISO, however, had cardiac muscle fibers with significantly fewer inflammatory cells (Figure 4(c)).

4. Discussion

To our knowledge, our study is the first to demonstrate the cardioprotective effect of WSLEt. The experimental animal hearts revealed a significant increase in both their absolute and relative weights following ISO administration, although the body weight remained relatively unchanged. The increase in heart weight may be due to increased water accumulation with edematous intramuscular spaces in heart tissue and increased protein content [10], which is also confirmed by the histopathological findings. It has been proposed that myocardial function may be reduced by approximately 10% due to an increase in myocardial water content by 1% [26]. Increased membrane permeability in the pathogenesis of cardiac muscle cell injury following catecholamine toxicity is purported to be one of the main contributing factors to water accumulation in the heart [11]. Catecholamines are important regulators of myocardial contractility and metabolism. However, it has been long known that excess levels of catecholamines are responsible for cellular damage, as observed in clinical conditions such as angina, transient myocardial hypoxia, acute coronary insufficiency, and subendocardial infarct. The administration of ISO has effects on mitochondrial LPO, antioxidants, TCA cycle enzymes, and respiratory marker enzymes. Animals develop infarct-like lesions when injected with ISO, a potent synthetic catecholamine [27]. Increased generation of cytotoxic free

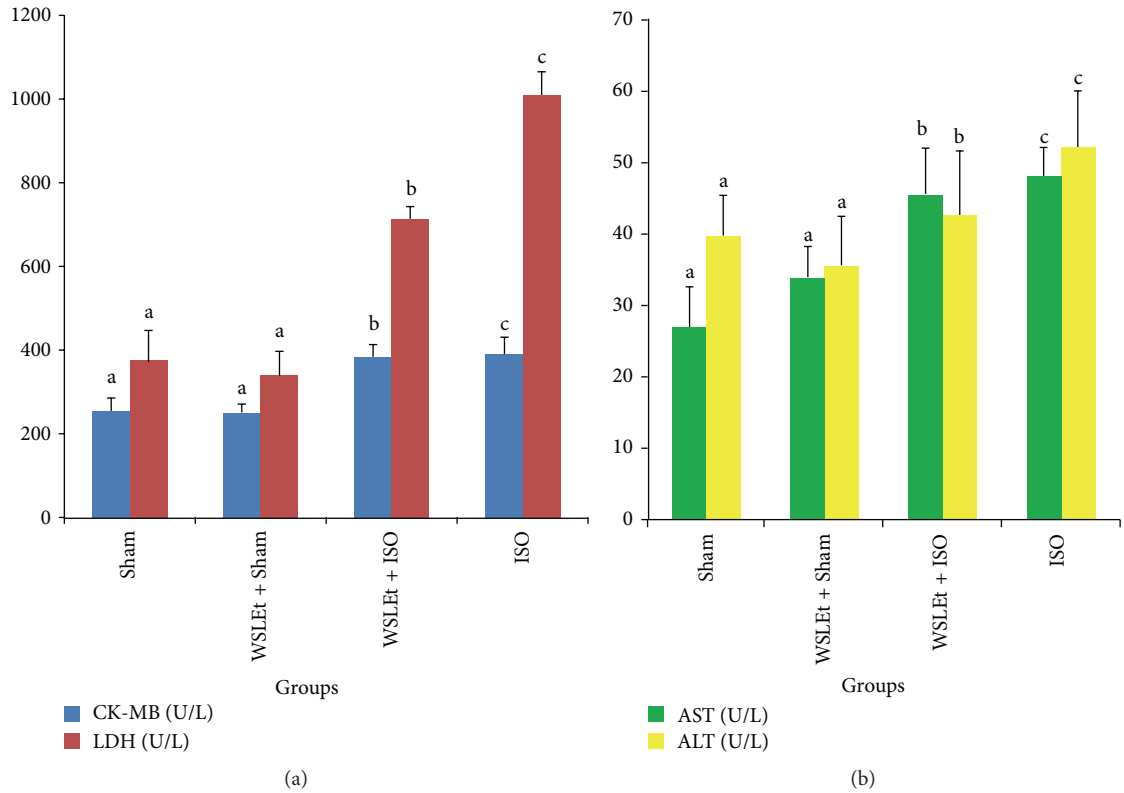

Figure 3: WSLEt ameliorates the oxidative damage caused by ISO as demonstrated by the changes in the cardiac marker enzyme activities. (a) The effects of WSLEt on CK-MB and LDH levels and (b) the effects of WSLEt on AST and ALT levels. Bars represent mean values ± SD ($n = 10$); bars with different letters represent significantly different mean values at $p < 0.05$.

radicals as a result of the autooxidation metabolic products of ISO is one of the well-recognized mechanisms of ISO-induced myocardial necrosis [28]. Pretreatment with WSLEt, however, significantly decreased the absolute and relative heart weights, bringing them close to their normal values, which indicates the protective effect of the WSLEt on the myocardium against infiltration or accumulation with water.

Cardiac troponin is a low molecular weight protein which is a constituent of the myofibrillary contractile apparatus of the cardiac muscle. cTnI has been shown to be a highly sensitive and specific marker of myocardial cell injury; it is usually absent in serum in normal individuals and released only after myocardial necrosis [29]. In this study, an increased level of serum cTnI in ISO-treated rats was observed relative to the control group. The increased level of cTnI may be attributed to the ISO-induced cardiac damage. Animals treated with ISO following pretreatment with WSLEt, however, exhibited a significant reduction in cTnI levels when compared to ISO-treated rats without the WSLEt pretreatment. Our results are consistent with those from a previous study reported by Priscilla and Prince [14]. Pretreatment with WSLEt significantly decreased serum cTnI levels in ISO-treated cardiotoxic rats. It is assumed that WSLEt may preserve the structural and functional integrity of the contractile apparatus, which prevents cardiac damage and leakage of troponins from the heart into the blood. Nevertheless, further research is essential to elucidate the exact mechanisms underlying the cardioprotective effect of WSLEt.

The myocardium contains high concentrations of diagnostic markers of MI; once it is metabolically damaged, it releases its contents into the extracellular fluids [30]. Of all the macromolecules leaked from the damaged tissue, myocardial enzymes are the best markers of tissue damage because of their tissue specificity and catalytic activity. When myocardial cells are damaged or destroyed due to a deficiency in the oxygen supply or glucose, the cardiac membrane becomes permeable or may rupture entirely, resulting in the leakage of enzymes [14]. The activity assay for CK-MB in serum is an important diagnosis because of the marked abundance of this enzyme in myocardial tissue and its virtual absence from most other tissues and its consequent sensitivity. CK-MB isoenzyme activity is useful as an index for the early diagnosis of not only myocardial infarction, but also any type of myocardial injury. Leakage of cytosolic enzymes including CK-MB, LDH, AST, and ALT (which serve as diagnostic markers from the damaged tissue) into the blood stream may occur when cell membranes become more permeable or rupture. The amounts of these cellular enzymes in the serum reflect the alterations in plasma membrane integrity and/or permeability [8]. Furthermore, the amount of the enzymes appearing in serum is reported to be proportional to the number of necrotic cells [31], which also reflects a nonspecific alteration in the plasma membrane integrity

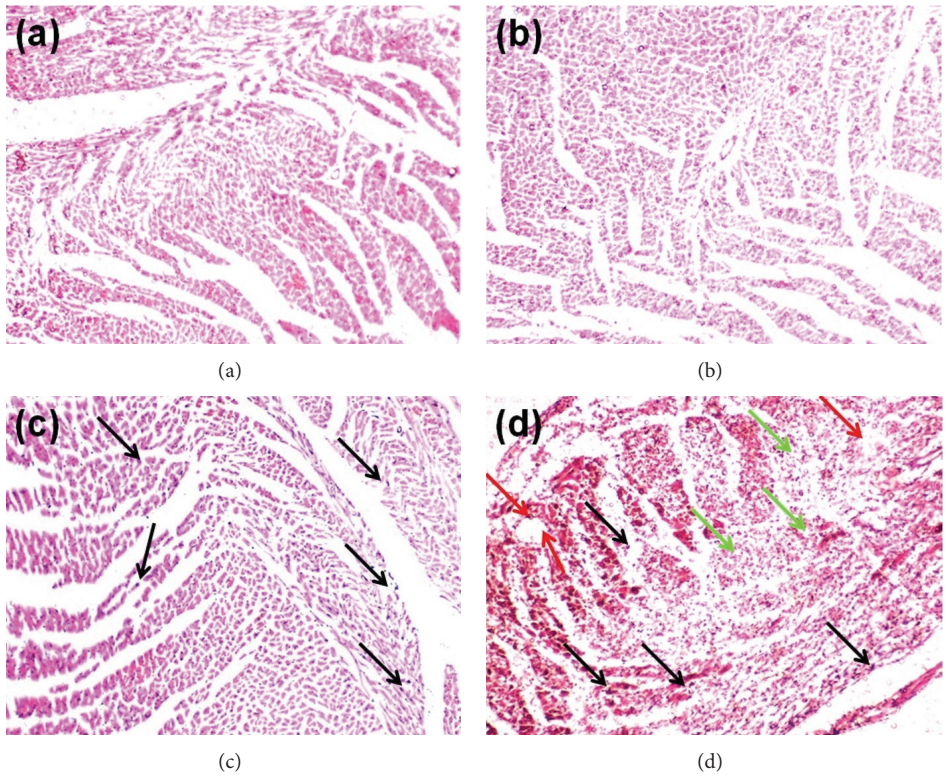

Figure 4: (a) Sham group: normal control heart showing normal cardiac muscle fibers. (b) WSLEt + Sham group: WSLEt-treated (100 mg/kg) heart showing normal muscle fibers without any pathological changes. (c) WSLEt + ISO group: WSLEt-treated (100 mg/kg) + ISO-treated (85 mg/kg) heart showing no edematous intramuscular space and fewer inflammatory cells (black arrows). (d) ISO group: ISO-treated (85 mg/kg) heart showing cardiac muscle fibers with muscle separation (green arrows), edematous intramuscular space (red arrows), and inflammatory cells (black arrows).

and/or permeability as a response to β-adrenergic stimulation [16]. In the present study, rats administered with ISO showed significant increases in the levels of all these marker enzymes in serum, in line with the results from previous reports, indicating ISO-induced necrotic damage of the myocardium and leakiness of the plasma membrane [14, 22, 32]. Pretreatment with WSLEt, however, resulted in lowered activities of all marker enzymes in the serum, indicating that WSLEt helps in maintaining the membrane integrity, thereby restricting the leakage of these enzymes. Phenolic acids such as gallic acid, syringic acid, vanillic acid, and p-coumaric acid and flavonoids such as catechin and naringenin are important constitutive antioxidants found in WSLEt, as in our study and that reported by Alam et al. [5]. Tanvir et al. [33] and Afroz et al. [34] speculated that antioxidant compounds present in their sample confer protective effects on liver by preserving the membrane integrity. Therefore, it is plausible that the presence of these antioxidants may help protect against oxidative cardiac injury, thus restricting the leakage of these enzymes from the myocardium. For instance, Arts et al. [18] evaluated the effects of catechin intake on the health risks of high levels of body fat and the incidence of IHD and stroke in a cohort of elderly men; according to the study, catechin, whether from tea or other sources, may reduce the risk of IHD mortality. It is suggested that flavonoids decrease the risk of CDV by improving coronary vasodilation, decreasing the ability of platelets in the blood to clot, and preventing low-density lipoproteins from oxidizing [35].

Lipids play an important role in CVD, not only by contributing to the development of atherosclerosis but also by modifying the composition, structure, and stability of the cellular membrane. High levels of circulating cholesterol and its accumulation in heart tissue have been associated with cardiovascular damage [36]. Rats treated with ISO showed a significant increase in serum levels of TC, TGs, and VLDL-C, as previously reported [37]. Generally, the mechanism of actions of lipolytic hormones, including ISO, on fat cells are believed to be mediated by the cAMP cascade, in which lipolytic hormones activate adenylate cyclase, thereby increasing cAMP formation. Subsequently, cAMP promotes lipolytic activity by activating cAMP-dependent protein kinase, which phosphorylates hormone-sensitive lipase [38]. This results in the hydrolysis of stored triacylglycerol, which may contribute to hyperlipidemia [39]. High levels of LDL-C and VLDL-C have been positively correlated with MI but are negatively correlated with HDL-C. HDL-C inhibits the uptake of LDL-C by the arterial walls and facilitates the transport of cholesterol from peripheral tissues to the liver,

where it is catabolized and excreted from the body [40]. Pretreatment with WSLEt, however, significantly ameliorates these changes, thereby maintaining the normal fluidity and function of the myocardial membrane. Polyphenols, particularly gallic acid and catechin, have been reported to inhibit cholesterol esterase [41]. In general, pancreatic cholesterol esterase plays an important role in hydrolyzing dietary cholesterol esters, which liberates free cholesterol in the lumen of the small intestine [42]. Therefore, the inhibition of cholesterol esterase is expected to limit the absorbance of dietary cholesterol, resulting in reduced cholesterol absorption. Moreover, polyphenols can also bind with bile acids to increase their fecal excretion, which has been hypothesized as a possible mechanism for the lowering of plasma cholesterol levels by polyphenols [41].

LPO is a well-established mechanism of cellular injury and has been used as an indicator of oxidative stress that leads to the pathogenesis of MI [7]. The degree of LPO has been evaluated by estimating TBARS, lipid hydroxides, and the presence of conjugated dienes [7]. Lipid peroxide-mediated myocardial damage has been observed in ISO-treated myocardial-infarcted rats. The myocardial necrosis observed in the rats receiving ISO can be attributed to peroxidative damage, as it has been previously reported that ISO generates lipid peroxides [43]. In our study, ISO treatment resulted in a significant increase in the levels of LPO products in the heart tissue. Increased LPO appears to be the initial stage of the pathogenesis making heart tissue more susceptible to oxidative damage. WSLEt pretreatment significantly reduces the levels of lipid peroxides in ISO-treated rats. Thus, it is plausible that some constituents present in WSLEt with antioxidant activities scavenge the LPO products produced excessively by ISO and confer protection to the cardiac tissue.

The oxidative stress may be exerted through quinone metabolites of ISO that react with oxygen to produce superoxide anions and other reactive oxygen species (ROS) that interfere with antioxidant enzymes [19]. The presence of the endogenous antioxidant enzymatic defense is highly important for the neutralization of oxygen-free-radical-mediated tissue injury [44]. SOD, catalase (CAT), and GPx, which are the primary free radical scavenging enzymes, are involved in the first-line cellular defense against oxidative injury, decomposing oxygen (O_2) and hydrogen peroxide (H_2O_2) before their interaction to form the more reactive hydroxyl radical [45]. In this study, significantly lower activities of SOD and GPx were observed in the heart tissues of ISO-treated rats when compared to control rats. The observed decreases in the activities of these enzymes may be due to their increased utilization for scavenging ROS and their inactivation by excessive ISO oxidation [16]. Treatment with WSLEt, however, improved the activities of SOD and GPx by scavenging superoxide and H_2O_2 produced by ISO. The two enzyme levels were also higher in WSLEt alone treated group when compared with the control group which is a clear indication that WSLEt not only scavenges the oxidative stress but also boosts the activity of few antioxidant enzymes during normal physiological conditions. GRx is an antioxidant enzyme involved in the reduction of GSSG (an end product of the GPx reaction) to GSH [21].

In ISO-treated rats, there was a marked reduction in GPx activity, leading to a reduced availability of substrate for GRx, thereby decreasing its activity. Oral treatment with WSLEt in ISO-treated rats restored the activity of GRx, which accelerates the conversion of GSSG to GSH. A phase II enzyme such as GST not only catalyzes the conjugation of both hydroquinones and epoxides of polycyclic aromatic hydrocarbons with GSH for their excretion, but also shows lower activity towards organic hydroperoxides for their detoxification from cells/tissues [19]. In ISO-treated rats, there was a marked reduction in GST activity, but the activity of this phase II enzyme was restored in WSLEt-treated rats. More interestingly GST levels doubled in WSLEt alone treated group; that is, WSLEt can show a strong potential to enhance GST activity in healthy individuals. It is plausible that the upregulation of the activity or expression of Nrf2, a transcription factor released from its repressor (Keap1) under oxidative or xenobiotic stress [46], is considered as possible mechanism through which WSLEt pretreatment restores antioxidant enzyme functions as also suggested by Erejuwa et al., 2011 [47]. The released Nrf2 binds to the antioxidant response element of cytoprotective genes and induces their expression as well as the subsequent expression of free radical scavenging enzymes to neutralize and eliminate the cytotoxic oxidants [46].

A histopathological examination of the myocardial tissue of normal control rats clearly illustrated the integrity of the myocardial cell membrane. The histopathology of the WSLEt-pretreated myocardial-infarcted heart samples showed a near normal morphology of cardiac muscle with the absence of necrosis when compared to ISO-treated samples without WSLEt pretreatment, which further confirms the biochemical findings. Similar histopathological findings were observed in ISO-treated rats for gallic acid [14], which also has strong antioxidant properties. Overall, the results of this study offer scientific evidence of the importance of WSLEt in cardioprotection against CVD, a set of diseases whose pathogenesis has long been associated with oxidative stress. Further studies should be conducted to elucidate the exact mechanism of the cardioprotective effect of WSLEt.

5. Conclusion

The present biochemical and histopathological findings confirm that WSLEt preserves the integrity of myocardial cell membrane by maintaining the activities of cTnI and marker enzymes in the serum and heart of ISO-treated cardiotoxic rats. This may be due to the antilipoperoxidative and antioxidant effects of WSLEt. We conclude that *W. somnifera* leaves have the potential to be used as cardioprotective agents by protecting cardiac tissue against oxidative damage.

Conflict of Interests

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

Acknowledgments

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Research Article

Activation of Endocannabinoid System Is Associated with Persistent Inflammation in Human Aortic Aneurysm

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Human aortic aneurysms have been associated with inflammation and vascular remodeling. Since the endocannabinoid system modulates inflammation and tissue remodeling, we investigated its components in human aortic aneurysms. We obtained anterior aortic wall samples from patients undergoing elective surgery for aortic aneurysm or coronary artery disease as controls. Histological and molecular analysis (RT-qPCR) was performed, and endocannabinoid concentration was determined using LC-MRM. Patient characteristics were comparable between the groups except for a higher incidence of arterial hypertension and diabetes in the control group. mRNA level of cannabinoid receptors was significantly higher in aneurysms than in controls. Concentration of the endocannabinoid 2-arachidonoylglycerol was significantly higher, while the second endocannabinoid anandamide and its metabolite arachidonic acid and palmitoylethanolamide were significantly lower in aneurysms. Histology revealed persistent infiltration of newly recruited leukocytes and significantly higher mononuclear cell density in adventitia of the aneurysms. Proinflammatory environment in aneurysms was shown by significant upregulation of M-CSF and PPARγ but associated with downregulation of chemokines. We found comparable collagen-stained area between the groups, significantly decreased mRNA level of CTGF, osteopontin-1, and MMP-2, and increased TIMP-4 expression in aneurysms. Our data provides evidence for endocannabinoid system activation in human aortic aneurysms, associated with persistent low-level inflammation and vascular remodeling.

1. Introduction

Aneurysms of the thoracic aorta include a wide range of genetic, degenerative, and acquired disease conditions and may result in a life-threatening Stanford type A dissection, needing emergency cardiac surgery [1]. Conservative therapy options are limited during the development of aortic aneurysm, and therefore surgical replacement of the ascending aorta or endovascular therapy for descending aorta is the treatment of choice [2]. In the last few years, an increased number of clinical and experimental studies contributed to a better understanding of the mechanisms involved in the development of aortic aneurysms. Genetic diseases such as Marfan syndrome, Ehlers-Danlos syndrome, and Loeys-Dietz syndrome are well described, but relatively rare [3, 4]. A combination of causes is assumed in patients with bicuspid aortic valve, which have a higher risk of aortic dilation or dissection, probably due to altered regional hemodynamics and structural anatomy of the aortic wall [5, 6]. Inflammation has also been suggested in the development of abdominal aortic aneurysms, since cellular infiltration via vasa vasorum was observed in the aortic wall and associated with expression of inflammatory mediators [7, 8]. Because of differences in aetiologies [9], these findings from abdominal aneurysms

cannot be directly projected into thoracic aneurysms, where we lack an evidence for inflammation. Nevertheless, involvement of mediators of vascular remodeling, for example, metalloproteinases 2 and 9 (MMP-2 and MMP-9), as well as oxidative stress has also been shown in studies investigating aortic aneurysm specimen [10–12].

The role of the endocannabinoid system in homeostasis and pathology has been established in most of the organs and body systems [13]. Cannabinoid CB2 receptor and its ligands anandamide and 2-arachidonoyl-glycerol have been associated with the regulation of inflammatory response in several conditions [14]. An experimental study postulated an antifibrotic role for the CB2 receptor in a model of liver fibrosis [15]. Also, the endocannabinoid anandamide has been associated with the regulation of pulmonary vascular resistance [16]. Furthermore, the CB2 receptor acts in cardioprotective manner and influences myocardial remodeling in a murine model of ischemic cardiomyopathy [17]. Our recent work showed the activation of the endocannabinoid system and its association with persistent inflammatory reaction in human myocardial hypertrophy of patients with aortic stenosis [18].

We therefore investigated cannabinoid receptors and their ligands and their association with persistent inflammation and vascular remodeling in human aortic aneurysms.

2. Materials and Methods

2.1. Patient Data. The ethics committee of the Medical School at the University of Bonn approved the study protocol and the investigation is conformed to the principles outlined in the Declaration of Helsinki. All patients gave an informed consent to participate in this study. Samples of the anterior aortic wall were collected from patients undergoing elective surgery for aortic aneurysm, defined as diameter of ascending aorta >5.0 cm ($n = 19$) or for coronary artery disease as controls ($n = 73$). Small buttons of aortic tissue from coronary artery bypass grafting (CABG) patients were collected from a single patient for histological evaluation ($n = 24$) or pooled for molecular analysis ($n = 30$) and mass spectrometry ($n = 25$), because of the small tissue amount available from one button. In order to exclude possible differences between the control patients for each specific analysis, we performed statistical analysis not only between total CABG and aneurysms, but also between the CABG subgroups, as well as every subgroup versus aneurysms (Table 1). Patients with a positive family history, chronic dissection, penetrating aortic ulcer, Marfan syndrome, or other genetic disorders, as well as tumour disease, were excluded.

