BioMed Research International

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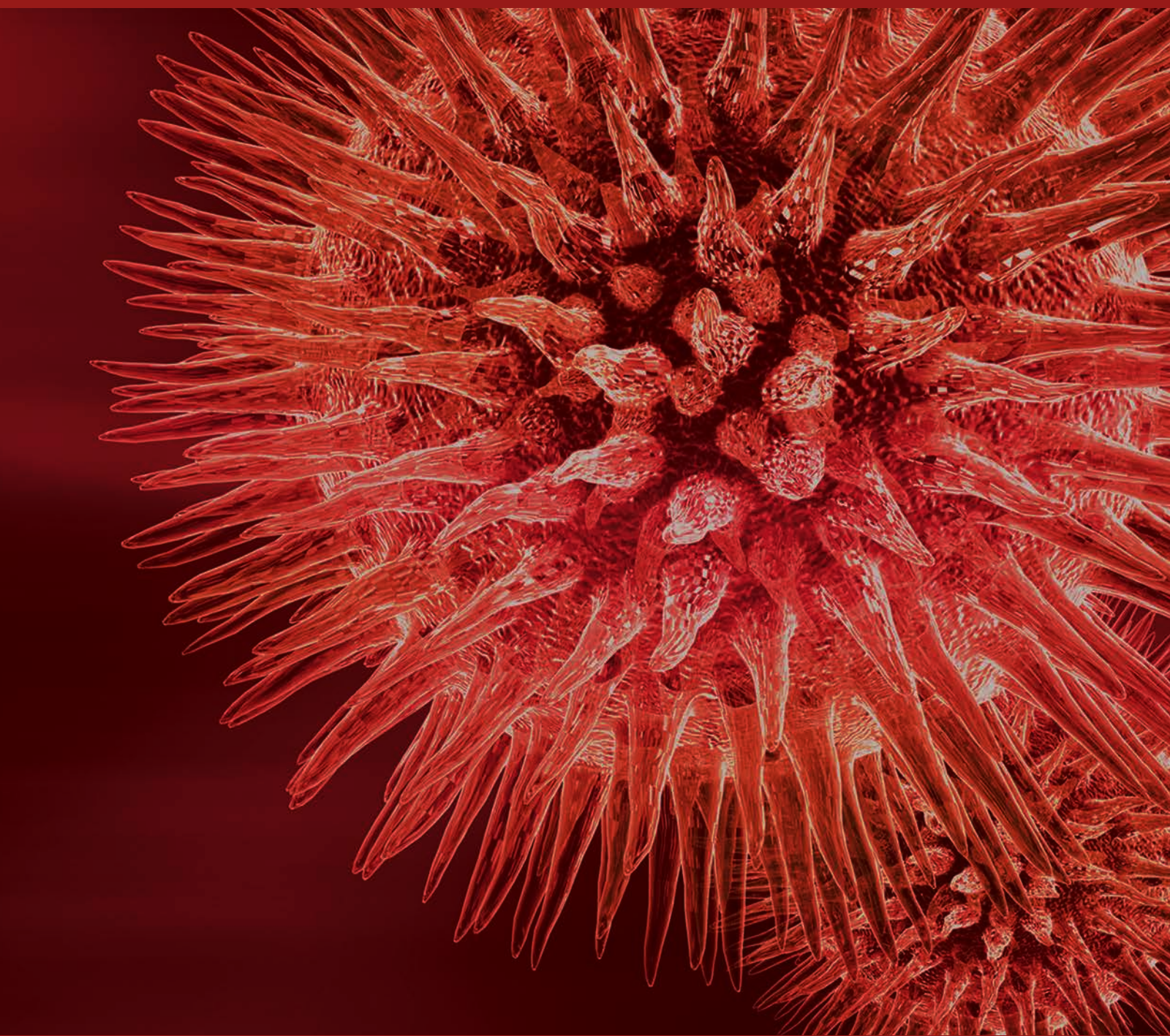
Guest Editors: Raffaele Serra, Stefano de Franciscis, and Laurent Metzinger



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Editorial

Roles and Clinical Applications of Biomarkers in Cardiovascular Disease

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Due to the high rates of mortality and morbidity caused by Cardiovascular and Peripheral Vascular Disease (CPVD), great efforts have been made in order to improve diagnosis and primary and secondary prevention and treatment, even by discovering and using related biomarkers. As such, the articles contained in the present issue include both reviews and research articles focused on characterizing several biomarkers in order to evaluate their role in the field of CPVD.

The review by S. de Franciscis et al. considers the current evidences of novel biomarkers with clear implications in the risk assessment, prevention strategies, and medical decision making in the main fields of CPVD, such as Hypertension, Coronary Heart Disease, Arterial Aneurysms, Carotid Artery Disease, Peripheral Artery Disease, Chronic Venous Disease, and Venous Thromboembolism.

The study by Y. Hao et al. shows multiple metabolites that associated with risk of developing Hypertension (HYT). Specifically, low amino acid levels and gut microbiome seem to play an important role in the pathogenesis of this disease.

J. Xu et al. confirm the already known association of genetic polymorphisms of ATP2B1 with the susceptibility to HYT in the Han Chinese population, particularly in the females, and found that the interaction of high BMI and ATP2B1 variants increased even more the susceptibility to Hypertension.

Acute heart failure (AHF) is the most common reason of hospitalization in patients aged 65 and older, with mortality rate up to 30–40% within one year, and, in this context, Y. Wang et al. show that sequential monitoring of changes of N-terminal probrain natriuretic peptide (NT-proBNP) within the first days after acute heart failure (AHF) may be helpful for guiding clinical management of AHF patients.