The analysis of control subgroups showed no significant differences in any parameter between the three subgroups. Patients in the total control group had significantly lower incidence in aortic regurgitation and bicuspid aortic valve than in aneurysms (Table 1). Also, patients in total control group had significantly higher incidence of arterial hypertension and diabetes mellitus, as well as higher white blood cell count, despite being in lower normal range. Based on surgery reports, we found mild to moderate signs of atherosclerosis with calcifications in 57.80% (11/19) of aneurysms, while palpable aortic calcifications were reported in 61.64% (45/73) of patients in the control group. All other parameters were comparable between the total controls and aneurysms. For significant differences between each control subgroup and aneurysms, please refer to Table 1. The perioperative data is summarized in Table 2. The control group patients underwent either solitary CABG or combined CABG and aortic valve replacement. The patients with aortic aneurysm underwent replacement of the ascending aorta with either aortic valve repair using partial excision of the noncoronary part of the aortic bulbus or aortic valve replacement using a bioprosthesis. The postoperative stroke rate and laboratory parameters except for troponin were not significantly different between the groups.

2.2. Endocannabinoid Quantification by Liquid Chromatography-Multiple Reaction Monitoring

2.2.1. Chemicals and Standard Solutions. Anandamide (AEA), 2-arachidonoyl glycerol (2-AG), arachidonic acid (AA), and their deuterated analogues AEA-d4, 2-AG-d5, and AA-d8 were obtained from Cayman Chemicals (Ann Arbor, MI, USA). Water, acetonitrile (ACN), formic acid (FA), ethyl acetate, and hexane (all from Fluka LC-MS grade) were obtained from Sigma-Aldrich.

2.2.2. Endocannabinoid Extraction. For eCBs extraction, heart tissues were first weighted in the cold room and transferred to precooled 2 mL Precellys tubes containing cold ceramic beads. Spiking solution of deuterated eCBs in acetonitrile (50 μL) was mixed with 450 μL ethyl acetate/hexane (9 : 1, v/v) and added to the tissue samples, followed by 0.1 M FA (e.g., 600 μL of 0.1 M FA and 400 μL of 0.1 M FA were added for aorta tissue and control tissue, resp.). Samples were homogenized for 2 min at 5000 rpm with Precellys 24 (Bertin Technologies, Montigny-le-Bretonneux, France). Homogenates were then centrifuged at 10000 g and 4°C for 10 min then at 16000 g for another 10 min and then kept for 10 min at −20°C to freeze the aqueous phase. The upper organic phase was recovered and evaporated to dryness and the extracts were reconstituted in 50 μL water : acetonitrile (1 : 1, v/v) for further LC/MRM analysis. Throughout the extraction procedure, the tubes, plates, beads, and so forth were invariably precooled and kept at 4°C. The samples were as well invariably kept on ice throughout the entire extraction procedure to prevent artificial alterations of endogenous eCB levels originating from enzymatic or chemical degradation and/or *ex vivo* synthesis of eCBs. The amounts of internal standards and concentration range of calibration curves were selected using test heart tissues.

2.2.3. Equipment. Liquid chromatography-multiple reaction monitoring (LC-MRM) analyses were performed on a LC-MS/MS system consisting of a 5500 QTrap triple-quadrupole linear ion trap mass spectrometer equipped with a Turbo V Ion Source (AB SCIEX, Darmstadt, Germany), an Agilent 1200 series LC system (degasser, pump; Agilent, Waldbronn, Germany), and a CTC HTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland). Data acquisition and analysis

Table 1: Preoperative patient data.

	CAD	CAD subgroups			AAA
	Total	Histology	PCR	LC-MRM	
	$n = 73$	$n = 24$	$n = 30$	$n = 25$	$n = 19$
Age (yrs)	69.29 ± 1.19 $p = 0.29$	71.42 ± 1.77 $p = 0.15$	66.67 ± 2.05 $p = 0.82$	70.24 ± 1.86 $p = 0.29$	65.42 ± 2.99
Female gender (%)	21.92 (16/73) $p = 0.38$	29.17 (7/24) $p = 1.0$	20.00 (6/30) $p = 0.50$	24.00 (5/25) $p = 0.74$	31.58 (6/19)
BMI	27.24 ± 0.72 $p = 0.06$	27.69 ± 3.87 $^{*}p = 0.03$	27.96 ± 4.29 $^{*}p = 0.02$	27.08 ± 0.81 $p = 0.16$	27.60 ± 0.46
Coronary artery disease (%)	100.00 (73/73) $p < 0.01$	100.00 (24/24) $p < 0.01$	100.00 (30/30) $p < 0.01$	100.00 (25/25) $p < 0.01$	15.79 (3/19)
Mean aneurysm diameter (cm)					5.40 ± 0.14
AAA (%)	0.00 (0/73) $p < 0.01$	0.00 (0/24) $p < 0.01$	0.00 (0/30) $p < 0.01$	0.00 (0/25) $p < 0.01$	100.00 (19/19)
Aortic valve regurgitation (%)	9.59 (7/73) $^{*}p < 0.01$	8.34 (2/24) $^{*}p < 0.01$	6.67 (2/30) $^{*}p < 0.01$	12.00 (3/25) $^{*}p < 0.01$	57.90 (11/19)
Aortic valve stenosis (%)	13.70 (10/73) $p = 0.48$	16.67 (4/24) $p = 1.0$	16.67 (5/30) $p = 0.72$	4.00 (1/25) $p = 0.15$	21.05 (4/19)
Bicuspid aortic valve	1.37 (1/73) $^{*}p < 0.01$	0.00 (0/24) $^{*}p = 0.03$	1.37 (1/30) $p = 0.07$	0.00 (0/25) $^{*}p = 0.03$	21.05 (4/19)
Ejection fraction (%)	55.32 ± 1.35 $p = 0.84$	58.92 ± 2.48 $p = 0.4$	54.17 ± 2.13 $p = 0.6$	52.96 ± 1.80 $p = 0.33$	55.89 ± 2.43
Atrial fibrillation (%)	19.18 (14/73) $p = 1.0$	29.17 (7/24) $p = 0.47$	13.33 (4/30) $p = 1.0$	12.00 (3/25) $p = 1.0$	15.79 (3/19)
Hypertension (%)	93.15 (68/73) $^{*}p < 0.01$	83.33 (20/24) $p = 0.30$	96.67 (29/30) $^{*}p = 0.01$	100 (25/25) $^{*}p < 0.01$	68.42 (13/19)
Stroke (%)	20.55 (15/73) $p = 0.18$	12.50 (3/24) $p = 0.62$	23.33 (7/30) $p = 0.13$	20.00 (5/20) $p = 0.21$	5.26 (1/19)
Diabetes (%)	41.10 (30/73) $^{*}p < 0.01$	16.67 (4/24) $p = 0.36$	50.00 (15/30) $^{*}p < 0.01$	44.00 (11/25) $^{*}p = 0.01$	5.26 (1/19)
COPD (%)	5.48 (4/73) $p = 1.0$	0.00 (0/24) $p = 0.44$	6.67 (2/30) $p = 1.0$	8.00 (2/25) $p = 1.0$	5.26 (1/19)
Renal dysfunction (%)	16.44 (12/73) $p = 0.29$	8.33 (2/24) $p = 1.0$	13.33 (4/30) $p = 0.64$	24.00 (6/25) $p = 0.12$	5.26 (1/19)
Creatinine (mg/dL)	1.05 ± 0.04 $p = 0.06$	0.97 ± 0.05 $p = 0.37$	1.05 ± 0.05 $p = 0.74$	1.15 ± 0.08 $p = 0.17$	1.02 ± 0.04
Troponin (ng/dL)	0.39 ± 0.17 $p = 0.09$	0.18 ± 0.097 $p = 0.13$	0.65 ± 0.44 $p = 0.08$	0.23 ± 0.12 $p = 0.78$	0.03 ± 0.01

Table 1: Continued.

	CAD	CAD subgroups			AAA
	Total	Histology	PCR	LC-MRM	
	$n = 73$	$n = 24$	$n = 30$	$n = 25$	$n = 19$
Hemoglobin (g/dL)	13.77 ± 0.18	13.87 ± 0.3	13.63 ± 0.3	13.50 ± 0.35	14.27 ± 0.27
	$p = 0.19$	$p = 0.33$	$p = 0.14$	$p = 0.11$	
WBC count (G/L)	7.8 ± 0.26	8.09 ± 0.4	7.7 ± 0.41	7.43 ± 0.49	6.45 ± 0.3
	$^*p = 0.01$	$^*p < 0.01$	$^*p = 0.03$	$p = 0.11$	
CRP (mg/L)	6.60 ± 1.87	4.19 ± 0.87	4.18 ± 1.01	6.04 ± 2.09	4.80 ± 2.12
	$p = 0.14$	$p = 0.28$	$p = 0.29$	$p = 0.46$	

Renal dysfunction was defined with serum creatinine above the normal range of 0.6–1.3 mg/dL. Normal range of laboratory parameters are troponin <0.05 ng/mL, haemoglobin 12.0–15.4 g/dL, WBC count 3.9–10.2 G/L, and CRP < 3 mg/L. CAD: coronary artery disease, AAA: aneurysm of ascending aorta, BMI: body mass index, COPD: chronic obstructive pulmonary disease, WBC: white blood cell, and CRP: C-reactive protein. Data presented as mean ± SEM. $^*p < 0.05$ versus AAA.

Table 2: Surgery and postoperative patient data.

	CAD	CAD subgroups			AAA
	Total	Histology	PCR	LC-MRM	
	$n = 73$	$n = 24$	$n = 30$	$n = 25$	$N = 19$
Aortic prosthesis (%)	0.00	0.00	0.00	0.00	100.00
	(0/73)	(0/24)	(0/30)	(0/25)	
	$^*p = 0.01$	$^*p = 0.01$	$^*p = 0.01$	$^*p = 0.01$	
Aortic valve replacement (%)	15.07	16.67	13.33	12	42.10
	(11/73)	(4/24)	(4/30)	(3/25)	(8/19)
	$^*p = 0.02$	$p = 0.09$	$^*p = 0.04$	$^*p = 0.04$	
Aortic valve repair (%)	0.00	0.00	0.00	0.00	15.79
	(0/73)	(0/24)	(0/30)	(0/25)	(3/19)
	$^*p = 0.01$	$^*p = 0.01$	$^*p = 0.01$	$^*p = 0.01$	
CABG surgery (%)	100	100	100	100	10.50
	(73/73)	(24/24)	(30/30)	(25/25)	(2/19)
	$^*p < 0.01$	$^*p < 0.01$	$^*p < 0.01$	$^*p < 0.01$	
Perioperative stroke (%)	0.00	0.00	0.00	0.00	0.00
	(0/73)	(0/24)	(0/30)	(0/25)	(0/19)
	$p = 1.0$	$p = 1.0$	$p = 1.0$	$p = 1.0$	
Hemoglobin (g/dL)	10.48 ± 0.16	10.64 ± 0.29	10.33 ± 0.25	10.59 ± 0.24	10.84 ± 0.31
	$p = 0.3$	$p = 0.64$	$p = 0.21$	$p = 0.52$	
Creatinine (mg/dL)	1.15 ± 0.04	1.02 ± 0.07	1.17 ± 0.08	1.21 ± 0.09	1.01 ± 0.07
	$p = 0.14$	$p = 0.27$	$p = 0.17$	$p = 0.11$	
Troponin (ng/dL)	0.68 ± 0.22	0.43 ± 1.11	0.96 ± 0.47	0.73 ± 0.44	1.09 ± 0.34
	$^*p = 0.02$	$^*p = 0.02$	$p = 0.21$	$p = 0.07$	
WBC count (G/L)	9.64 ± 0.30	10.26 ± 0.56	9.44 ± 0.36	9.34 ± 0.61	9.47 ± 0.87
	$p = 0.82$	$p = 0.44$	$p = 0.97$	$p = 0.89$	
CRP (mg/L)	70.84 ± 6.00	70.3 ± 11.7	68.00 ± 8.33	69.59 ± 9.53	80.53 ± 9.36
	$p = 0.43$	$p = 0.51$	$p = 0.33$	$p = 0.43$	

Surgery data and laboratory data obtained 72 h postoperatively. Normal range of laboratory parameters are creatinine 0.6–1.3 mg/dL, troponin <0.05 ng/mL, haemoglobin 12.0–15.4 g/dL, WBC count 3.9–10.2 G/L, and CRP < 3 mg/L. CAD: coronary artery disease, AAA: aneurysm of ascending aorta, CABG: coronary artery bypass grafting, WBC: white blood cell, and CRP: C-reactive protein. Data presented as mean ± SEM. $^*p < 0.05$ versus AAA.

were performed using Analyst software (version 1.6.1; AB SCIEX).

2.2.4. LC-MRM Quantification. 20 μL of the solution of extracted eCBs was injected and separated on a Phenomenex Luna 2.5 μm C18(2)-HST column, 100 mm × 2 mm, combined with a precolumn (C18, 4 mm × 2 mm; Phenomenex, Aschaffenburg, Germany), by increasing acetonitrile containing 0.1% formic acid over 2 min from 55% to 90% and maintaining it at 90% for 5.5 min. Throughout the analysis,

samples were maintained at 4°C in the LC autosampler. The separated eCBs were flow-through analyzed using MRM. Positive ions (of AEA, 2-AG, and PEA) and negative ions (of AA) were simultaneously analyzed using the "positive-negative-switching" mode. The following MRM transitions were monitored for quantification of eCBs: AEA, m/z 348.3 to m/z 62.3; AEA-d4, m/z 352.3 to m/z 62.1; 2-AG, m/z 379.1 to m/z 287.2; 2-AG-d5, m/z 384.1 to m/z 287.2; PEA, m/z 300.2 to m/z 62.1; PEA-d4, m/z 304.2 to m/z 62.1; AA, m/z 303.05 to m/z 259.1; and AA-d8, m/z 311.04 to m/z 267.0. Endocannabinoid concentrations were normalized to the tissue weight.

2.3. Histology and Immunohistochemistry. Basic histological evaluation of paraffin-embedded 5 μm slices was performed using hematoxylin-eosin staining. Collagen area was calculated on picrosirius red stained samples as previously described [17]. Briefly, photographic images were taken (DP70 camera, Olympus, Münster, Germany) and planimetric analysis of collagen-stained area as percentage of the total aortic wall area was performed using the Software Cell-F (Olympus Soft Imaging Solutions, Münster, Germany).

In order to investigate specific cell types, we used the following primary antibodies cross-reacting with human tissue for immunohistochemistry: MAC-387 monoclonal mouse anti-human antibody for newly recruited leukocytes (NatuTec, Frankfurt, Germany) and CD11b monoclonal rabbit anti-human antibody for monocytes (clone: EP1345Y (ab52478); Abcam, Cambridge, UK). An appropriate Vectastain Elite ABC kit and diaminobenzidine (both from AXXORA, Lörrach, Germany) were used for immunohistochemistry. Cell density was calculated using cell count/mm^2.

2.4. Molecular Analysis. mRNA of the aortic wall samples was isolated using standard phenol/chloroform extraction (TRIzol, Invitrogen, Karlsruhe, D). First-strand cDNA was synthesized using the high capacity cDNA transcription kit (Applied Biosystems, Foster City, CA, USA) with random hexameric primers as described by the manufacturer protocol. Expression of mRNA was analysed using the TaqMan real time quantitative PCR system (RT-qPCR; Applied Biosystems). RT-qPCR was performed and analysed on an ABI Prism 7900HT Sequence Detection System and SDS2.2 Software (Applied Biosystems) with 1/10 diluted cDNA following manufacturer's instructions. Target gene-expression was normalized to an internal control and housekeeping gene GAPDH using comparative ΔΔCT method. All primers were measured using FAMTAMRA chemistry and relative standard curve method. Dissociation curve analysis was performed in order to ascertain the amplification.

2.5. Statistical Analysis. Sample size calculations were performed using G*Power (V3.1) and depending on the expected difference of the given parameter resulted in a minimum of 15 patients needed for each group. The poststatistical calculation confirmed this value. Data are reported as mean ± SEM and tested for normal distribution using D'Agostino and Pearson omnibus normality test. Differences were analysed in Prism 6.0 software (GraphPad, La Jolla, CA, USA) using Mann-Whitney test or unpaired t-test and considered statistically significant when $p < 0.05$.

3. Results

3.1. Activated Endocannabinoid System in Human Aortic Aneurysms. Experimental data suggested dynamic regulation of endocannabinoids and their receptors in the vascular system [16]. Therefore, we investigated the components of the endocannabinoid system in human aorta and found significantly higher mRNA levels of the cannabinoid receptors CB1 (Figure 1(a)), CB2 (Figure 1(b)), TRPV1 (Figure 1(c)), and GRP55 (Figure 1(d)) in the aneurysms as compared to the samples from controls. Expression of related factors 5HT1A and PPARα was comparable between the groups (data not shown). Mass spectrometry measurements of endocannabinoids showed a significantly lower level of anandamide (Figure 1(c)) and a significantly higher level of 2-arachidonoyl glycerol in aneurysms (Figure 1(d)). Interestingly, aneurysm samples contained a significantly lower amount of the endocannabinoid degradation product arachidonic acid (Figure 1(e)) and palmitoylethanolamide (Figure 1(f)) than the control samples. Therefore, aortic aneurysm showed not only increased level of cannabinoid receptors, but also a different amount of ligands and decreased level of their degradation products suggesting differentiated, persistent action of endocannabinoids in the aortic wall.

3.2. Cellular Infiltration of the Aortic Wall and Associated Mediators of Inflammation. Animal studies and our previous work in human myocardial hypertrophy showed association between activation of endocannabinoid system and persistent inflammatory reaction [14, 18]. Therefore, we investigated the infiltration of the aortic wall with inflammatory cells. We quantified newly recruited leukocytes stained with MAC387 and found a significantly higher cell density of them in the adventitia of both groups when compared to the respective tunica media (Figures 2(a)–2(c)). We observed a comparable density of newly recruited leukocytes between the aneurysms and controls in the adventitia, indicating persistent accumulation of inflammatory cells into the aortic wall in both groups. Since mononuclear cells differentiate into macrophages after tissue invasion and macrophages act as an important source of different inflammatory or tissue remodeling mediators, we evaluated their infiltration into aortic wall using CD11b staining. Mononuclear cell density was significantly higher in adventitia of aneurysms not only when compared to controls, but also when compared to the media (Figures 2(d)–2(f)). At the same time, control samples showed no significant difference in mononuclear cell density between complete wall and adventitia, but significantly more cells in the adventitia alone. Taken together, the aneurysms and controls showed persistent infiltration of aortic wall with newly recruited leukocytes, but the aneurysms presented with a significantly higher density of tissue remodeling relevant mononuclear cells.

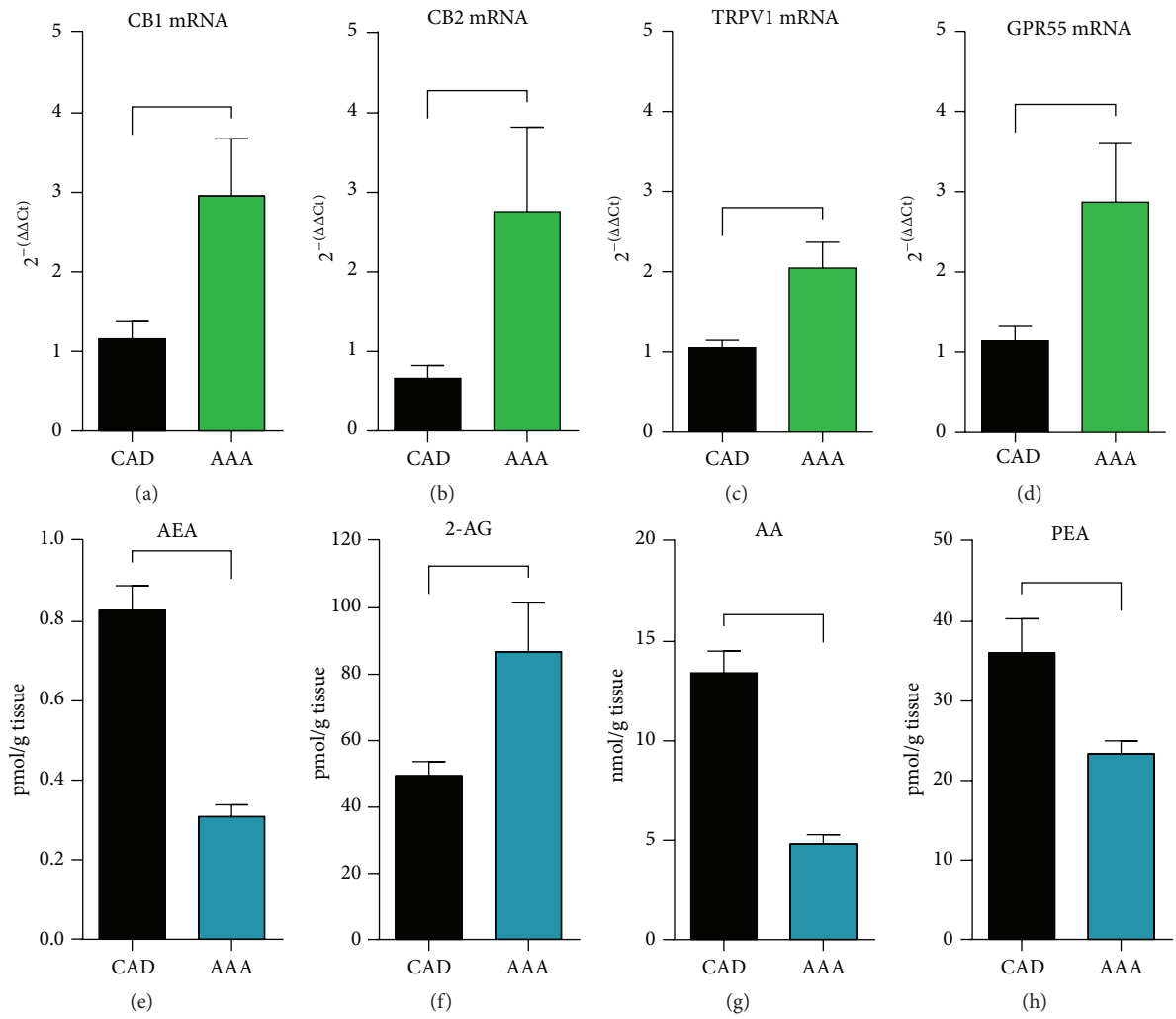


Figure 1: Activation of the endocannabinoid system in aortic aneurysms. mRNA levels of cannabinoid receptors (a) CB1, (b) CB2, (c) TRPV1, and (d) GPR55 showed a significant increase in aneurysm of ascending aorta (AAA) as compared to the normal aorta of patients with coronary artery disease (CAD). Tissue concentration of the endocannabinoids (c) anandamide (AEA) and (d) 2-arachidonoyl glycerol (2-AG), as well as their degradation products (e) arachidonic acid (AA), and (f) palmitoylethanolamide (PEA) in comparison between the groups. $n = 19$ in the AAA group, $n = 30$ in the CAD RT-qPCR, and $n = 25$ in the CAD LC-MRM group. mRNA levels in RT-qPCR are related to controls and GAPDH using comparative ΔΔCt method. Bracket indicates $p < 0.05$.

In the next step, we measured the molecular expression of inflammatory mediators associated with this cellular infiltration pattern. Interestingly, we found no significant difference in mRNA expression of proinflammatory cytokines TNFα, IFNγ, and IL-1β (Figures 3(a)–3(c)), or anti-inflammatory cytokine IL-10 (Figure 3(g)). We measured a significantly lower expression of the cytokine IL-6 (Figure 3(d)) and significantly higher expression of macrophage colony-stimulating factor (M-CSF) (Figure 3(e)) and peroxisome proliferator-activated receptor (PPAR)γ (Figure 3(f)) in aneurysms than in controls. In addition, we measured expression of potent mononuclear chemoattractants and found that chemokine CCL2 had significantly lower expression in aneurysms than in controls, while the chemokine CCL4 showed only a tendency to a lower expression in aneurysms (Figures 3(h), 3(i)). We also measured mRNA expression of reactive oxygen scavengers heme oxygenase 1 and glutathione peroxidase 1 and found no difference between the two groups (data not shown). Therefore, the persistent cellular infiltration of the adventitia of aneurysms was associated with an increased M-CSF and PPARγ expression, while the aorta of patients with coronary artery disease showed higher level of chemokine CCL2 and cytokine IL-6.

3.3. Collagen and Markers of Remodeling in Aortic Aneurysm. Several experimental and clinical studies showed association between endocannabinoids and tissue remodeling [15, 17], and we therefore compared collagen and related remodeling markers between the aneurysms and controls. Collagen was visualised using picrosirius red staining in the aortic wall and its planimetric evaluation revealed a comparable

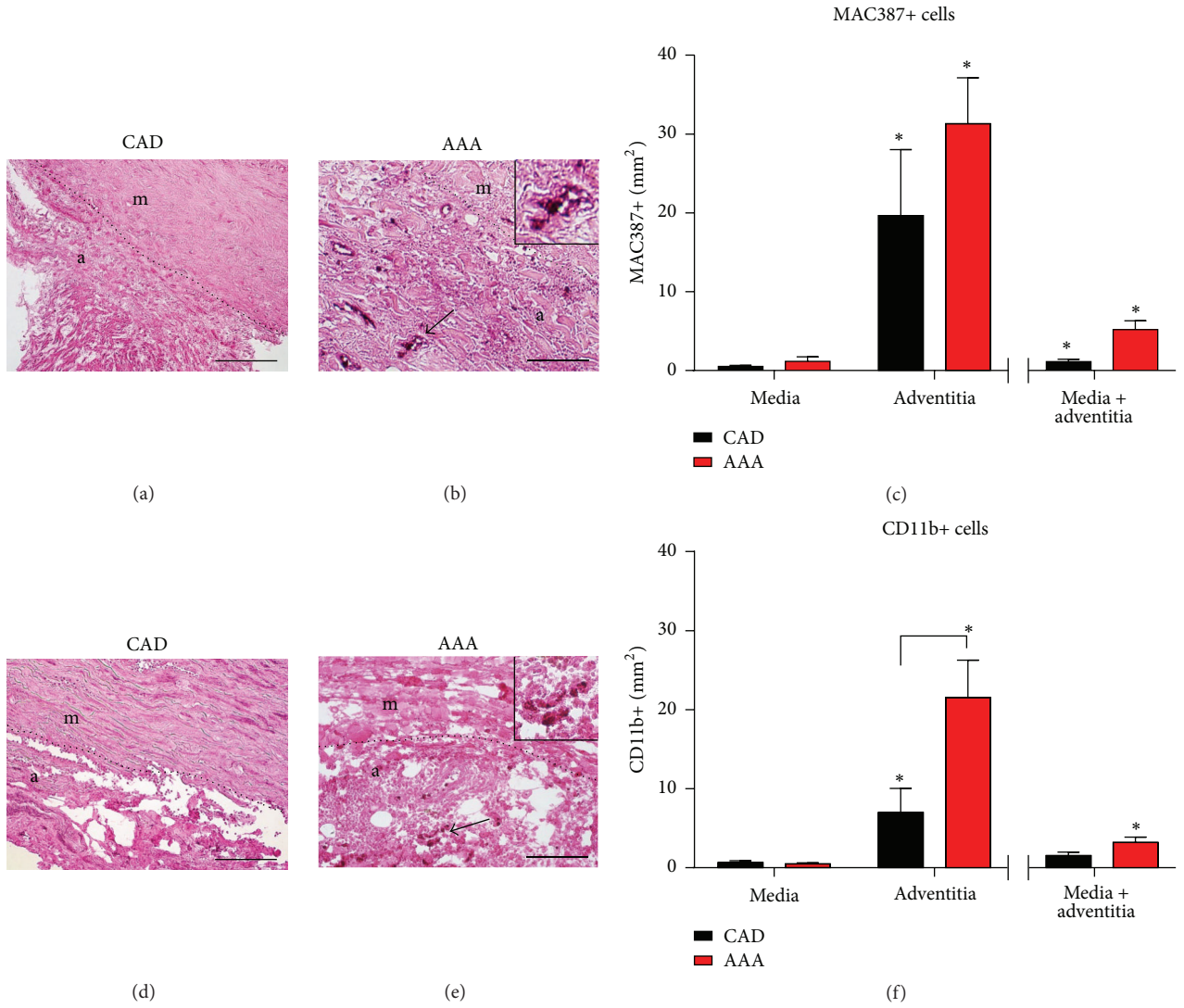


Figure 2: Infiltration of aortic wall with inflammatory cells. Representative image of newly recruited leukocytes stained with MAC-387 antibody (a) in aorta from patients with coronary artery disease (CAD) and (b) from aneurysm of the ascending aorta (AAA). Arrows indicate the stained leukocytes and the dotted line delineates the adventitia (a) from the media (m). (c) Quantitative analysis of newly recruited leukocytes showed comparable cell density between the groups with significantly higher density in adventitia than in the media. Representative image of mononuclear cells stained using CD11b antibody in (d) aorta from CAD and (e) from AAA patient. (f) Quantitative analysis of mononuclear cells showed significantly higher cell density in adventitia of AAA samples than in CAD samples or respective media of the aortic wall. Scale bar in (a), (b), (d), and (e): 200 μm. $n = 19$ in the AAA group and $n = 24$ in the CAD histology group. Bracket indicates $p < 0.05$ between the groups; $*$ indicates $p < 0.05$ versus media.

collagen-stained area between both groups (Figures 4(a)–4(c)). We also differentiated between the inner and the outer tunica media, since the thinner aneurysmatic wall may have less collagen, but we found no difference between the two layers in both groups. Next, we measured mRNA expression of related mediators and found a significantly lower level of connective tissue growth factor (CTGF) and osteopontin-1 in aneurysms (Figures 4(d), 4(e)). At the same time, the expression of early remodeling marker tenascin C was comparable between the groups (Figure 4(f)). We also measured the mRNA expression of transforming growth factor- (TGF-) β1 and found no difference between the groups (Figure 4(g)). Since tissue remodeling is always associated with induction of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs), we measured the level of MMP-1, MMP-2, MMP-9, and MMP-14 (Figures 5(a)–5(d)), as well as TIMP-1, TIMP-2, and TIMP-4 (Figures 5(e)–5(g)). We found only a significantly lower mRNA expression of MMP-2 in aneurysms, whereas higher expression of MMP-9 in aneurysms was not significant between the groups as well as the expression of MMP-1 and MMP-14. At the same time, we found no difference in expression of TIMP-1 and TIMP-2. The significantly higher expression of TIMP-4 seems to be responsible for the lower MMP-2 expression and this is also reflected in their significantly increased MMP-2/TIMP-4 ratio (Figure 5(h)). Other MMP/TIMP

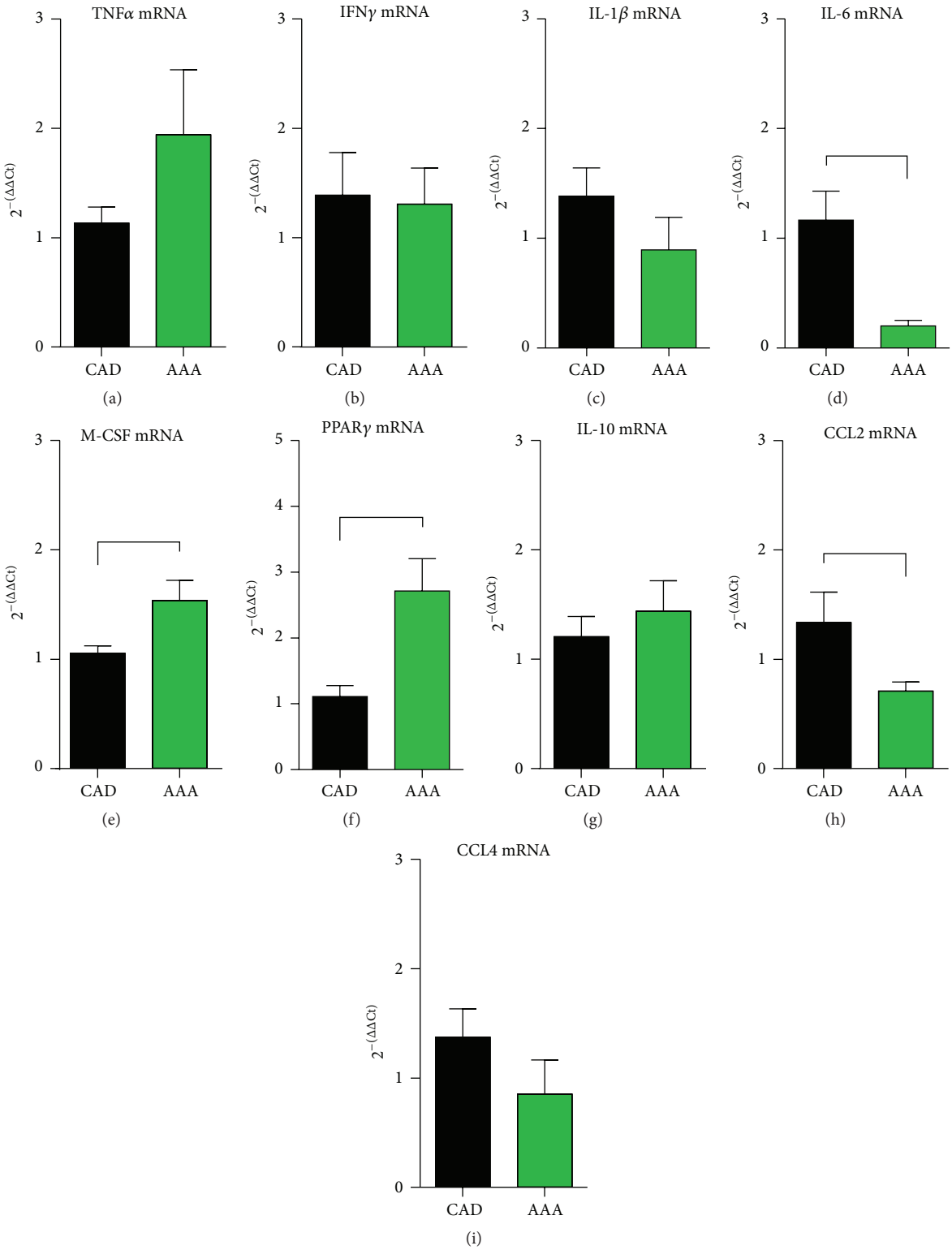


Figure 3: mRNA expression of cytokines and chemokines in aortic wall. The mRNA expression of proinflammatory cytokines (a) TNFα, (b) IFNγ, and (c) IL-1β was comparable between ascending aortas from aneurysms (AAA) and patients with coronary artery disease (CAD). The mRNA expression of (d) IL-6 was lower, while (e) macrophage colony-stimulating factor (M-CSF) and (f) peroxisome proliferator-activated receptor (PPAR)γ were significantly higher in AAA than in CAD aortic tissue. The expression of (g) the anti-inflammatory cytokine IL-10 was comparable between the groups. (h) A significantly lower expression of potent mononuclear chemoattractants CCL2 and (i) nonsignificantly lower level of related chemokine CCL4 were found in AAA samples. $n = 19$ in the AAA group and $n = 30$ in the CAD RT-qPCR group. mRNA expression in RT-qPCR is related to controls and GAPDH using comparative $\Delta\Delta$Ct method. Bracket indicates $p < 0.05$.

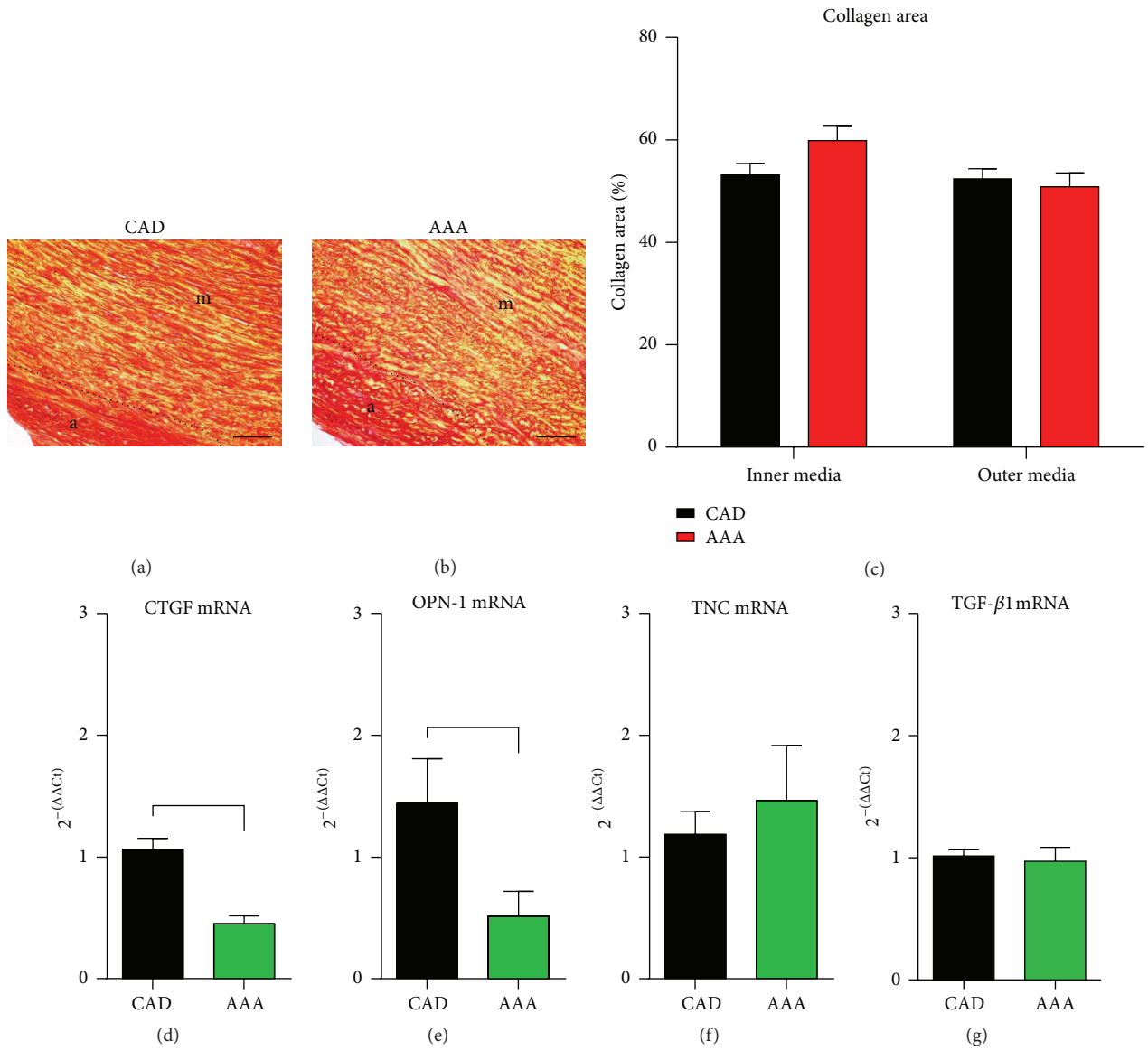
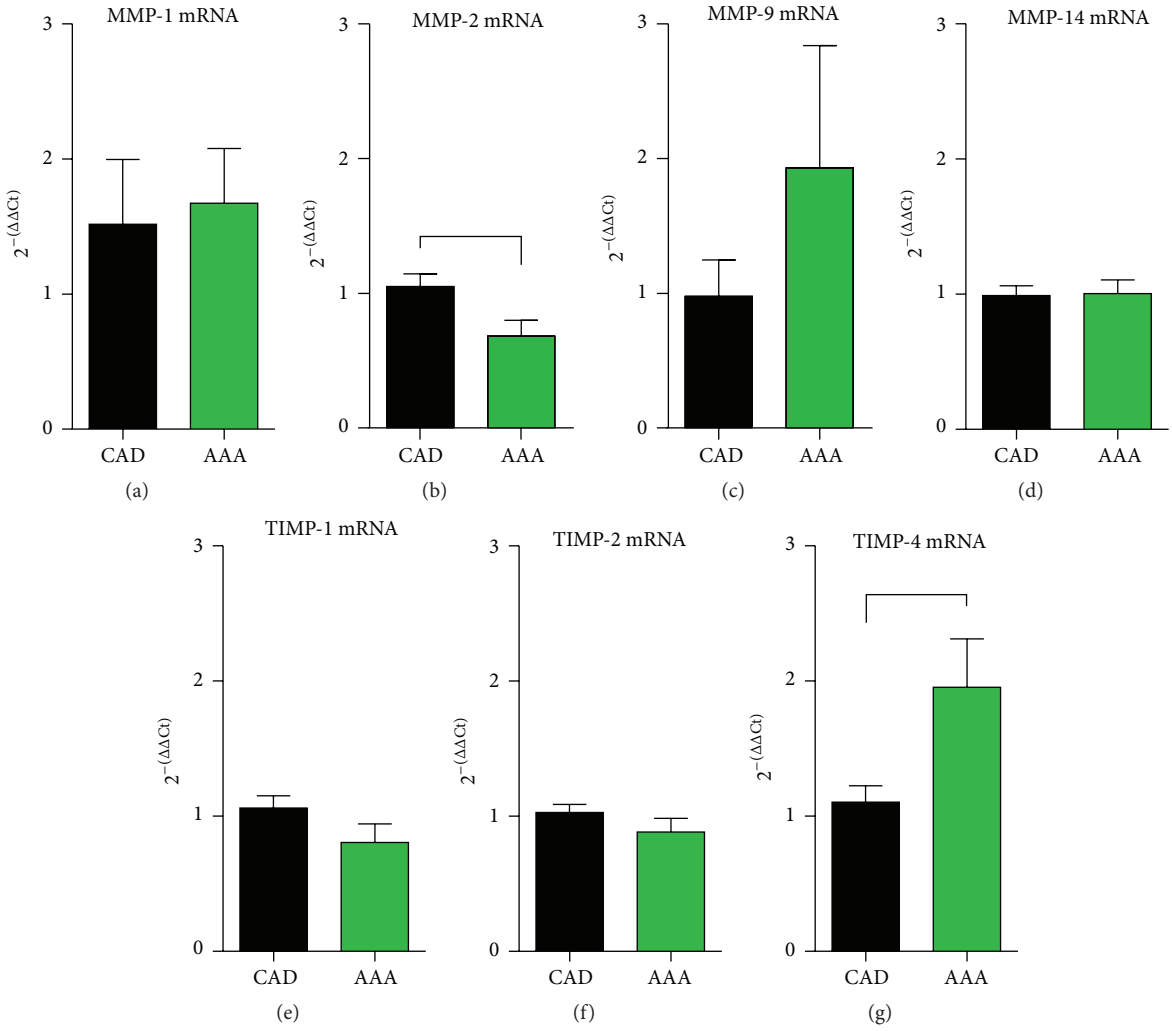


Figure 4: Collagen area and expression of remodeling related factors in aortic aneurysm. Representative images of picrosirius red staining show comparable collagen-deposition pattern in media (m) and adventitia (a) of (a) aorta from patient with coronary artery disease (CAD) and (b) aneurysm of the ascending aorta (AAA). (c) The planimetric evaluation of the collagen-stained area in the inner and outer media showed comparable results between both groups. The mRNA expression of (d) the collagen-related connective tissue growth factor (CTGF) and (e) extracellular matrix remodeling related factor osteopontin-1 is significantly lower in AAA when compared to the CAD aortic samples. (f) The mRNA expressions of early remodeling factor tenascin C and (g) transforming growth factor- (TGF-) β1 are comparable between the groups. $n = 19$ in the AAA group, $n = 24$ in the CAD histology, and $n = 30$ in the CAD RT-qPCR group. mRNA expression in RT-qPCR is related to controls and GAPDH using comparative $\Delta\Delta$Ct method. Bracket indicates $p < 0.05$.

ratios were not significantly different. These findings indicate a finely regulated low-level extracellular matrix remodeling being driven predominantly by MMP-2/TIMP-1 interaction in aortic aneurysms, when compared to controls.

4. Discussion

Several studies investigated mechanisms in pathogenesis of human aortic aneurysm. Beside the genetically well-described diseases [3, 4], there are a growing number of studies reporting molecular mediators and cellular interactions in the aortic aneurysmatic wall. Inflammation has been postulated as a major factor during development of abdominal aortic aneurysms [7] and has not yet been reported in aneurysms of the ascending aorta. Since regulation of inflammatory response and tissue remodeling are the major effects mediated by the endocannabinoid system [14, 15], we investigated it in aneurysms of ascending aorta.



Ratios	Control	AAA	p value
MMP-1/TIMP-1	1.71 ± 0.54	2.93 ± 0.91	n.s.
MMP-2/TIMP-1	1.03 ± 0.09	1.07 ± 0.19	n.s.
MMP-2/TIMP-2	1.04 ± 0.10	0.81 ± 0.08	n.s.
MMP-2/TIMP-4	1.14 ± 0.15	0.58 ± 0.14	$p = 0.01$
MMP-9/TIMP-1	1.64 ± 0.41	2.75 ± 1.11	n.s.
MMP-14/TIMP-2	1.00 ± 0.08	1.39 ± 0.21	n.s.

(h)

Figure 5: Expression of matrix metalloproteinases and their tissue inhibitors in aortic aneurysm. The mRNA expression of (a) matrix metalloproteinase- (MMP-) 1, (b) MMP-2, (c) MMP-9, and (d) MMP-14 was not significantly different in aorta from patients with aneurysm of the ascending aorta (AAA) compared to coronary artery disease (CAD) except for MMP-2. The mRNA expression of (e) tissue inhibitor of matrix metalloproteinase- (TIMP-) 1, (f) TIMP-2, and (g) TIMP-4 was not significantly different in aorta from patients with AAA compared to CAD except for TIMP-4. (h) Ratios of different MMP/TIMP showed only a significant difference for MMP-2/TIMP-4 between AAA and CAD group. $n = 19$ in the AAA group and $n = 30$ in the CAD RT-qPCR group. mRNA expression in RT-qPCR is related to controls and GAPDH using comparative ΔΔCt method. Bracket indicates $p < 0.05$.

With respect to the published data, we here provide novel evidence for the activation of the endocannabinoid system in human aortic aneurysms. The higher mRNA levels of the four receptors (CB1, CB2, TRPV1, and GRP55) show together with differential levels of their ligands in the tissue an activated endocannabinoid system in human aneurysms. The lower tissue amount of arachidonic acid, a degradation product of both endocannabinoids, also supports this assumption, indicating prolonged demand for endocannabinoids in aneurysmatic tissue. Since the published data suggest a regulatory

role of AEA in inflammatory response, the lower AEA tissue amount could be associated with lower expression of IL-6 and CCL2. Still, our data also show higher M-CSF and PPARγ expression, and in the lack of experimental evidence we can only speculate whether this finding is linked to the increased 2-AG tissue amount. To our knowledge, a distinct association of the two known endocannabinoids with specific mediators of inflammatory response has not yet been dissected. The endocannabinoid-mediated fine-tuning of inflammatory reaction involves several mediators acting on different cell types [14]. Our data on the newly recruited leukocytes show no difference between aneurysms and controls but reveal a constant low-level infiltration of them into the adventitia of the aortic wall. This action of inflammatory cells underlines their role in homeostatic regulation of the aortic wall tissue, representing not only inflammation, but also tissue remodeling or regeneration. While previous studies reported mostly on the CD68-positive mature macrophages or T-cells [19], our study also provides evidence for an increased infiltration of the aortic wall by CD11b-positive mononuclear cells. In the context of the endocannabinoid system, the mononuclear cell infiltration is important because of a CB2 receptor-dependent differentiation of M2a subpopulation of macrophages acting at the junction between inflammation and remodeling, as we recently described [17]. In the same study, we also showed a CB2 receptor-dependent regulation of potent inflammatory mediator for mononuclear cells M-CSF in murine heart *in vivo*. Our present study shows a significantly higher level of M-CSF and PPARγ associated with a lower level of IL-6 and no significant difference in other cytokines. Still, we found a significantly lower expression of potent chemotactic mediator chemokine CCL2, which may reflect a negative feedback regulation of leukocyte infiltration in order to keep the inflammation at a very low level. This finding is very interesting since experimental studies have suggested beneficial effects of chemokine inhibition on formation of aneurysms [8], but future studies specifically targeting chemokines should clarify their contribution to this pathology. Another interesting aspect regarding production of reactive oxygen species has also been associated with human aortic aneurysms [10]. Even though we did not measure reactive oxygen species production, we found no difference in the expression of their scavenger enzymes heme oxygenase and glutathione peroxidase. So far, all of these facts indicate a very finely tuned inflammatory process in human aortic aneurysms and therefore the recently postulated prognostic value for phosphorylation of c-kit and downstream targets in aneurysm formation fits well in this concept [20]. In this context, recent studies reported that the inhibition of cytokine IL-1β attenuates experimental development of aortic aneurysm [21], as well as activation of TGF-β signalling [22]. Future studies should address the exact characterization and cellular imaging of the inflammatory cells, that is, T-cells, macrophage subpopulations, and so forth, in order to better understand their specific contribution during development of human aneurysms.

The homeostasis of vascular tissue is associated with constant remodeling in order to preserve the normal function of the blood vessels. Tissue remodeling involves several mediators acting in extracellular matrix and specific cells, for example, smooth muscle cells or fibroblasts. As a result, in aneurysms, the thickness of the vessel wall changes and this may lead to dissection or rupture. The nature of acquisition of aortic buttons in bypass surgery did not allow measurement of the exact aortic wall thickness. We therefore concentrated on matrix components and investigated the collagen-stained area in the tunica media of the aorta. Induction of CTGF, a major collagen-deposition related factor, was reported in vascular smooth muscle cells after oxidative burst in thoracic aneurysms *ex vivo* [10]. A recent study reported higher level of CTGF, osteopontin-1, and collagen in human thoracic aneurysms when compared to CABG controls without significant difference in incidence of hypertension or diabetes [23]. Their results are in contrast to our data, probably due to the differences in the control group. Our controls have a higher incidence of hypertension and diabetes, which is expected in patients having CAD, as well as WBC count, thus supporting the inflammatory nature of the underlying atherosclerosis. Despite this difference, our aneurysm samples show a higher level of M-CSF, PPARγ, and mononuclear cells than in controls, thereby indicating a specific modulation of vascular remodeling. Even though we report no difference in collagen-stained area between the two groups, we measured significantly lower expression of the CTGF, indicating a lower turnover of the collagen in the aneurysms. Our findings on CTGF are further supported by the lower expression of osteopontin-1, which has been associated with general deposition of matricellular components and tissue remodeling. Expression of tenascin C, an early remodeling marker, is comparable between the two groups and this additionally indicates absence of novel extracellular matrix deposition, but rather a low grade of matrix turnover. Our previous experimental work showed a CB2 receptor-mediated regulation of smooth muscle cell function, where myofibroblasts were not able to induce tenascin C, MMP-9, and TIMP-1 under hypoxic cell culture conditions [17]. In the same study, we observed an increased MMP-9/TIMP-1 ratio and lower myofibroblast density in CB2 receptor-deficient mice in our *in vivo* model of myocardial ischemia. These findings strongly indicate an important role for endocannabinoid system in function of smooth muscle cells and extracellular matrix remodeling. Previous studies reported activation of MMP-2 and MMP-9 being associated with formation of aortic aneurysms [11, 12]. While some experimental data are associated with dissection of the abdominal aorta [24], other studies provide evidence for structural differences between the thoracic and abdominal aorta which in addition to different biophysical stimulus makes them not well comparable [25]. A recent study showed attenuation of experimental aortic aneurysm formation in mice after use of unsaturated fatty acid causing reduced expression of MMP-2 and MMP-9 [26]. Our data describe a significantly lower expression of MMP-2 and higher expression of TIMP-4 in aneurysms with no significant difference in other MMPs or TIMPs. Since TIMP-4 is an important counter actor for MMP-2 in achieving a balanced matrix turnover, we assume that the observed difference in MMP-2/TIMP-4 ratio reflects this balance in our aneurysm patients. Our MMP-2 findings are in

contrast to some of the published data [11, 12] and we assume this difference to be based on practically unknown stage of disease development in our patients and other human studies, abdominal versus thoracic aortic tissue, differences between animal and human tissue, or even different extent of inflammation in the aorta in experimental settings including dissection. Future studies are needed to clarify these differences.

This study has a relatively small patient number and in the lack of an experimental model it offers only descriptive data regarding the underlying mechanisms. Therefore, examination of suitable genetically or pharmacologically engineered animal models without dissection should be performed in future studies. Endocannabinoids have been associated in experimental studies with arterial pressure regulation, which by now did not yield a specific pharmacological target [27], whereas its association in metabolic homeostasis and diabetes development offers some promising cues [28]. Despite effects on hypertension or diabetes and inability to clearly delineate them from other pathologies, the short action of endocannabinoids due to their fast degradation makes us confident to claim the observed results as representative for the endocannabinoid level in the aortic wall. Our separate analysis of each CAD subgroup showed only a few nonessential differences between the subgroups, and this strengthened the basis for our data interpretation. We have chosen patients undergoing coronary bypass surgery as controls, due to the availability of vital tissue needed for lipid measurements and because patients with aortic aneurysm and age >40 years frequently present with atherosclerotic lesions. Still, our data provides approximation in the extent of atherosclerotic changes in the aorta of control patients when compared to the aneurysms, where both groups showed signs of atherosclerosis in more than 50% of patients. Obviously, aorta from normal healthy subjects would be a "proper" control, but since it is only available postmortem it is not usable for mRNA or endocannabinoid extraction. We could only investigate the tissue at the time point of surgery and therefore one cannot make any assumptions regarding the dynamic changes in tissue remodeling and formation of aortic aneurysms. Therefore, novel imaging techniques and molecular markers in the blood stream are needed in order to better understand the temporal resolution of this process. The translation of the published data into clinical setting has not been achieved yet and therefore the clinical morbidity and outcome of the modern therapy options remain unchanged [29].

5. Conclusions

Our study provides evidence for differential regulation and activation of the endocannabinoid system in human aortic aneurysms. This was associated with a low level of inflammatory cell infiltration and induction of M-CSF and PPARγ accompanied by downregulation of cytokine IL-6 and chemokine CCL2. At the same time, vascular remodeling of the aneurysmatic aortic wall was characterized by a lower expression of CTGF, osteopontin-1, and MMP-2, together with increased TIMP-4, all suggesting a possible low collagen and matrix turnover. These findings provide a basis for future pharmacological and experimental studies and may contribute to the development of novel therapeutic targets.

Conflict of Interests

There is no conflict of interests declared.

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Research Article

Detection of Differentially Expressed MicroRNAs in Rheumatic Heart Disease: miR-1183 and miR-1299 as Potential Diagnostic Biomarkers

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This study compared microRNA (miRNA) expression profiles between rheumatic heart disease (RHD) patients and healthy controls to investigate their differential expression and help elucidate their mechanisms of action. Microarray analysis was used to measure miRNA expression, and a total of 133 miRNAs were shown to be significantly upregulated in RHD patients compared with controls, including miR-1183 and miR-1299. A total of 137 miRNAs, including miR-4423-3p and miR-218-1-3p, were significantly downregulated in RHD patients. Quantitative real-time-PCR confirmed microarray findings for miR-1183 and miR-1299 in both tissue and plasma. Bioinformatic predictions were also made of differentially expressed miRNAs as biomarkers in RHD by databases and GO/pathway analysis. Furthermore, we investigated miR-1183 and miR-1299 expression in RHD patients with secondary pulmonary hypertension (PAH). Our findings identified an important role for miR-1299 as a direct regulator of RHD, while the observed difference in expression of miR-1183 between RHD-PAH patients with high or low pulmonary artery pressure suggests that miR-1183 overexpression may reflect pulmonary artery remodeling. miR-1183 and miR-1299 appear to play distinct roles in RHD pathogenesis accompanied by secondary PAH and could be used as potential biological markers for disease development.

1. Introduction

MicroRNAs (miRNAs) are a class of single-stranded endogenous noncoding RNA molecules [1, 2], approximately 22 nucleotides (nt) in length, that negatively regulate gene expression by targeting the 3′-untranslated region of specific mRNAs for degradation or translational repression [3, 4]. The rapid growth in miRNA studies has demonstrated that they play an important role in a range of biological processes and are viewed as critical regulators in immune cell lineage commitment, differentiation, maturation, and immune signaling pathways [5, 6]. Additionally, deregulated miRNA expression patterns have been documented in many human diseases including inflammatory and autoimmune diseases [7–9].

Early miRNA studies focused on their role in cancer [10–13]; however, more recently, there has been a shift of attention to their possible impact on cardiovascular development and diseases [14]. Indeed, they are important in tissue development and influence the pathological processes of many cardiovascular diseases, including acute myocardial infarction, heart failure, coronary artery disease, stroke, and hypertension [1, 2]. To the best of our knowledge, the effects of miRNAs on the development of rheumatic heart disease (RHD) in either healthy tissues or circulating plasma have not yet been investigated.

RHD is primarily an autoimmune sequela of an acute rheumatic fever [15, 16], which occurs as a result of beta-hemolytic streptococcal infection [17, 18]. RHD can cause

chronic inflammation of the endocardium and myocardium, leading to valvular dysfunction and hemodynamic changes [19] and, commonly, heart failure, stroke, or other serious related complications. Unfortunately, because of the lack of a specific method of detecting RHD, many patients have been diagnosed with irreversible valvular dysfunction, for which valvular surgery is one of the main treatments. RHD continues to be a burden in several developing countries such as India and China, though it is reasonably rare in western countries—probably because of the widespread use of antibiotics [20–22]. Therefore, the identification of a biomarker of characteristic RHD pathophysiology will be valuable to aid early detection and enable patients to avoid surgery by starting effective treatment at an early stage.

Pulmonary arterial hypertension (PAH) is a common complication of many RHD patients. Because PAH is characterized by the enhanced proliferation and reduced apoptosis of pulmonary artery smooth muscle cells [23], and as some miRNAs are also associated with the regulation of cell proliferation and apoptosis, it is hypothesized that they might be implicated in the etiology of PAH [22–26].

Therefore, in this study, we analyzed the miRNA expression profiles of RHD patients using microarray and confirmed our findings using quantitative real-time- (qRT-) PCR. We screened the roles of differentially expressed miRNAs in RHD with secondary PAH and used bioinformatics to predict and analyze their target genes as potential biomarkers of RHD. We also propose new directions for their potential therapeutic use in RHD.

2. Materials and Methods

2.1. Patients. A total of 100 subjects were selected for the study from the Inpatient Clinic of Ningbo Medical Center, Lihuili Hospital (Ningbo, China), between March 2012 and October 2013. Of these subjects, 50 were RHD patients (case group), and the remaining 50 were normal healthy adults (control group) with no medical history of congenital heart disease, cardiomyopathy, or liver or renal diseases. The inclusion criteria of the RHD group are as follows: (i) every patient diagnosed with mitral valve prolapse because of mitral chordae tendineae fracture and mitral insufficiency and scheduled for mitral valve replacement; (ii) left ventricular ejection fraction (EF) > 50%; (iii) left ventricular end-diastolic diameter (LVEDD) < 55 mm. RHD cases and their controls were well matched based on the following details: (iv) same gender; (v) difference of age < 5 years; (vi) other physiological indexes from physical check in close. All human materials were obtained in accordance with the hospital's regulations and hence were approved by the Ethics Committee of Lihuili Hospital. Written informed consent was also obtained from all subjects in advance.

2.2. Sample Collection. Blood samples were collected in EDTA tubes for plasma collection from the 50 RHD cases and stored at −80°C. All blood samples of cases and controls were collected by the same investigators. Left ventricular papillary muscles were obtained from 12 cases of resected mitral valves from RHD cases. They were later transferred into a physiological saline solution and then into liquid nitrogen and stored at −80°C. Six normal tissues for comparison were obtained from donors who had died from trauma.

2.3. RNA Isolation and Characterization. Peripheral blood was coagulated at room temperature for 30 min then centrifuged at 3000 rpm for 15 min to completely remove cell debris. It was stored at −80°C until required for miRNA detection. Total RNA was extracted from 625 μL of plasma using the mirVana PARIS kit (Ambion, USA) according to the manufacturer's instructions. Total RNA was also extracted from 10–50 mg left ventricular papillary muscles using the mirVana isolation kit according to the manufacturer's protocol (Ambion) [27–29]. The final elution volume of all RNA samples was 100 μL, and concentrations were determined by the ultramicro nucleic acid ultraviolet tester (NANODROP 1000, Wilmington, USA). RNA was reverse-transcribed into cDNA using the TaqMan microRNA reverse transcription kit (Applied Biosystems, Foster City, CA) using miRNA-specific primers provided by the manufacturer in Applied BioSystems 9700 Thermocycler. All cDNAs were stored at −20°C.

2.4. Quantitative Real-Time-PCR. Quantitative real-time-PCR (qRT-PCR) was performed as previously described [30]. Each reaction was performed in a final volume of 10 μL containing 4.5 μL cDNA, 5 μL TaqMan Universal PCR Master Mix (No AmpErase), and 0.5 μL TaqMan miRNA Assay (Applied Biosystems). The thermal cycle was set as start with 10 min template denaturation at 95°C, 40 cycles of denaturation at 95°C for 15 s, and combined primer annealing/elongation at 60°C for 1 min. Each sample was run in triplicate, and noncoding small RNA RNU6B was used as the internal control gene, according to the Applied Biosystems Application Note. RNU6B has previously been demonstrated to have both abundant and stable expression across 38 different human tissues and organs. It is regarded as one of the control genes with the least variability for miRNA assays and has been widely used in different fields, including cardiovascular research. We used Taqman microRNA assay for the qRT-PCR. The primer sequences were searched on the ABI official website, shown as follows.

The assay ID of miR-1183 assay is 002841; hsa-miR-1183 mature miRNA sequence is CACUGUAGGUGAUGGUGAGAGUGGGCA. The assay ID of miR-1299 assay is 241065_mat; hsa-miR-1299 mature miRNA sequence is UUCUGGAAUUCUGUGUGAGGGA. The assay ID of RNU6B assay is 001093; control RNU6b sequence is CGCAAGGATGACACGCAAATTCGTGAAGCGTTCCATATTTTT.

2.5. miRNA Microarray Analysis. To study the differential expression of miRNAs in RHD patients, we performed miRNA expression profiling on plasma samples using the miRCURY LNA Array system because of its comprehensive profiling and high sensitivity and specificity (see S2_Fig in Supplementary Material available online at http://dx.doi.org/10.1155/2015/524519). Here we selected 3 patients named numbers 1, 2, and N1-3 for microarray assay

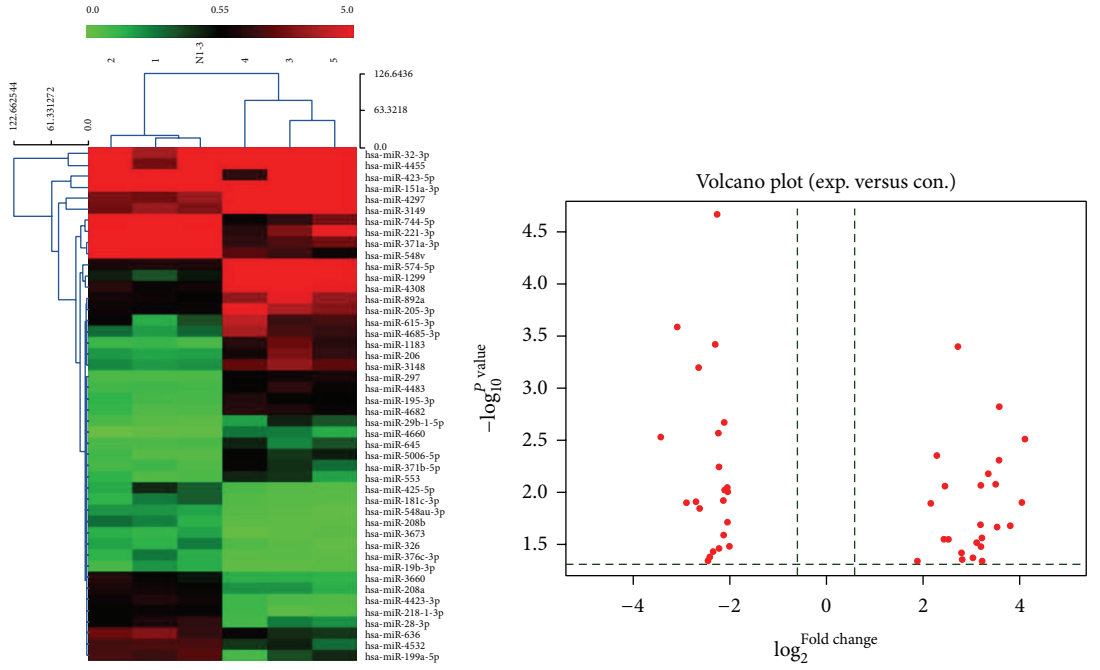


Figure 1: MicroRNA microarray expression data from plasma samples of rheumatic heart disease ($n = 3$) and healthy control subjects ($n = 3$). (a) Heat map of microRNA microarray expression data from plasma samples of rheumatic heart disease ($n = 3$) and healthy control subjects ($n = 3$). The expression of miRNA is hierarchically clustered on the y-axis, and RHD plasma samples or healthy control plasma samples or healthy control plasma samples are hierarchically clustered on the x-axis. The legend on the right indicates the miRNA represented in the corresponding row. The relative miRNA expression is depicted according to the color scale shown on the right. Red indicated upregulation; blue indicated downregulation; numbers 1, 2, and N1-3 indicate healthy control samples; numbers 3, 4, and 5 indicate RHD plasma samples. (b) Volcano plot from gene chips represents different fold changes from many upregulated miRNAs and downregulated miRNAs.

from the above 50 RHD patients. Meanwhile, we also selected 3 healthy controls named numbers 3, 4, and 5 from 50 control groups, which are also evaluated for microarray assay. Microarray analysis was performed by Kangcheng Bio-tech Inc. (Shanghai, China). miRNAs selected for investigation in our study were further filtered on the basis of expression levels and previously published data [31]. The RNA of each individual was analyzed on a separate chip. Data were analyzed in Genepix Pro 6.0 and saved as EXCEL files. GeneSpring 7.2 was used for further data analysis. Fold changes in miRNAs between groups, either twofold greater or less, were considered to represent differential expression.

2.6. Statistical Analysis. Experimental data were analyzed using SPSS19.0 statistical software. RNA concentration and Ct value levels were presented as means ± SD.

3. Results

3.1. RNA Concentration of RHD Patients in Plasma and Tissue. First, we validated the feasibility of miRNA detection from plasma in all subjects. No differences in RNA concentrations were observed among the different groups (plasma RHD group: 17.74 ± 5.59 ng/μL; plasma control group: 16.43 ± 4.32 ng/μL; tissue RHD group: 40.95 ± 27.90 ng/μL; tissue control group: 52.28 ± 28.21 ng/μL) (S1_Fig). However, the lower RNA yield from plasma hampered the actual RNA quantification compared with that from tissue (S1_Fig).

3.2. Genome-Wide Expression Profiling of Plasma miRNAs by Microarray Analysis. Hundreds of miRNAs showed differential expression between RHD cases and controls (S2_Fig). A total of 133 miRNAs, including miR-1299 and miR-1183, were significantly upregulated (>2-fold), while 137 were significantly downregulated (>2-fold), including miR-4423-3p and miR-218-1-3p (S3_Fig). We selected several significantly differentially expressed miRNAs for further study. Gene chips represents different fold changes from many upregulated miRNAs and downregulated miRNAs, either by heat map of microRNA microarray expression data (Figure 1(a)) or volcano plot (Figure 1(b)). Chip results suggested that miR-1299 and miR-1183 expressed in RHD were significantly upregulated (>10-fold), whereas miR-4423-3p and miR-218-1-3p expressed in RHD were significantly downregulated (>5-fold) in RHD cases compared with controls (Table 1).

3.3. Plasma and Tissue miR-1183 and miR-1299 Expressions Are Potential Biochemical Marker for RHD by Quantitative Real-Time-PCR. To verify the accuracy of the microarray-based miRNA measurements, expression levels of hsa-miR-1183, hsa-miR-1299, and RNU6B were assessed using qRT-PCR [32]. All miRNAs including RNU6B showed reliable

Ct values in most samples (Figure 2), and fluorescent signals failed to reach the set threshold after 40 cycles in very few assays. Results are shown in Figures 2 and 3; qRT-PCR revealed significant differences in the expression of miRNA-1183, miRNA-1299, and RNU6B in both tissue and plasma samples of RHD patients compared with healthy controls, which was consistent with microarray data. Ct values in RHD tissue for miR-1183 were 34.62 ± 2.23, 31.81 ± 2.46 for miR-1299, and 28.33 ± 3.48 for RNU6B.

Pulmonary artery systolic pressure (PASP) is a key factor to evaluate the severity of PAH, so we next divided RHD patients with secondary PAH into two groups: those with high PASP (RHD-PAH [PASP higher >40 mmHg]) and those with low PASP (RHD-PAH [PASP lower <40 mmHg]) (Table 2). The expression of miR-1183 was significantly upregulated in both RHD plasma samples (Figure 2(a), $P = 0.012$) and the subset of RHD cases with high PASP (Figure 2(b), $P = 0.021$). By contrast, although the expression of miR-1299 was significantly upregulated in RHD plasma samples (Figure 2(c), $P = 0.011$), no significant difference was observed between the subsets with high and low PASP (Figure 2(d), $P = 0.566$). Moreover, the overexpression of miR-1183 in high versus low PASP cases could reflect the pulmonary artery remodeling of PAH secondary to RHD, meaning that it plays a more important role in secondary PAH complications than primary disease. Although the same trend was also observed when comparing miRNA-1183 in tissue samples between the two groups by qRT-PCR (Figure 3(a)), the difference was not significant. Interestingly, the expression of miRNA-1299 is significantly higher in the RHD group compared with the non-RHD group for both tissue and plasma (Figure 3(b), $P < 0.05$).

3.4. Bioinformatic Analysis and Predicted miRNA Molecular Targets Identified in RHD Patients. Those miRNAs showing significantly differential expression by microarray were analyzed by bioinformatics, including target gene prediction, gene ontology (GO) analysis, and pathway analysis, with the aim of investigating target genes and regulatory mechanisms. Target genes of differential miRNA expression were predicted using three algorithms: miRBase (http://www.mirbase.org/), miRanda (http://www.microrna.org/), and TargetScan (http://www.targetscan.org/) (Figure 4). Only genes identified by all three algorithms were considered to be the predicted targets for each miRNA.

Targets were found to be predominantly involved in the regulation of cellular and biological processes, including SOX family members and *MEF2A*. Hundreds of target genes were predicted and were mainly involved in transcription coactivator activity, RNA polymerase II transcription coactivator activity, histone-lysine N-methyl transferase activity, protein-lysine N-methyl transferase activity (GO molecular function), the positive regulation of biological processes, histone-lysine methylation, protein alkylation (GO biology process), and intracellular roles (GO cellular component) (Figure 5, S4_Fig). To assess the possible biological impact of the differentially expressed miRNAs, we undertook pathway analyses of the predicted target genes, revealing that the gene set was mostly involved in biological pathways including the pentose phosphate pathway, glutamatergic synapse, and the MAPK signaling pathway (Figure 6).

Because of the differences in miR-1183 and miR-1299 expression, we separately predicted the target genes for these two miRNAs. *Bcl-2* was predicted to be an important target gene of miR-1299, which may influence cardiomyocyte apoptosis. Considering RHD as an autoimmune sequela of an acute rheumatic fever, it can cause chronic inflammation. PBMC and THP-1 cells as classical immune cells are studied to dig deep into the mechanism study including both miR-1183 and miR-1299 mimic and inhibitor study. Series of studies are in progress including identifying the changes of target genes Bcl-2 and EGFR mRNA expression and related cytokines (data not shown). Up to now, we have already further studied the relationship of miR-1299 mimic, inhibitor, and Bcl-2 expression levels. It seems that miR-1299 mimics can upregulate miRNA level and induce cardiomyocyte apoptosis (data not shown). Meanwhile, CXCR4, EGF, and EGFR were also predicted to be important target genes of miR-1183. However, the following mechanism study including miR mimic was still in progress.

4. Discussion

Several studies have shown that a variety of miRNAs are implicated in cardiovascular diseases, so exploratory research has been conducted into the possibility of using them as biological markers based on their expression in plasma or tissue [33–35]. However, this has not been tested in RHD. The present miRNA array results suggested that miR-1299, miR-1183, and so forth are significantly upregulated, while miR-4423-3p, miR-218-1-3p, and so forth are significantly downregulated in RHD. qRT-PCR confirmed that the expression of miR-1299 and miR-1183 in RHD tissue and plasma was significantly higher in RHD cases than healthy controls. Moreover, the enhanced levels of miR-1183 and miR-1299 expression in plasma are consistent with those in tissues, suggesting that they could be used as potential biological markers in RHD. Besides, some other miRNAs determined to be expressed differently in RHD compared to healthy controls can be dug deeper, like miR206, miR208a, miR208b, and miR574-5p which have already been studied in the regions of other cardiovascular diseases [36–38]. To explore the possible functions on RHD of these miRNAs may provide us ideas whether they are potent therapeutic targets for cardiac hypertrophy, fibrosis, dysfunction, and so forth.

Pulmonary arterial hypertension is a debilitating condition with progressive remodeling of the pulmonary resistance vessels [22]. It is characterized by excessive vascular resistance and smooth muscle cell proliferation in small pulmonary arteries and finally causes elevation of pulmonary vascular resistance, right ventricular failure, and death [39, 40]. Variations in PASP in RHD patients are likely to influence the expression of different miRNAs in RHD-PAH.

We identified an important role for miR-1299 as a direct regulator of RHD. Meanwhile, the difference in expression of miR-1183 between RHD cases with high and low PASP

Table 1: List of the hsa-miRNAs with at least 2-fold changes.

Upregulated miRNA (>10 folds)			Downregulated miRNAs (>5 folds)		
miRNA	Folds	*P* value	miRNA	Folds	*P* value
miR-1299	17.13272	0.003132	miR-4423-3p	0.092672	0.002951
miR-1183	16.5075	0.012476	miR-218-1-3p	0.117514	0.000261
miR-4455	13.98099	0.020973	miR-744-5p	0.13412	0.01252
miR-3148	11.95398	0.001515	miR-4666a-5p	0.139837	0.018463
miR-4660	11.9266	0.004957	miR-208b	0.154499	0.012267
miR-3149	11.56086	0.021548	miR-199a-5p	0.159151	0.000643
miR-4682	11.37554	0.008373	miR-548v	0.162247	0.014474
miR-297	10.12601	0.006702	miR-3660	0.184096	0.045429
miR-206	9.313578	0.045881	miR-28-3p	0.188257	0.041912
miR-32-3p	9.312469	0.027754	miR-425-5p	0.196498	0.037533
miR-4308	9.191765	0.020532	miR-548-3p	0.203344	0.000383
miR-4483	9.155266	0.033305	miR-4532	0.209924	0.000217
miR-574-5p	9.132102	0.008565	miR-3673	0.211119	0.0027
miR-4685-3p	8.583969	0.03023	miR-221-3p	0.215457	0.005767
miR-4297	8.120556	0.042287	miR-181c-3p	0.216058	0.034691
miR-4722-5p	7.657944	0.002658	miR-636	0.226805	0.011922
miR-3591-5p	7.405006	0.020074	miR-376c-3p	0.227146	0.025902
miR-615-3p	7.082924	0.044276	miR-4798-3p	0.228792	0.038073
miR-195-3p	6.976838	0.039299	miR-208a	0.231132	0.002143
miR-4657	6.682702	0.00013	miR-371a-3p	0.233856	0.009664
miR-3657	6.671498	0.024803	miR-4708-5p	0.23388	0.010049
miR-5006-5p	6.639262	0.000398	miR-151a-3p	0.239967	0.00974
miR-4458	6.188047	$4.05E-06$	miR-4678	0.240886	0.004481
miR-29b-1-5p	5.735279	0.028064	miR-423-5p	0.240937	0.009271
miR-205-3p	5.521096	0.008749	miR-326	0.243204	0.019519
miR-498	5.497149	0.017617	miR-4703-3p	0.244857	0.021779
miR-4450	5.491613	0.026159	miR-19b-3p	0.24777	0.033327
miR-371b-5p	5.378559	0.028034	miR-3612	0.250328	0.017766
miR-2113	5.371673	0.025333	miR-4748	0.252973	0.026891
miR-3689a-5p	5.342335	0.015381	miR-186-5p	0.253236	0.045289
miR-3120-5p	5.221742	0.00234	miR-320b	0.253705	0.029743
miR-4681	5.200976	0.01057	miR-550b-2-5p	0.253829	0.045176
miR-3690	5.145123	0.015582	miR-346	0.254841	0.021562
miR-4804-3p	5.03806	0.013518	miR-130a-3p	0.256733	0.04919
miR-4447	4.964015	0.001608	miR-4650-5p	0.256936	0.02673
miR-892a	4.895473	0.004484	miR-2682-5p	0.257933	0.008486
miR-4795-5p	4.698387	0.000309	miR-219-5p	0.263898	0.011573
miR-3158-3p	4.618894	0.003654	miR-337-3p	0.265439	0.016578

Table 2: Baseline characteristics of different severe pulmonary arterial hypertension complication on RHD patients and healthy control subjects.

Groups	Group 1: high pulmonary artery pressure group in RHD patients ($n = 10$)	Group 2: low pulmonary artery pressure group in RHD patients ($n = 10$)	Group 3: control ($n = 10$)
Age, years	54.2 ± 5.73	59.2 ± 6.27	49.6 ± 7.0
Sex, males/females	3/7	4/6	5/5
NYHA grades	II~III	IV	/
PASP (mmHg)	88.3 ± 5.376	33.1 ± 4.55	/

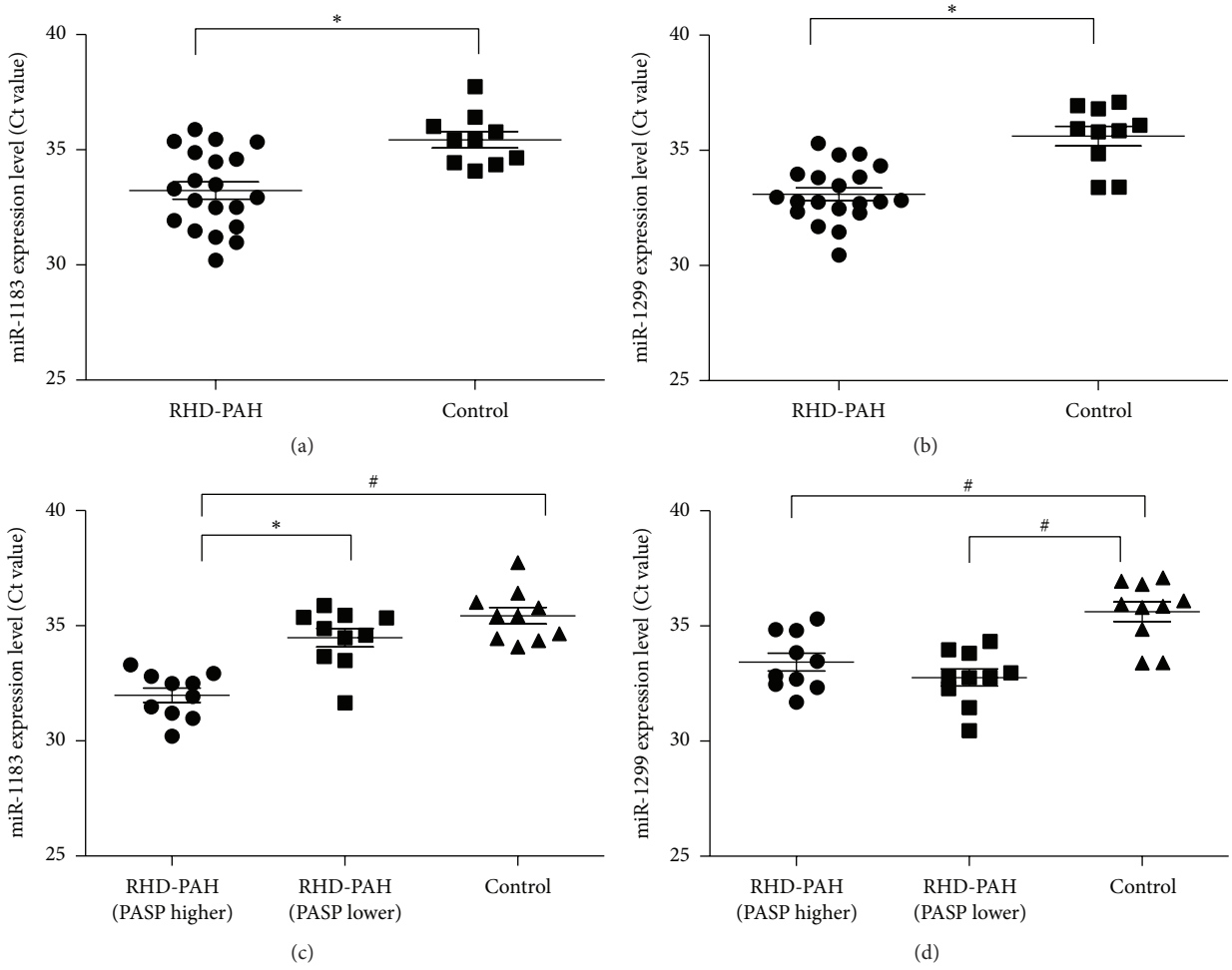


Figure 2: Plasma level of representative differentially expressed miR-1183 and miR-1299 in 20 RHD patients and 10 normal controls ($^{*}P < 0.05$, $^{\#}P < 0.01$).

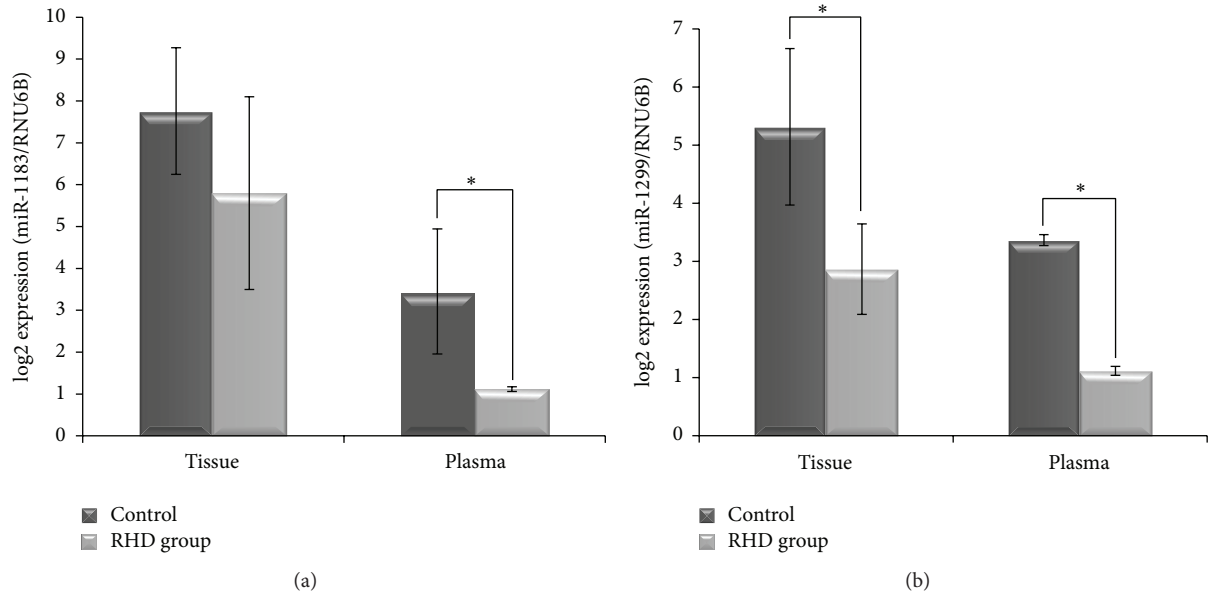
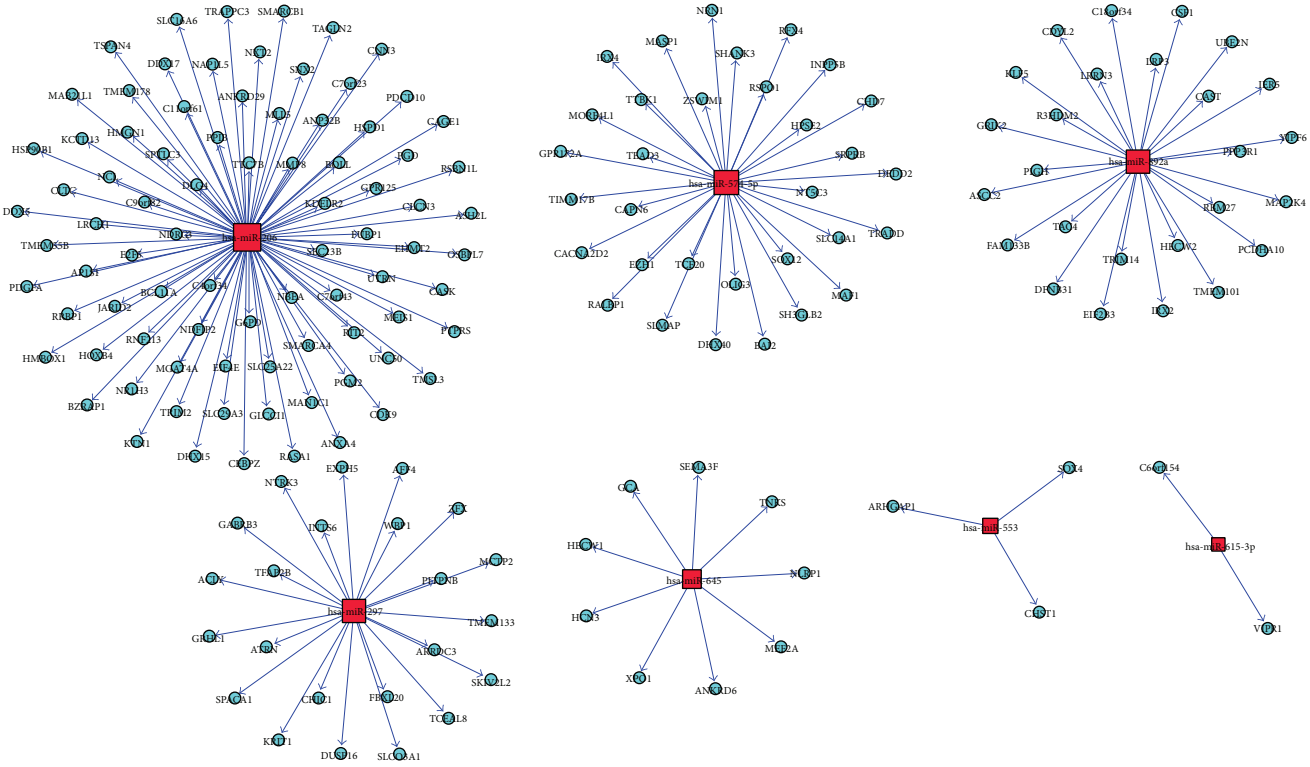
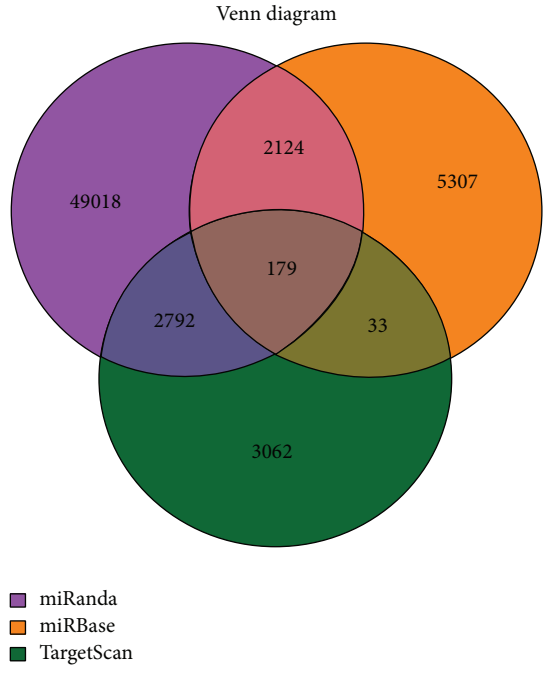


Figure 3: Upregulation of microRNA-1183 and microRNA-1299 expression in rheumatic heart disease tissues and plasma by real-time RT-PCR. (a) The differential expression of miRNA-1183 in the tissue and plasma of RHD patients and normal controls. (b) The differential expression of miRNA-1299 in the tissue and plasma of RHD patients and normal controls ($^{*}P < 0.05$).

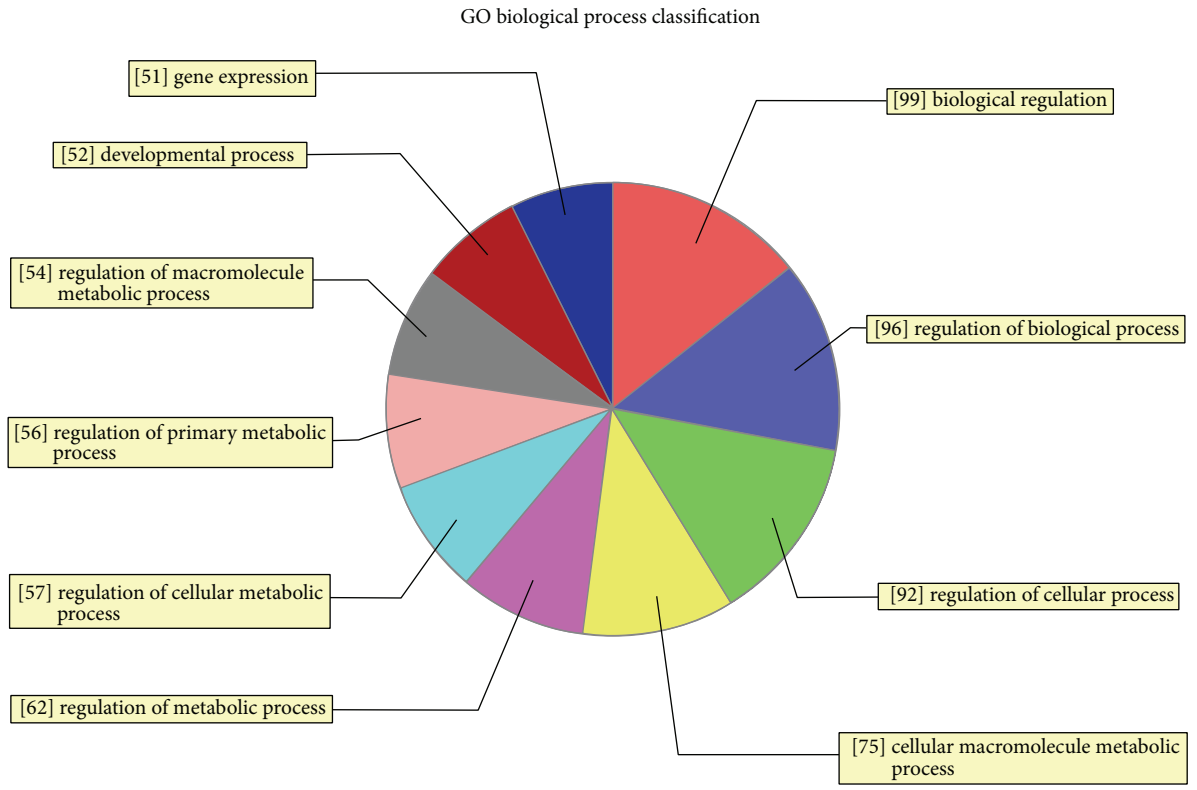


(a)

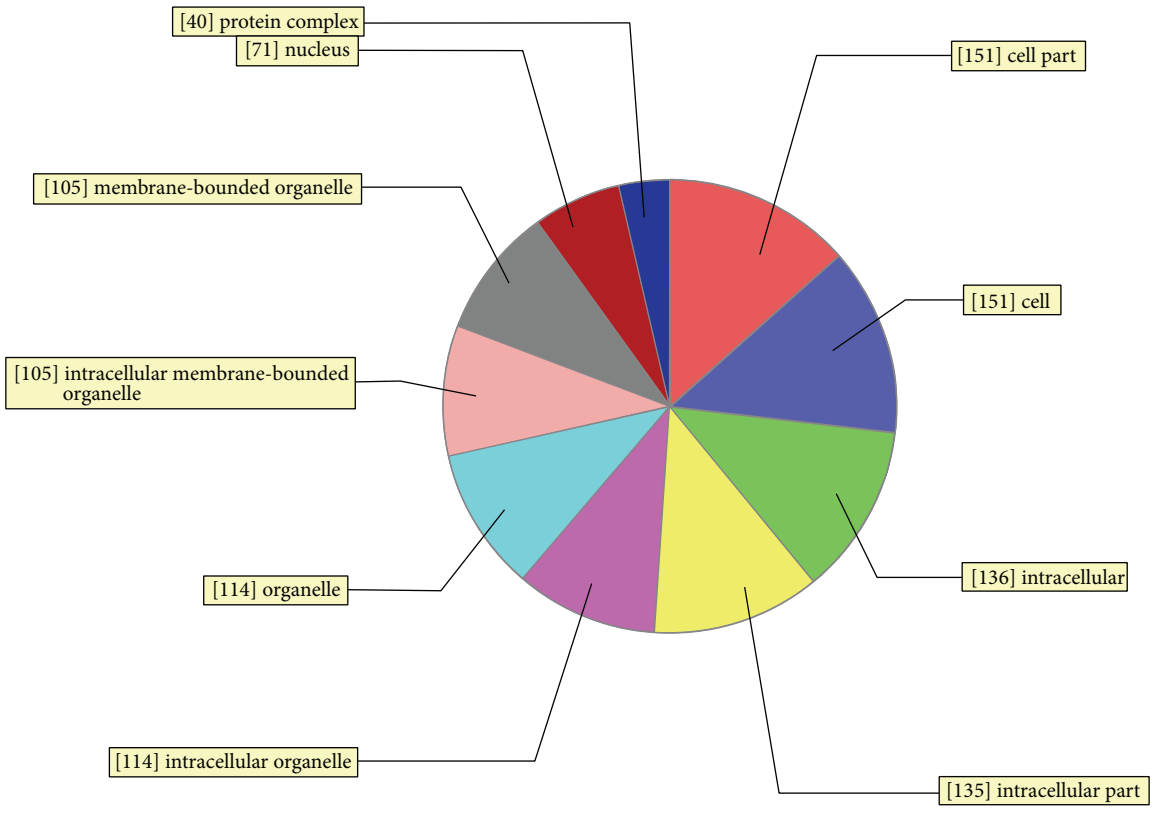


(b)

Figure 4: Target genes of differential miRNA expression predicted. (a) MicroRNA–mRNA-Gene-Network of several representative miRNAs with their predicted target genes. (b) Overlapping data of three databases from the target summary by miRBase (http://www.mirbase.org/), miRanda (http://www.microrna.org/), and TargetScan (http://www.targetscan.org/).



(a)



(b)

Figure 5: Continued.

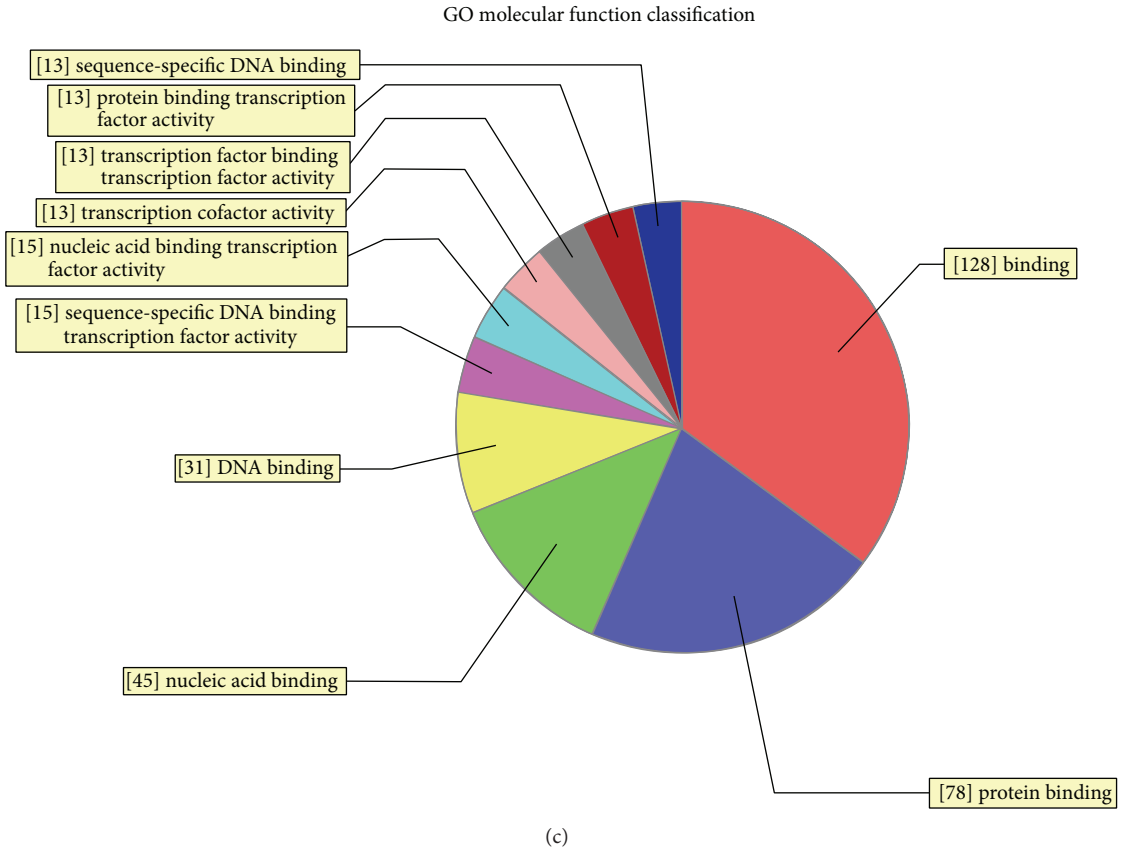


Figure 5: GO analysis classifications of predicted target genes regulated by differentially expressed microRNAs (miRNAs) in rheumatic heart disease. GO analysis was performed on genes predicted to be targets of differentially expressed miRNAs.

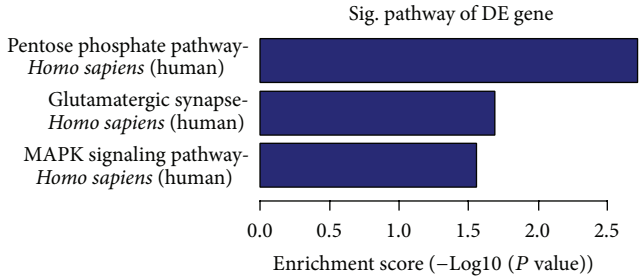


Figure 6: Pathway classifications of predicted target genes regulated by differentially expressed microRNAs (miRNAs) in rheumatic heart disease. Pathway analysis was performed on genes predicted to be targets of differentially expressed miRNAs. The negative log of the P value (log 10 P) is plotted on the x-axis.

suggests that its overexpression is caused by the pulmonary artery remodeling of PAH secondary to RHD, meaning that it plays a more important role in secondary complications than primary disease. miR-1183 and miR-1299 may therefore have an independent effect on the disease processes of RHD and PAH. We searched the literature for mechanistic articles on miR-1183 or miR-1299 and found them to be extremely limited. miR-1183 was previously reported to be downregulated in tick salivary glands [41], while another study reported that it has a relationship with a functional polymorphism in the EpCAM gene [42]. miR-1183 is also known to be upregulated in locally advanced rectal cancer patients [43]. Information about miR-1299 is even more limited, so further detailed studies are required for a greater understanding of the molecular mechanisms of miR-1183 and miR-1299.

Based on the work which we have done for screening differentially expressed miRNAs (e.g., miR-1183 and miR-1299) of RHD, most importantly, evaluation of the stability and effect of miRNA-based therapeutics is of great importance for the comprehensive understanding of the miR-1183 and miR-1299 functions in rheumatic heart disease. The bioinformatics analysis is often used to further study the mechanism of the differential expression of miRNAs [44–46] and to predict and analyze their target genes with the aim of understanding regulatory mechanisms in the future [47, 48]. Our analysis predicted hundreds of target genes, and pathway and GO analyses demonstrated that the gene set was mostly involved in biological pathways and cellular processes such as the pentose phosphate pathway, glutamatergic synapse, and MAPK signaling pathway. Our findings also suggest that the regulation of cellular and metabolic processes may influence the release of miR-1299 and miR-1183 into the peripheral blood following their overexpression in the tissue. The *Bcl-2* gene and its influence on cardiomyocyte apoptosis may be

an important factor in association with miR-1299 expression. However, more detailed studies are needed for a greater understanding of this process.

5. Conclusion

Taken together, our data revealed the differential expression of specific miRNAs in RHD accompanied by secondary PAH. miR-1183 and miR-1299 appear to play a distinct role in disease pathology and so could be potential biological markers for both RHD and PAH. Future functional and mechanistic studies on the dynamic changes of miRNA expression in RHD may improve our understanding of the regulatory role of miRNAs in RHD.

Abbreviation

RHD:	Rheumatic heart disease
miR:	MicroRNAs
PAH:	Pulmonary hypertension
QRT-PCR:	Quantitative real-time-PCR
PASP:	Pulmonary artery systolic pressure
nt:	Nucleotides.

Disclosure

Ni Li and Jiangfang Lian are co-first authors of this work.

Conflict of Interests

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

Acknowledgments

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Review Article

Role and Function of MicroRNAs in Extracellular Vesicles in Cardiovascular Biology

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Intercellular communication mediated by extracellular vesicles is crucial for preserving vascular integrity and in the development of cardiovascular disease. Extracellular vesicles consist of apoptotic bodies, microvesicles, and exosomes that can be found in almost every fluid compartment of the body like blood, saliva, and urine. In the recent years, a lot of reports came up suggesting that major cardiovascular and metabolic pathologies like atherogenesis, heart failure, or diabetes are highly influenced by transfer of microRNAs via extracellular vesicles leading to altered protein expression and phenotypes of recipient cells. The following review will summarize the fast developing field of intercellular signaling in cardiovascular biology by microRNA-containing extracellular vesicles.

1. Introduction

Extracellular Vesicles. Intercellular communication is essential for the maintenance of tissue homeostasis and disease development. Long known mechanisms of intercellular communication include direct cell-cell contact or the transfer of secreted molecules. In the last two decades, a third mechanism for intercellular communication has emerged that involves intercellular transfer of extracellular vesicles (EVs) [1].

EVs are small membrane vesicles, which are released by most cell types in the extracellular space. By containing and transferring various bioactive molecules to target cells, like proteins, RNAs, or microRNA (miR), EVs affect molecular pathways and biological behavior of recipient cells. EVs are heterogeneous in size and are released from cells under physiological and pathological conditions [2]. According to current definitions, EVs consist of three subgroups: exosomes (20–100 nm), microvesicles ((MVs) 0.1 μm–1 μm), and apoptotic bodies (0.5 μm–2 μm). Whereas exosomes are released continuously from cells, MVs and apoptotic bodies are predominantly liberated from activated or apoptotic cells [2, 3]. Exosomes are released from cells via the endolysosomal pathway. In contrast, MVs and apoptotic bodies are formed by budding from the plasma membrane. Therefore, the membrane composition of MVs or apoptotic bodies reflects that of the parent cell more closely than does the membrane composition of exosomes.

In 2007, it was first shown that EVs contain and transfer genetic information, in form of mRNAs and miRs, between mast cells regulating protein expression of recipient cells [4]. The notion that EVs might function as vector to transfer genetic information being able to regulate gene expression in target cell opened up a completely new field of research regarding intercellular communication mechanisms. Today, there is increasing evidence that the effect of EVs on target cells is mainly dependent on their intravesicular miR expression [5, 6]. By transferring miRs to target cells, EVs are now established as a novel layer in intercellular gene regulation [7].

microRNA. miRs are small, noncoding RNAs that provide posttranscriptional regulation of gene expression and control many (patho)physiological processes in cardiovascular health and disease [8]. miRs are short (18–25 nucleotides) noncoding RNAs transcribed in the nucleus and cleaved by the RNAse III enzyme Drosha to precursor hairpin miR (pre-miR). After transportation into the cytoplasm, the pre-miR is further processed into 18–25 nucleotide mature miR

duplexes. Mature miRs can be loaded by the enzyme Dicer into the RNA-induced silencing complex (RISC), where protein expression of specific mRNA targets can be prevented by mainly two mechanisms. At sites with broad pairing complementarity, miRs can induce Argonaute-catalyzed mRNA cleavage [9]. More commonly, miRs direct translational repression, mRNA destabilization, or a combination of both including inhibition of translation initiation and poly(A) shortening [10]. Importantly, miRs not only exert their function intracellularly, but also can be exported from cells in the extracellular space via EVs or bound to proteins like Ago-2 or HDL [11–13]. In contrast to RNA, extracellular miRs show a high stability in fluids and are reliably detectable in the blood [14]. Therefore, miRs have emerged as a novel class of biomarkers for many diseases, such as cardiovascular disease [15]. The dual function of miRs as active effector of gene expression on one hand and as stable biomarker on the other hand opens up fascinating opportunities to improve the understanding and diagnosis of cardiovascular diseases. Of note, increasing evidence suggests that the stability of miRs in fluids is mediated by the protection of miRs from circulating RNAses through EVs [13]. Depending on the condition of the releasing cells, miR-incorporating EVs have been shown to regulate a multitude of divers functions in target cells mediating the maintenance of cardiovascular hemostasis or inducing cardiovascular pathologies, which will be the focus of the next paragraphs.

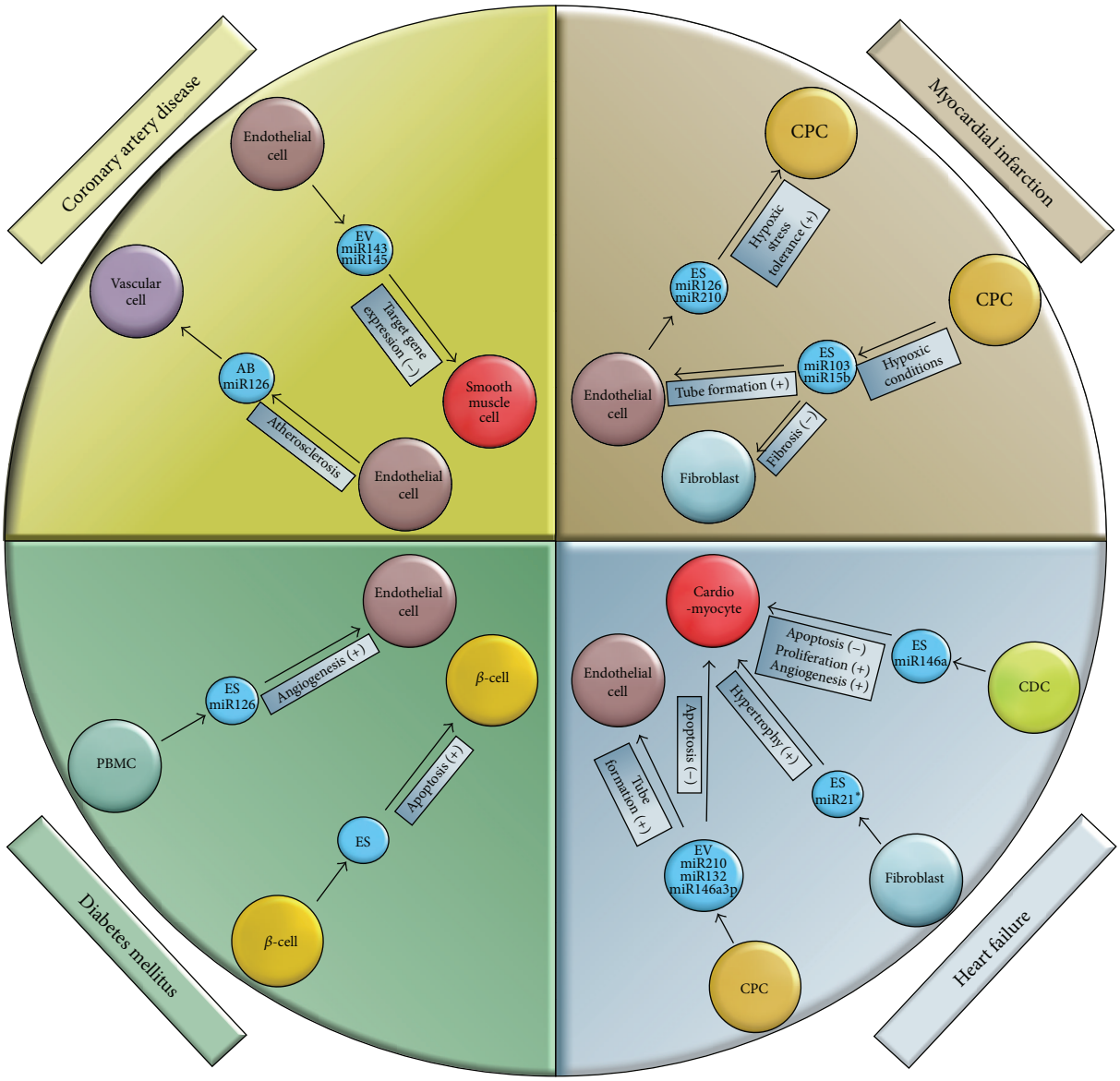
EV microRNAs in Intercellular Communication. See Figure 1.

2. EV microRNAs: The Maintenance of Cardiovascular Homeostasis

EVs containing miRs are shed from a variety of cells under physiologic and pathophysiologic conditions. Considering the fact that most cellular mechanisms like cell growth, differentiation, apoptosis, and proliferation are regulated by miRs shows that they orchestrate the maintenance of cardiovascular homeostasis and thereby are promising targets for development of new therapeutical approaches [16]. There are several reports about beneficial biological effects of EVs-bound miRs. EVs from endothelial cells reduced atherosclerotic lesion formation in the aorta of $ApoE^{-/-}$ mice and limited atherogenesis, improved plaque stability, and promoted incorporation of progenitor cells [17]. EVs from cardiac progenitor cells (CPC) inhibited apoptosis in mice-cardiomyocytes and injection of these CPC-EVs into infarcted hearts led to reduced cardiomyocyte apoptosis and improved LV ejection fraction [18]. Exosomes from CPCs are reported to improve cardiac function in a rat ischemia-reperfusion injury model and exosomes from endothelial cells increase tolerance to hypoxic stress in recipient CPCs (Figure 1) [18, 19]. Intravascular injection of endothelial MVs containing miR-126 accelerated reendothelialization after electric denudation of the endothelium in vivo [20]. Taken together, these findings illustrate the fundamental relevance of miR-delivering by EVs for preserving physiologic conditions in the body and emphasize the relevance of further investigations in this field aiming at a broadened understanding of their functions and development of new personalized drugs in the future.

3. Vascular Diseases

3.1. Coronary Artery Disease. Coronary artery disease (CAD) is the most common cause of death in the industrialized world [21]. In the last 5 years, many studies provided insights into the important role of miRs in the development of atherosclerosis by influencing inflammatory state, proliferation, and regeneration of vascular cells. miRs transfer by EVs contributes to maintenance of arterial homeostasis on the one hand but leads to inflammatory state in the interplay of endothelial cells (EC), smooth muscle cells (SMC), and immune cells on the other hand [16]. In the last years, a lot of reports have been published that show a close relationship between development of atherosclerosis and altered miR expression. For example, a recent publication showed that ApoE promotes the increase of miR-146a expression and thereby reduced NF-κB signaling in monocytes and macrophages. In vivo, it could be pointed out that intravascular application of miR-146a mimetics leads to reduced atherogenesis [22]. Meiler et al. gained additional interesting results about the successful reduction of atherosclerotic plaque size. They proved that miR-302a regulates the expression of ATP-binding cassette (ABC) transporter ABCA1 mRNA and protein in primary macrophages. Moreover, they found that in vivo application of an anti-miR-302a in a low-density lipoprotein receptor deficiency ($Ldlr^{-/-}$) mouse model leads to an increase in ABCA1 in the aorta as well as an increase in circulating plasma high-density lipoprotein levels by 35%. The anti-miR-302a-treated mice also displayed reduced atherosclerotic plaque size by 25% and a more stable plaque morphology with reduced signs of inflammation [23]. Zhang et al. examined that expression of miR-26a is reduced in the aortic intima of $ApoE^{-/-}$ mice and treatment of human aortic endothelial cells with oxidized low-density lipoprotein (ox-LDL) suppressed miR-26a expression. In contrast, overexpression of miR-26a inhibited endothelial apoptosis and overexpression of TRPC6, a target of miR-26a, abolished the antiapoptotic effect of miR-26a [24]. A recent publication of our working group revealed that EMPs promote anti-inflammatory effects in vitro and in vivo by reducing endothelial ICAM-1 expression via the transfer of functional miR-222 into recipient cells and that EMP-mediated miR-222-dependent anti-inflammatory effects are reduced in pathological hyperglycaemic conditions [25]. Another study showed that exposing ECs to high glucose activates transcription of miR-503. Furthermore, it points out that miR-503 is packed into EMPs and delivered to vascular pericytes, resulting in reduced expression of EFNB2 and VEGFA in these cells, followed by impaired migration and proliferation [26]. Another publication discussing the involvement of miRs in the development of atherosclerosis was given by Di Gregoli et al. By investigating human coronary atherosclerotic plaques, they observed that increased matrix metalloproteinases-(MMP-) 14 protein expression in foam cell macrophages was



EV: extracellular vesicle
AB: apoptotic bodies
ES: exosome
miR: microRNA
PBMC: peripheral blood mononuclear cells
β-cell: pancreatic beta-cells
CPC: cardiac progenitor cells
CDC: cardiosphere-derived cells

Figure 1: Intercellular signaling mechanisms via EV-bound microRNAs.

associated with lesions exhibiting histological characteristics associated with an unstable phenotype. Added to that, they examined that microRNA-24 expression in these atherosclerotic plaques was inversely related to MMP-14 protein expression, that stable plaques contained higher microRNA-24 levels than unstable plaques, and that microRNA-24 colocalized with foam cell macrophages exhibited low MMP-14 protein expression. In ApoE$^{-/-}$ mice, they found that microRNA-24 inhibition leads to increased plaque size and macrophage MMP-14 expression [27]. Schober et al. were able to observe that administration of miR-126-5p prevents atherosclerotic lesion formation by Notch1 inhibitor delta-like 1 homolog (Dlk1) suppression in an ApoE$^{-/-}$ mouse model [28]. Sun et al. expanded our knowledge about influences of miRs

on atherosclerotic development by several major findings. Firstly, they showed that miR-181b expression is reduced in the aortic intima and plasma in apolipoprotein E-deficient mice. Secondly, they determined that circulating miR-181b is markedly reduced in the plasma of human subjects with coronary artery disease. Moreover, they could show that systemic delivery of miR-181b suppressed NF-κB signaling, reduced target gene expression in the aortic arch in apolipoprotein E-deficient mice, and significantly inhibited atherosclerotic lesion formation, proinflammatory gene expression, and the influx of lesional macrophages and CD4+ T cells in the vessel wall [29]. Hergenreider et al. found that Krüppel-like factor 2 (KLF2), a shear-responsive transcription factor, leads to upregulation of the miR-143/145 cluster in EC, while it is known that miR-143/145 controls SMC-phenotypes. They used KLF2-transduced or shear stress-stimulated human umbilical vein endothelial cells (HUVEC) to obtain miR-143/145 enriched EVs. Coculturing of HUVECs with SMCs induced reduced target gene expression in recipient SMCs (Figure 1). Furthermore, the authors could show that in an in vivo ApoE$^{-/-}$ mice model EVs derived from KLF2-expressing EC reduced atherosclerotic lesion formation in the aorta [17]. Zernecke et al. gave another example for participation of EVs in development of atherosclerotic lesions. It is known that in response to tissue damage, the CXC chemokine CXCL12 and its receptor CXCR4 counteract apoptosis and recruit progenitor cells. On this basis they found that EC during atherosclerosis shed apoptotic bodies containing miR-126, leading to production of CXCL12 in recipient vascular cells (Figure 1). They examined miR-126 to repress the function of regulator of G protein signaling 16, an inhibitor of G protein-coupled receptor (GPCR) signaling, as underlying mechanisms. This leads to a feedback loop initiated by CXCR4, a GPCR, resulting in an increased production of CXCL12. Furthermore, they detected that treatment with apoptotic bodies, isolated from EC limited atherosclerosis, improved plaque stability and promoted the incorporation of Sca-1+ progenitor cells in a mouse model of atherosclerosis in an miR-126-dependent manner [30]. Another example for miR-participation in cellular processes involved in CAD development was given by Climent et al. They proved that SMCs transfer miR-143 and miR-145 to ECs, thereby modulating angiogenesis by reducing the proliferation of ECs and targeting hexokinase II (HKII) and integrin β 8 (ITGβ8). In vivo studies showed that transforming growth factor (TGF) β and vessel stress initiated miR-143/145 transfer from SMCs to ECs by nanotubes [31]. Exploring the prognostic value of EV-bound miRs in a clinical study, our group pointed out that increased expression of miR-126 and miR-199a in circulating MVs was associated with a significantly lower major adverse CV event rate. EC and platelets were discovered as the major sources by generating an expression profile of miRs in MVs of 181 patients with stable coronary artery disease. In an experimental setting, we found that vascular endothelial repair is promoted by delivery of miR-126 containing endothelial microvesicles (EMV) and that these effects are altered under hyperglycaemic conditions, as occurring in diabetic patients [20, 32]. Another interesting mechanism was reported by Rautou et al., who observed that MVs isolated from human atherosclerotic plaques contain ICAM-1 and transfer this adhesion molecule to EC-membrane. ECs with increased ICAM-1 in the cell membrane in turn showed increased endothelial monocyte adhesion in cell culture and in isolated perfused mouse carotid. This mechanism probably contributes to atherosclerotic plaque progression [33]. In the last few years, many publications have been published about miR-regulation in endothelial cells caused by shear stress. Wang et al. showed that endothelial cells exposed to pulsatile shear stress for 24 h show altered miR-expression profiles. They demonstrated that the miR-23b cluster (miR-23b and miR-27b) is upregulated by pulsatile stress, whereas the miRs of miR-17–92 cluster (miR-17, miR-19b, miR-20a, miR-20b, and miR-92a), miR-16 cluster (miR-15b and miR-16), and miR-221 cluster (miR-221 and miR-222) are downregulated [34]. Another report revealed that shear stress leads to increased miR-27a/b expression in endothelial cells and that overexpression of miR-27a and miR-27b leads to increased endothelial cell sprouting [35]. Another report showed that miR-712 is upregulated by disturbed flow in endothelial cells, resulting in proatherogenic responses, endothelial inflammation, and permeability. Furthermore, they could demonstrate that miR-712 silencing by an anti-miR-712 prevents atherosclerosis in murine models of atherosclerosis [36]. Vion et al. identified shear stress exposition of endothelial cells as a regulator of microparticle release. They observed that low shear stress stimulates EMP release by activation of ERK1/2 pathways and Rho kinases, whereas high shear stress limits EMP release by regulating ABCA1 in a NO-dependent expression and by cytoskeletal reorganization [37]. There are several reports about shear stress-induced regulation of leukocyte adhesion to endothelial cells. Endothelial cells exposed to oscillatory shear stress show increased miR-21 and augmented VCAM-1 expression accompanied with higher adhesiveness of THP-1 cells, which could be attenuated by anti-miR-21 [38]. Endothelial cells exposed to low shear stress show increased inflammation caused by upregulation of miR-92a expression. Besides, overexpression of miR-92a leads to lower NO synthesis and enhanced monocyte adhesion to the endothelium [39–41]. Moreover, several publications show influences of shear stress on endothelial miR expression. Regarding miR-10a, it is reported that shear stress leads to increased expression of miR-10a resulting in inhibited NF-κB activation and reduced endothelial inflammation [42]. miR-21 upregulation in endothelial cells can be achieved by exposing them to oscillatory shear stress (OSS). Upregulation of miR-21 results in reduced peroxisome proliferator-activated receptor (PPAR) α-translation, causing endothelial inflammation by inducing VCAM-1 and CCL2 expression [43]. Endothelial cells exposed to disturbed flow show miR-712 upregulation, promoting endothelial inflammation, and higher permeability of the endothelial barrier [36]. Interestingly, influences on atherosclerotic development by shear stress-induced miRs have been recently reported. For example, one report points out that expression of miR-19a is upregulated under shear stress exposition, resulting in cell-cycle arrest at G1-phase by cyclin D1 targeting [44]. Overexpression of miR-145 in

SMCs leads to higher plaque stability and reduced plaque size in an in vivo mouse model [45]. Endothelial cells exposed to low shear stress combined with atherogenic oxLDL show upregulation of miR-92a expression. Application of an miR-92a antagomir in a ApoE$^{-/-}$-mouse model leads to decreased atherosclerosis in antagomir-treated group compared to controls. Furthermore, atherosclerotic plaques of antagomir-treated mice showed higher collagen content, suggesting that lower expression of mir-92a leads to development of more stable plaques [39]. In a clinical study, it has been examined that miR-21 plasma levels of patients with diabetes type 2 are reduced compared to controls [46]. In circulating angiogenic progenitor cells (APCs) of patients with CAD, increased miR-21 levels could be observed compared to controls [47]. In vivo studies were able to point out that miR-10a is upregulated in endothelial cells from athero-protected sites of the aorta, while in endothelial cells from atheroprone sites of the aorta miR-21, miR-92a, mir-103, and miR-221 are upregulated [42, 48]. In the last years, several articles reported that miRs are involved in endothelial dysfunction. Transfection of endothelial cells with miR-221 and miR-222 reduced cell migration and overexpression of miR-221 and miR-222 resulted in reduced eNOS expression in endothelial cells [49, 50]. Xu et al. performed miRNA expression analysis of serum samples from atherosclerotic CAD patients. They observed that atherosclerotic CAD patients have increased expression levels of miR-135b-5p and miR-499a-3p in serum. Additionally, they identified miR-135b-5p and miR-499a-3p to repress myocyte enhancer factor 2C (MEF2C), leading to enhanced EC and VSMC proliferation and migration [51]. Cheng et al. activated endothelial cells by stimulating them with the proinflammatory cytokines IL-1β and TNF-α and observed upregulation of miR-146a and miR-146b. Next, they overexpressed miR-146a in endothelial cells and observed decreased expression of the inflammatory genes of VCAM-1, ICAM-1, SELE, and MCP-1 and an increased expression of eNOS mRNA, leading to decreased leukocyte adhesion. The authors concluded that miR-146a promotes decreased endothelial activation. Moreover, they observed that miR-146 negatively regulates proinflammatory NF-κB-, MAP kinase-pathway, and downstream EGR transcription factors. Finally, they report that HuR, an RNA binding protein that promotes endothelial activation by suppressing expression of endothelial nitric oxide synthase (eNOS), is an miR-146 target [52]. In one publication of our own working group we generated EMP from HCAEC exposed to high glucose concentrations, defined as "injured" EMP (iEMP). It could be detected that iEMP injection significantly impaired endothelial function in ApoE$^{-/-}$ mice, leading to increased macrophage infiltration and adhesion protein expression in atherosclerotic lesions of iEMP-treated ApoE$^{-/-}$ mice by phosphorylation of p38 into its biologically active form phospho-p38 [53]. All these publications indicate the importance of miRs in maintaining vascular homeostasis on the one hand, but on the other hand they demonstrate that dysregulation of miRs in cardiovascular diseases gives rise to the development of atherosclerotic lesions. Further investigations are required to expand the knowledge about influences of miRs on vascular cell function and their role in the progression of atherosclerotic lesions in order to place the basis for development of new drugs and treatments of atherosclerosis.

3.2. Myocardial Infarction. In myocardial infarction (MI), a sudden thrombotic occlusion of a coronary vessel causes reduced oxygen supply to myocardial cells resulting in cell death. There are some studies elucidating the role of miRs and EVs in development of MI. A study of Gray et al. showed that CPCs secrete proregenerative exosomes under hypoxic conditions enhancing tube formation of ECs and decreasing profibrotic gene expression in TGF-β-stimulated fibroblasts. Microarray analysis of exosomes secreted by hypoxic CPCs identified 11 miRNAs that were upregulated compared with exosomes secreted by CPCs grown under normoxic conditions. Treatment of ECs and fibroblasts with exosomes from hypoxic CPCs revealed elevated miR-103 and miR-15b levels in recipient cells. Next, exosomes were used after ischemia-reperfusion injury in a rat model. The exosomes from hypoxic CPCs improved cardiac function, reflected by improved fractional shortening and reduced fibrosis (Figure 1) [18]. These findings indicate that hypoxia triggers a regenerative response by the delivery of exosomes from cardiac progenitor cells transferring antifibrotic miRs to fibroblasts. These findings were fostered by Ong et al., who examined that EC-derived exosomes can in turn be taken up by CPCs promoting antiapoptotic effects by transferring miR-126 and miR-210. They used a mouse model to prove that intramyocardial codelivery of a nonviral minicircle plasmid carrying HIF1 (MC-HIF1) together with CPCs leads to better survival of CPCs, when given after MI. Additionally, codelivery of MC-HIF1 with CPCs leads to improved echocardiographic ejection fraction. In vitro experiments revealed that EC produced exosomes that were internalized by recipient CPCs and that these exosomes overexpressing HIF1 had elevated contents of miR-126 and miR-210. These miRs activated prosurvival kinases and induced a glycolytic switch in recipient CPCs, providing them with increased tolerance to hypoxic stress in vitro. The inhibition of both of these miRs blocked the protective effects of the exosomes (Figure 1) [19]. Another very interesting report about the positive effects transduced by EVs was published by Lai et al. They treated mice after myocardial ischemia/reperfusion injury with exosomes gathered from medium of mesenchymal stem cells (MSC), leading to reduced infarct size [54]. Boulanger et al. were able to expand the knowledge about effects of MVs under MI condition by treating rat aortic rings with endothelium, using MV isolated from patients with acute MI. They observed that endothelium-dependent relaxation by acetylcholine was impaired in endothelium of aortic rings treated with MVs from patients with MI by impairing the endothelial nitric oxide transduction pathway [55]. Summarized, these reports show that EVs play a fundamental role in the reorganization of the heart muscle after MI and thus imply an enormous potential of interventional possibilities by admitting or decreasing miR-containing EVs.

4. Heart Diseases

4.1. Heart Failure. Heart failure (HF) is the result of cardiac remodeling caused by stress through a lot of adverse conditions, like CAD, atrial fibrillation, elevated blood pressure, and valvular heart disease. Cardiac remodeling leads to hypertrophy of cardiomyocytes and fibrosis of the heart muscle. In 2006, one study investigated increased expression of miRs in two mouse models (thoracic aortic banding (TAB); Tg mice expressing activated calcineurin A (can)) for cardiac hypertrophy and in hearts collected from patients with HF. Increased expression of miR-23, miR-24, mir-125b, miR-195, miR-199a, and miR-214 could be observed in hearts from patients with HF. Next, the authors used adenoviral vectors to overexpress these miRs in cultured myocytes. Overexpression of miR-23a, miR-23b, miR-24, miR-195, and miR-214 in cultured myocytes caused hypertrophic growth in vitro. In an in vivo mouse model overexpression of miR-195 promoted cardiac hypertrophy [56]. An intercellular communication mechanism between cardiac fibroblasts and cardiomyocytes was first shown by Bang et al. They revealed that miR transfer by exosomes plays an important role in the development of hypertrophy of cardiomyocytes. For example, they found that cardiac fibroblasts secrete exosomes containing miR passenger strands ("star" miRNAs), which are normally degraded intracellularly. Further investigations pointed out that miR-21_3p (miR-21*), the passenger strand of miR-21, induces cardiomyocyte hypertrophy by targeting sorbin, SH3 domain-containing protein 2 (SORBS2), and PDZ and LIM domain 5 (PDLIM5). Furthermore, pharmacological inhibition of miR-21* reduced the development of cardiac hypertrophy in a Ang II mouse model. Taken together, this study could prove that fibroblast-derived exosomes enriched with miR-21* act as paracrine signaling mediator of cardiomyocyte hypertrophy (Figure 1) [57]. Another study showed that 16K PRL, a 16 kDa N-terminal prolactin fragment, induces miR-146a expression in ECs, leading to reduced angiogenesis by downregulation of NRAS and release of miR-146a-loaded exosomes. Absorption of these exosomes by cardiomyocytes resulted in decreased expression of Notch1, Erbb4, and Irak1. In a mouse model for peripartum cardiomyopathy (PPCM) with a cardiomyocyte-restricted Stat3 knockout (CKO mice) elevated cardiac miR-146a expression and simultaneously downregulated of *Erbb4*, *Nras*, *Notch1*, and *Irak1* were found, which could be attenuated when miR-146a was blocked with locked nucleic acids or antago-miR. Measurement of miR-146a in hearts and plasma levels of PPCM patients revealed elevated miR-146a levels [58]. In conclusion, this study presents miR-146a-loaded exosomes shed by ECs as important messengers in the development of PPCM, which can be attenuated by antago-miRs. Furthermore, it delivers evidence that miR-146a-loaded exosomes could play an important role as biomarker for diagnosis of PPCM in the future. Barile et al. described that medium from CPCs inhibited apoptosis in cardiomyocytic mouse cells and enhanced tube formation in HUVECs by EVs containing miR-210, miR-132, and miR-146a-3p (Figure 1). miR-210 downregulated ephrin A3 and PTP1b, which caused inhibition of apoptosis in cardiomyocytic cells, whereas miR-132 inhibited RasGAP-p120, resulting in increased tube formation in ECs. Besides, they demonstrated that injection of EVs from CPCs into infarcted hearts was associated with less cardiomyocyte apoptosis, enhanced angiogenesis, and improved LV ejection fraction compared with a control group [59]. Also, cardiosphere-derived cells (CDCs) are involved in exosome secretion, which causes antiapoptotic and enhanced proliferative and angiogenic effects on cardiomyocytes. For that reason, exosomes were injected in injured mouse hearts and could recapitulate the regenerative effects of CDC transplantation, while blocking of exosome production by CDCs attenuated these effects. Exosomes of CDCs showed high levels of miR-146a and administration of an miR-146a mimic showed some benefits of CDC exosomes (Figure 1) [60]. In conclusion, the presented studies display the important role of miR transfer by EVs in the development of heart failure and the protection against undesired modifications of the heart muscle. Further investigations are necessary for a more detailed understanding of the effects of EVs and to enable the development of new extracellular vesicle based therapies in the future.

4.2. Cardiac Hypertrophy. Several reports show that miRs play a functional role in cardiac hypertrophy. Roncarati et al. created a miR profile of 41 hypertrophic cardiomyopathy (HCM) patients. HCM patients were diagnosed by transthoracic echocardiography and cardiac magnetic resonance and healthy people, who were matched by age and sex, were used as control.

In plasma of HCM patients 12 miRs were significantly increased, but only miR-29a was significantly associated with hypertrophy and fibrosis. The authors concluded that miR-29a is a potential biomarker for HCM assessment [61]. Further reports about generating miR-expression profiles from patients with cardiac diseases can be found. For instance, Nair et al. analyzed miR-expression profile of three different kinds of patients with dilated cardiomyopathy (DCM): patients with DCM and isolated diastolic dysfunction, patients with stable compensated DCM, and patients with decompensated congestive heart failure secondary to DCM (DCM-CHF). The expression profile showed that mir-142-3p was decreased in patients with DCM and DCM- CHF, while miR 124-5p was increased in patients with DCM [62]. A similar report compared expression profile of miRs in patients with Takotsubo cardiomyopathy, healthy individuals, and ST-segment elevation acute myocardial infarction (STEMI) patients. They examined that patients with Takotsubo cardiomyopathy had special miR-1, miR-16, miR-26a, and miR-133a profile compared with the other groups, which could be used as a biomarker to distinguish Takotsubo cardiomyopathy patients from STEMI patients [63].

The presented studies are of particular interest, as the planning of an adequate therapy strategy in treatment of diseases is of great importance to enable the separation of low- and high-risk patients. Due to their different underlying therapy strategies, the necessity increases to distinguish between MI and Takotsubo cardiomyopathy in clinical daily life.

5. Diabetes Mellitus

Diabetes mellitus (DM) is a metabolic disorder characterized by dysfunction of insulin-secreting pancreatic beta-cells with great importance for the development of CAD. It is well known that miRs regulate beta-cell activity, but recently some studies reported that these miRs are also transferred from beta-cells to other recipient cells via exosomes. For instance, cytokine-treated MIN6B1 cells secrete exosomes containing miRs that are transferred to neighboring beta-cells, leading to apoptosis. MIN6B1 cells were treated with cytokines (IFNγ, TNF-α, and IL-1β) and exosomes were isolated from the culture media. Giving these exosomes to MIN6B1 or mice islet cells leads to apoptosis in recipient cells (Figure 1). Furthermore, they showed that miRs released in MIN6B1 exosomes do not simply reflect the content of the cells of origin, but a subset of miRs was preferentially released in exosomes, while others were selectively retained in the cells. Interestingly, exposition of MIN6B1 cells to inflammatory cytokines changed the release of several miRs [64]. This study gave new insights into the contribution of exosomes to the vanishing of insulin-secreting pancreatic beta-cells and thereby include the possibility to develop new therapies on this pathomechanism. Additional knowledge about mechanisms taking place in diabetic conditions was gained by Barutta et al. They reported that urinary exosomes from patients with microalbuminuria contain increased concentrations of miR-130a and miR-145, while the amount of miR-155 and miR-424 is reduced. In an animal model of early experimental diabetic nephropathy urinary exosomal miR-145 levels were increased while simultaneously miR-145 within the glomeruli was overexpressed. In addition, cultured mesangial cells exposed to high glucose showed increased miR-145 content in mesangial cells and their associated exosomes [65]. In 2010, Zampetaki et al. generated an expression profile of miRs in plasma of patients with DM. They observed lower plasma levels of miR-20b, miR-21, miR-24, miR-15a, miR-126, miR-191, miR-197, miR-223, miR-320, and miR-486 in prevalent DM, but a modest increase of miR-28-3p. Added to that, they were able to point out a reduction of miR-126 content in endothelial apoptotic bodies under high glucose concentrations [46]. The next study provides an interesting insight into the way miR-containing EMPs influence inflammatory effects in diabetic state. EMVs promote anti-inflammatory effects in vitro and in vivo by reducing endothelial ICAM-1 expression as they transfer functional miR-222 into recipient cells. Intriguingly, anti-inflammatory effects were reduced under hyperglycaemic conditions due to reduced miR-222 content of generated EMP [25]. Mocharla et al. were able to detect that $CD34^+$ peripheral blood mononuclear cells (PBMC) shed higher levels of miR-126 containing exosomes than CD34- PBMC subsets and that the exosomes containing higher miR-126 levels had higher proangiogenic effects on ECs than lower miR-126 level containing exosomes (Figure 1). At the same time, they also reported that treatment of $CD34^+$-PBMC with anti-miR-126 or inhibition of their release lowered their proangiogenic effects. Beyond that, they observed that treatment of $CD34^+$ PBMC with high-glucose levels and growing $CD34^+$ PBMC under diabetic conditions showed reduced miR-126 levels accompanied with impaired proangiogenic properties, which could be rescued by miR-mimic-126 treatment [66]. As the pancreatic rest function is often accompanied with the necessity of insulin injection in addition to oral therapy, the long-term conservation of pancreatic rest function in diabetic patients must be targeted. The presented studies offered new insights into mechanisms leading to apoptosis of insulin-secreting pancreatic beta-cells. Furthermore, new biomarkers for microalbuminuria were presented by a special miR-expression profile in the urine of patients with microalbuminuria, possibly helping to diagnose microalbuminuria in earlier stages in the future.

6. miR-Based Therapeutics

In general, there are two possible therapeutic approaches of using miRs. On the one hand, overexpressed miRs could be suppressed by application of miRs with a complementary sequence to the target miRs (anti-miRs) and, on the other hand, downregulated miRs with an appreciated effect could be precipitated by application of oligonucleotides mimicking endogenous miRs [67]. Recent reports have raised hope that targeting of miRs could serve as a new therapeutic approach. For example, one study reported that Miravirsen, a locked nucleic acid-modified DNA phosphorothioate antisense oligonucleotide, is able to suppress effects of miR-122, and by that it is able to reduce hepatitis C virus RNA [68]. Under hypoxic conditions from CPCs, generated exosomes improved cardiac function and reduced fibrosis in a rat ischemia-reperfusion injury model [18]. Fiedler et al. showed in a mouse model that antago-miRs were able to reduce endothelial apoptosis, enhance vascularization, decrease infarct size, and improve cardiac function after MI [69]. Kumarswamy et al. proved in a transaortic constriction (TAC) mouse model that miR-21 silencing antago-miRs were able to reduce cardiac dysfunction and fibrosis [70]. A mouse model of limb ischemia and MI indicated that intravenous application of an miR-92a antago-miR was able to induce enhanced angiogenesis and functional improvement of damaged tissue [71]. In vivo studies pointed out successful miR downregulation by adenovirus-transfer. Therefore, an arterial balloon injury in a rat model was combined with an adenovirus mediated transfer of miR-126-3p target sites, which was able to inhibit proliferation of vascular SMCs and to attenuate restenosis [72]. Taken together, there is a bunch of promising reports about positive effects of antago-miRs in a variety of diseases. But it should be concerned that a lot of obstacles have to be managed until miR- or antago-miR-based drugs will find broad way into the clinic.

6.1. Vesicle-Incorporated miRs as Novel Therapeutic Tool? EVs containing miRs represent a promising new therapeutic approach, because of their important natural roles in cellular processes like proliferation, differentiation, and apoptosis combined with high stability, tissue-specific expression pattern, and secretion to body fluids [73]. Among all EVs, exosomes seem to be the most suitable vehicle for miR delivery, because they physiologically target specific cells due

to the proteins contained in their membranes. This enables them to specifically bind to recipient cell receptors, providing the possibility to create exosomes that specifically target one desired cell type. This fact signifies a big step in the development of personalized medicine. Besides, they are flexible in cargo type, are nonimmunogenic, and maintain the cargo stable for delivery [73]. Meanwhile, there are several reports of exosomes used as therapeutic agents. For example, Lai et al. reported that mesenchymal stem cell (MSC) derived exosomes reduce infarct size in a mouse model of myocardial ischemia/reperfusion injury [54]. Added to that, it is reported that miR-150 is selectively packaged into MVs of monocytes and can be taken up by ECs leading to enhanced cell migration [74]. Besides therapeutic tools of miRs incorporated in extracellular vesicles, like exosomes, many reports using nanoparticles as a new approach to transport miRs to recipient cells have been published in the last few years. Cheng and Saltzman developed biodegradable polymer nanoparticles, which are coated with cell-penetrating peptides that can effectively deliver chemically modified oligonucleotide analogues to achieve gene regulation. This nanoparticle system could block the activity of the oncogenic miR-155, as well as attenuating the expression of the protooncogene, Mcl-1, leading to reduced cell viability and proapoptotic effects in the recipient cells [75]. Furthermore, Babar et al. inhibited miR-155 by delivery of antisense peptide nucleic acids encapsulated in polymer nanoparticles and thereby slowed down pre-B-cell tumors growth in vivo [76]. An integrin $\alpha v\beta 3$-targeted nanoparticle was used by Anand et al. to deliver anti-miR-132 to the tumor endothelium of human breast carcinoma in mice, causing restored p120RasGAP expression in the tumor endothelium and thereby suppressed angiogenesis and decreased tumor burden [77]. Chen et al. took a LPH (liposome-polycation-hyaluronic acid) nanoparticle formulation modified with tumor-targeting single-chain antibody fragment (scFv) for systemic delivery of miR-34a into lung metastasis of murine B16F10 melanoma, prompting significant downregulation of survivin expression in the metastatic tumor and reduced tumor load in the lung [78]. Su et al. reported that systemic delivery of a chemically stabilized anti-miR-122 complexed with interfering nanoparticles (iNOPs) effectively silences the liver-expressed miR-122 in mice. miR-122 is a liver-specific miRNA, with suggested roles in cholesterol, fatty acid, and lipid metabolism [79]. Although these articles focused on miR delivery using nanoparticles mainly as therapeutic tool to combat cancer, it is reasonable that nanoparticles can also be used to deliver miRs to recipient vascular cells for influencing inflammation and development of atherosclerosis.

6.2. Current Limitations and Future Perspective/Directions. Unfortunately, the usage of miRs in EVs as therapeutical approaches is limited by the amount of miRs that have to be transferred in order to have an effect on recipient cells. It could be shown that a lot of miRs did not show any detectable activity and that some miRs only had weak effect on mRNA silencing, because of an inappropriate target-to-miR ratio [80]. Besides this, the increase of their binding specificity, circulation time, and protection from cleavage by nucleases still needs to be managed. To handle these problems, miRs need chemical modifications, like locked nucleic acids (LNAs) or $2''''$-O-methylation and have to be packed into vehicles, like lipids, vectors, or polymers [67]. In perspective, circulating miRNAs could revolutionize diagnosis and estimation of prognosis of cardiovascular diseases by providing new biomarkers. But it should also be considered that no possible biomarker has been validated in large cohort studies until now [81]. Despite these promising results, a lot of issues need to be addressed until all advantages of EV-bound miRs can be used in full range. It will still need a lot of investigation and time to develop EVs that do not have any off-target effects and any immunogenicity and whose long-term effects are known [73]. Moreover, it must be assumed that miR expression profile changes in different disease states and thereby determination of appropriate endogenous controls will be complicated. Apart from that, the detection of suitable clinical methods to quantify circulating miRNA, also associated with upcoming costs, must be put in focus [81].

7. Conclusions

EV-driven miR transfer in CAD is a new promising field of research giving new insights in protein regulation, pathomechanisms of diseases, and modulation of cellular phenotypes and will possibly deliver new biomarkers and therapeutical approaches in the future.

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

Soluble Receptor for Advanced Glycation End Product: A Biomarker for Acute Coronary Syndrome

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The receptor of advanced glycation end products (RAGE) and its ligands are linked to the pathogenesis of coronary artery disease (CAD), and circulating soluble receptor of advanced glycation end products (sRAGE), reflecting the RAGE activity, is suggested as a potential biomarker. Elevated sRAGE levels are reported in relation to acute ischemia and this review focuses on the role of sRAGE as a biomarker for the acute coronary syndrome (ACS). The current studies demonstrated that sRAGE levels are elevated in relation to ACS, however during a very narrow time period, indicating that the time of sampling needs attention. Interestingly, activation of RAGE may influence the pathogenesis and reflection in sRAGE levels in acute and stable CAD differently.

1. Introduction

Worldwide, cardiovascular disease (CVD) is a prominent cause of increased morbidity and mortality with a heavy burden on the health care system [1]. Even though improvements in prevention, diagnosing, and treatment of the disease have increased substantially during the last decades, more attention is needed. It is believed that inflammatory mechanisms are involved in the development of CVD [2, 3], though the detailed pathogenic role of the inflammation system is still under investigation. The receptor of advanced glycation end products (RAGE) is found to play an important role in the development of CVD [4], and the soluble RAGE (sRAGE) may to some extent reflect RAGE activity, thus increasing the value of sRAGE as a biomarker [5, 6]. This review focuses on the role of sRAGE as a biomarker for the acute coronary syndrome (ACS).

2. Receptor for Advanced Glycation End Products (RAGE)

RAGE is a transmembrane receptor of the immunoglobulin superfamily composed of three domains: an extracellular domain binding to ligands, a hydrophobic membrane spanning domain, and a highly charged cytoplasmic domain essential for the intracellular signaling. RAGE is expressed in many cell types including endothelial cells, lymphocytes, monocytes, and vascular smooth muscle cells. RAGE expression is minimal under normal conditions but increases significantly during cellular stress [7, 8].

RAGE was first described as a receptor for advanced glycation end products (AGEs) and it was initially linked to hyperglycemia, diabetes, and diabetic complications [9]. However, RAGE is now characterized as a multiligand receptor [10], and, apart from AGEs, RAGE interacts with other ligands, such as the S100 proteins [11], high mobility group box 1 (HMGB1) [12, 13], and amyloids [14]. Ligand binding is described to increase RAGE activity [15, 16], which mediates proinflammatory responses [17–19] and generates oxidative stress [15, 18, 20] that may contribute to the pathogenesis of CVD. Still, the exact function in vascular pathogenesis is unclear.

Mechanistic studies showed that cardiomyocytes upregulated both RAGE and AGEs after exposure to hypoxia followed by reoxygenation. Furthermore, cardiomyocytes isolated from genetic RAGE knockout or from mice pretreated with sRAGE showed protection against cellular damage [21]. Similarly, RAGE expression increased in mice myocardium after a temporary occlusion of the left anterior descending artery compared to sham-operated animals and RAGE

colocalized with apoptotic cardiomyocytes [22]. The infarct sizes diminished in RAGE knockout mice hearts exposed to I/R injury [23] and precursors of RAGE ligands were reduced [24]. Furthermore, RAGE knockout mice or mice treated with RAGE inhibitors had less impaired cardiac function [12, 23–25] and diminished atherosclerosis [18, 26]. Moreover, administration of sRAGE reduced atherosclerotic lesions in atherosclerotic and diabetic mice models [4, 27, 28]. Inhibition or deletion of RAGE suppressed proinflammatory activity and oxidative stress [12, 24, 29, 30]. Additionally, RAGE ligands are reported to be involved in monocyte migration and cholesterol efflux from macrophages, and the effect was diminished through anti-RAGE antibodies or sRAGE [31, 32].

In human settings, RAGE was highly expressed in plaques, retrieved after carotid endarterectomy, from diabetic patients compared to plaques from the nondiabetic patients [33] and RAGE was primarily associated with apoptotic smooth muscle cells and macrophages together with an increased proinflammatory response [33, 34]. Furthermore, increased RAGE mRNA was found in mononuclear cells also from patients with premature CAD when compared to cells from healthy controls [35]. Together these experimental and morphological studies point towards RAGE activation in I/R injury and atherosclerosis.

3. Soluble RAGE (sRAGE)

Soluble isoforms of RAGE are found in the circulation and may act as regulators of RAGE activity by competitive inhibition. These isoforms lack the intramembranous and intracellular parts of the receptor, which devoid intracellular signaling [36]. Soluble RAGE is produced in two different ways, either as a splice variant, esRAGE, from a truncated RAGE mRNA [6, 37] or as a cleaved variant. Metalloproteinases cleave sRAGE from the full-length RAGE from the cell membrane [5, 38, 39]. So far, the concentrations of soluble RAGE have been determined as esRAGE or as the total amount of sRAGE, which are positively correlated [40, 41], and esRAGE constitutes 20% of total sRAGE [41, 42]. Different functions of the secreted and the cleaved sRAGE have not yet been demonstrated. Furthermore, sRAGE may reflect enhanced activity in the RAGE system since the effects of ligand stimulation mediate sRAGE upregulation [5] and sRAGE is secreted in parallel with RAGE [5, 6]. This property makes sRAGE a valuable biomarker.

4. sRAGE in Patients with Acute Coronary Syndrome (ACS)

sRAGE levels in patients with ACS, defined as unstable angina, non-ST-segment elevation myocardial infarction (STEMI), and STEMI, have been presented in twelve published cohorts with diverging results (Table 1). Cai et al. and Park et al. reported elevated levels of sRAGE in patients with ACS when compared with healthy controls [43, 44]. The study by Basta et al. did not find different sRAGE levels in non-STEMI patients compared to patients with stable CAD. However, sRAGE concentrations were higher in patients with elevated cardiac Troponin I (TnI), a specific and approved biomarker of acute myocardial infarction (AMI) [45]. In the study by Raposeiras-Roubín et al., plasma samples were collected within 12 hrs after symptoms in relation to percutaneous coronary intervention (PCI) [46], whereas Cai et al. and Basta et al. collected the blood samples at a later time point [43, 45]. Raposeiras-Roubín et al. reported similar sRAGE levels in STEMI and non-STEMI patients, but increased sRAGE levels were associated with poor in-hospital prognosis [46]. The time of blood sampling was not reported. Fukushima et al. found equal sRAGE levels in patients with ACS at baseline and at 8–12 months follow-up [47]. The time of baseline sampling is not described in detail, but one could speculate that blood was drawn at randomization for different statin treatment 72 hrs after PCI.

In two different cohorts of STEMI patients, samples were drawn within 12 hrs after onset of symptoms and before PCI. We recently reported a fourfold increase in sRAGE levels in these STEMI patients as compared to 100 healthy individuals (described in Figure 1) [48, 49]. Successive blood samples were drawn during and after treatment of one of the STEMI cohorts, and sRAGE levels reduced almost threefold the day after PCI and decreased even further two days after PCI [49]. Interestingly, the increase of sRAGE was seen prior to TnI. The rapid decrease in sRAGE levels the day after PCI may provide valuable information in relation to diagnosis of reinfarction. Our results support the fact that sRAGE levels are elevated particularly in relation to acute ischemia, which may indicate sRAGE as an additional biomarker of AMI. In addition, the repeated measurements elucidate that sRAGE levels change over a very narrow time span in relation to acute disease. Therefore, attention to time point of sRAGE measurement is important when interpreting the results.

In contrast to the above studies, Falcone et al. found significantly decreased sRAGE levels in patients with ACS as compared to stable angina [55]. Blood samples were collected before the revascularization. Similarly, McNair et al. found lower levels of sRAGE in patients with non-STEMI compared to the controls, and time of blood sample collection was not indicated [51–54].

Only few studies have evaluated the effect of diabetes on the sRAGE levels in patients with ACS. In a group of patients (50% with type 2 diabetes (T2D)), Park et al. reported higher plasma sRAGE levels in patients with AMI than in controls, however, regardless of the presence of diabetes [44]. Similarly, Fukushima et al. reported no differences in sRAGE levels in diabetic ACS patients (30%) compared to nondiabetics with ACS [47]. Our two studies of STEMI patients included 6% and 9% diabetics and no difference in sRAGE levels was found due to diabetes [48, 49].

The ambiguity in the studies may be explained by incomparable conditions between studies; of particular importance is the time point of blood sample collection and age. A different ratio of diabetic patients within the studies described in Table 1 may also contribute to the inconclusive results as the data in diabetics with ACS are sparse.

Table 1

Patients	sRAGE	Description of main results	Reference
STEMI treated with PCI with/without remote ischemic conditioning ($n = 191$)	↑	Increased sRAGE levels with higher NYHA classification. No effect of remote ischemic conditioning on sRAGE levels and association between sRAGE and salvage index.	Jensen et al., 2015 [48]
STEMI treated with PCI ($n = 80$)	↑	Consecutive samples show high sRAGE levels prior to and immediately after PCI followed by decreased levels day 1 and day 2 after PCI. sRAGE was an independent predictor of cardiac dysfunction assessed by decreased LVEF.	Jensen et al., 2015 [49]
ACS ($n = 208$)	→	No difference in sRAGE levels at baseline and after 8–12 months after PCI. Baseline sRAGE levels were not associated with plaque progression 8–12 months after PCI.	Fukushima et al., 2013 [47]
ACS ($n = 330$), stable angina ($n = 530$)	↓	Significantly decreased sRAGE levels in ACS compared with stable angina.	Falcone et al., 2013 [55]
STEMI ($n = 102$), non-STEMI ($n = 113$)	→	No difference in sRAGE levels between STEMI and non-STEMI. Elevated sRAGE level was associated with in-hospital cardiac events.	Raposeiras-Roubín et al., 2013 [46]
Non-STEMI ($n = 190$), stable angina ($n = 75$)	→↑	No difference in sRAGE levels between non-STEMI and stable angina. Increased sRAGE in patients with elevated TnI.	Basta et al., 2011 [45]
ACS ($n = 420$), stable angina ($n = 211$), controls ($n = 251$)	↑	Increased sRAGE levels in ACS compared with controls.	Cai et al., 2011 [43]
AMI ($n = 54$), controls ($n = 54$)	↑	Increased sRAGE levels in patients with AMI. Diabetic patients with AMI had higher sRAGE levels than diabetic patients without AMI.	Park et al., 2011 [44]
Non-STEMI ($n = 36$), controls ($n = 30$)	↓	Lower sRAGE levels in non-STEMI compared to controls. Negative correlation between sRAGE and cTnI.	McNair et al., 2011 [51] McNair et al., 2010 [52]
Non-STEMI ($n = 46$), controls ($n = 20$)		Lower sRAGE levels in non-STEMI compared to controls. Non-STEMI with post-PCI restenosis had lower post-PCI sRAGE levels than pre-PCI levels.	McNair et al., 2010 [53]
Non-STEMI ($n = 46$), controls ($n = 28$)	↓	Lower sRAGE levels in non-STEMI compared to controls. sRAGE levels were inversely associated with the number of diseased vessels.	McNair et al., 2009 [54]

ACS: acute coronary syndrome; AMI: acute myocardial infarct; NYHA: New York Heart Association classification; LVEF: left ventricular ejection fraction; PCI: percutaneous coronary intervention; sRAGE: soluble receptor of advanced glycation end products; STEMI: ST-segment elevation myocardial infarction; TnI: Troponin I.

The major source of sRAGE in relation to ACS is still not clear, but it is highly probable that it originates from the cardiomyocytes or the vascular cells in the damaged myocardium.

5. sRAGE in Patients with Stable CAD

Opposite to the patients with ACS, patients with stable CAD had low plasma sRAGE levels when compared to controls [35, 50, 56–58]. In a large population study including 2,571 individuals, high coronary calcium score, a risk marker of CVD, was more prevalent in the group with low sRAGE levels [59]. Furthermore, in a small prospective study of patients with suspected CAD, low sRAGE levels were predictive of future cardiovascular events after 48 months of follow-up [60]. In support, low sRAGE levels in the Atherosclerosis Risk in Communities (ARIC) Study were an indicator of future CAD after 18 years of follow-up [61]. One might speculate that the low levels of sRAGE in nondiabetic patients with stable CAD may reflect a local release of RAGE from the atherosclerotic vessels. sRAGE may capture RAGE ligands and thereby reduce measurable sRAGE in the circulation and furthermore reduce the activity in the RAGE axis.

Patients with T2D and stable CAD had significantly higher levels of sRAGE than the nondiabetic CAD patients [58, 62], and the T2D patients with high sRAGE concentrations had increased risk of CAD [63]. sRAGE levels were able to predict future CAD in T2D patients after approximately 4 years of follow-up [40]. In addition, high sRAGE levels were associated with an increased cardiovascular morbidity and mortality in T1D during follow-up [64, 65]. It may be speculated that the persistent high levels of sRAGE in diabetic patients may reflect an ongoing inflammatory and RAGE activity related to diabetes.

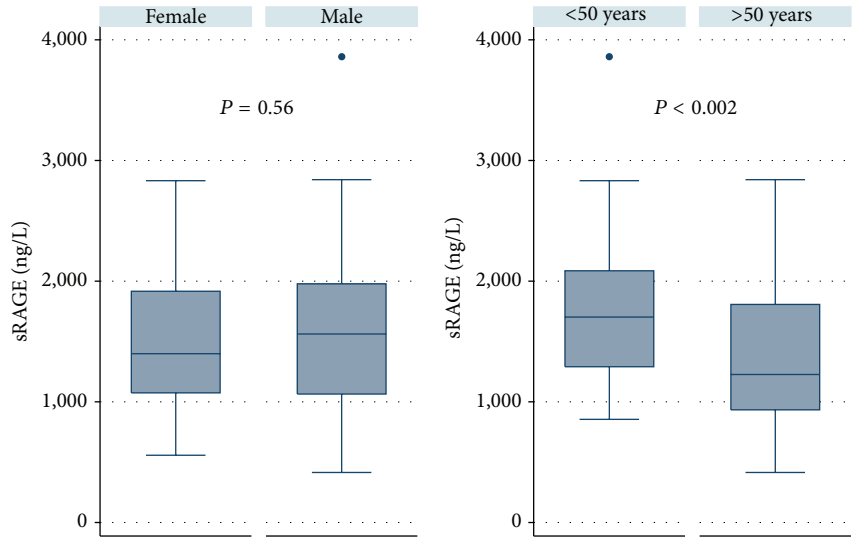

Figure 1: sRAGE concentrations in 100 healthy individuals. Healthy individuals are divided by gender (female ($n = 50$) and male ($n = 50$)) or by age (below 50 years ($n = 50$) and above 50 years ($n = 50$)).

6. Measurement and Variation in sRAGE Levels

As a potential biomarker, sRAGE may be influenced by detection methods and various factors such as gender, age, and ethnicity as well as diseases and medications. In the majority of published studies, sRAGE levels have been determined with a human sRAGE enzyme-linked immunosorbent assay (ELISA) (DRG00, R&D Systems). In the enclosed datasheet, mean of sRAGE levels was 1,655 (±693 (SD)) ng/L in EDTA plasma and 1,794 (±693 (SD)) ng/L in serum in apparently healthy volunteers. An extern validation revealed a stable assay with comparable concentrations in EDTA plasma and serum samples [66]. We validated a time-resolved immunofluorometric assay (TRIFMA) using commercial human RAGE antibodies (DY1145, R&D Systems) [49] and found comparable sRAGE concentrations by TRIFMA and ELISA. We detected plasma sRAGE levels of 1,533 ng/L (±61 (SD)) in 100 healthy individuals. No difference in sRAGE levels was seen according to gender ($P = 0.56$), but age introduced a difference since individuals below the age of 50 had significantly higher levels of sRAGE than those above the age of 50 (1,759 (±86 (SD)) ng/L versus 1,378 (±78 (SD)) ng/L, $P < 0.002$, Figure 1). A negative association between age and sRAGE levels was also observed in some diabetic cohorts [42, 63] and in a population study [59].

sRAGE levels remained stable when repeatedly measured within at least 3 years in patients with and without diabetes [40, 42, 67]. A negative association between sRAGE levels and body mass index (BMI) has been found in some cohorts [42, 61] and also in one of our studies [48]. Additionally, ethnicity is reported to influence the sRAGE levels as higher sRAGE levels are reported in white compared with black individuals [40, 61, 68]. sRAGE levels may be influenced by diseases other than CAD and diabetes, for example, cancer, inflammatory diseases, neurodegenerative diseases, or chronic kidney disease [69]. Furthermore, medications may also affect sRAGE levels [70, 71].

7. Conclusion

Several studies indicate that RAGE activation may influence the pathogenesis and reflection in sRAGE levels in acute and stable CAD differently. The current studies demonstrated that, in nondiabetic patients, sRAGE levels are elevated in relation to ACS and sparse data indicate that diabetes does not have an additive effect in ACS patients. On the contrary, in patients with stable CAD, sRAGE levels are low in nondiabetic patients but elevated in diabetic patients which may add predictive value to recognition of future CVD.

Current data on sRAGE levels in CAD are diverging and sRAGE may be influenced by several other factors, which is why precaution must be taken with the drawn conclusions. In relation to ACS, we found the time of sampling to be of importance, which is highly relevant for evaluation as a potential biomarker. Additional mechanistic studies are needed as well as investigations of the sources and functions of sRAGE. Further clinical studies are also needed to establish the value of sRAGE as a prognostic marker in patients with ACS.

Conflict of Interests

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

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