In ST-elevation myocardial infarction (STEMI) patients, it is pivotal to prevent AHF and recurrent thrombosis, and, in this context, M. Marinšek and A. Sinkovič show the beneficial effects of Ramipril and Losartan associated with dual antiplatelet therapy (DAPT) in respect to DAPT alone, by means of measuring the level of several biomarkers such as NT-proBNP, ejection fraction (EF), plasminogen-activator-inhibitor type 1 (PAI-1), and platelet aggregation by closure times (CT).

As atherosclerosis and vascular calcification are dynamic processes, T.-L. Chuang et al. studied the association between the bony microarchitecture score (trabecular bone score, TBS) and coronary artery calcification (CAC) in adult subjects undergoing health exams and they found that advanced age was significantly associated with high CAC, while increased TBS was associated with moderate CAC, independent of age and other risk factors, and they concluded that further evidences were needed to confirm their data.

Osteoprotegerin (OPG) and its ligands, receptor activator of nuclear factor κB ligand (RANKL) and TNF-related apoptosis-inducing ligand (TRAIL), are known players in osteoblastogenesis and osteoclastogenesis. In fact, they now represent also new biomarkers in the atherosclerosis and

vascular calcification fields, as described by S. Bernardi et al. with evidences pointing towards a proatherogenic role for OPG and an antiatherogenic role for TRAIL.

Misdiagnosis in Pulmonary Embolism (PE) may increase the related high mortality, and E. Gul et al. investigated the role of serum adiponectin levels and they found that adiponectin levels were significantly low in the patient group with PE compared to patients without PE, and they concluded that these findings may have important implications in the diagnosis of PE.

The current literature abundantly describes that microRNAs (miRNAs) and long noncoding RNAs are potential new biomarkers for most pathophysiological processes, including cardiovascular diseases and metabolic disorders. In this special issue, L. Louvet et al. show, using a human *in vitro* model of vascular smooth muscle cells, that several miRNAs are implicated in the preventive role of magnesium towards vascular calcifications. A. Carino et al. show that the levels of several circulating miRNAs are altered during the switch from clopidogrel to ticagrelor. Their results hint at the possibility of using miRNAs as noninvading biomarkers to determine primary end points in the CPVD field, although further work will be necessary to clearly establish that. Finally, the very recent discovery of long noncoding RNAs, defined as non-protein coding transcripts longer than 200 nucleotides, opens new horizons in the CPVD field, as evidenced by the paper of Y. Yan et al., that shows that the circulating level of the long noncoding RNA UCA1 is altered during AHF.

We hope that this special issue would throw light on the major issues in the area of biomarkers in CPVD and would attract the interest of scientific community in order to pursue further investigations leading to the discovery of novel biomarkers and to their rapid implementation into clinical practice.

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Research Article

Magnesium Attenuates Phosphate-Induced Deregulation of a MicroRNA Signature and Prevents Modulation of Smad1 and Osterix during the Course of Vascular Calcification

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Vascular calcification (VC) is prevalent in patients suffering from chronic kidney disease (CKD). High phosphate levels promote VC by inducing abnormalities in mineral and bone metabolism. Previously, we demonstrated that magnesium (Mg^{2+}) prevents inorganic phosphate- (Pi-) induced VC in human aortic vascular smooth muscle cells (HAVSMC). As microRNAs (miR) modulate gene expression, we investigated the role of miR-29b, -30b, -125b, -133a, -143, and -204 in the protective effect of Mg^{2+} on VC. HAVSMC were cultured in the presence of 3 mM Pi with or without 2 mM Mg^{2+} chloride. Total RNA was extracted after 4 h, 24 h, day 3, day 7, and day 10. miR-30b, -133a, and -143 were downregulated during the time course of Pi-induced VC, whereas the addition of Mg^{2+} restored (miR-30b) or improved (miR-133a, miR-143) their expression. The expression of specific targets Smad1 and Osterix was significantly increased in the presence of Pi and restored by coincubation with Mg^{2+}. As miR-30b, miR-133a, and miR-143 are negatively regulated by Pi and restored by Mg^{2+} with a congruent modulation of their known targets Runx2, Smad1, and Osterix, our results provide a potential mechanistic explanation of the observed upregulation of these master switches of osteogenesis during the course of VC.

1. Introduction

Vascular calcification (VC) is characterized by a pathological deposition of mineralized matrix in the vascular wall. VC is particularly associated with atherosclerosis, diabetes, and chronic kidney disease (CKD) and is rarely linked to genetic mutations [1, 2]. Clinically, VC is reflected in changes in parameters such as pulse pressure, coronary artery calcification, intima/media thickness, or pulse wave velocity [3]. The presence of VC leads to increased mortality rates in patients with CKD compared to the general population, due to increased intimal and medial calcifications of the large arteries [4]. VC is now described as a tightly regulated process sharing similarities with bone formation [5]. This active process involves the alteration of the contractile phenotype of vascular smooth muscle cells (VSMC) by specific exogenous stimulation. Indeed, exposure of VSMC to high phosphate and/or high calcium concentrations leads to an increase in mineralization, implying pathways involved in osteogenesis [5]. Furthermore, the specific upregulation of transcription factors such as Core-binding factor 1 α (Cbfa1)/Runt-related transcription factor 2 (Runx2), osterix (Osx), or transcription activators like Smad proteins are major features of both osteogenesis and VC [6].

Over the last decade, a novel class of regulators emerged as repressors of gene expression. Constituted of 18 to 25 nucleotides, microRNAs (miRs) are small, noncoding, regulatory RNAs. In the current canonical model [7], they act either by inducing an inhibition of the translation of their target mRNAs or via their degradation. A single miR is able to

Table 1: Selection of specific miRs, involved in VSMC phenotypic switch, osteogenesis, or VC, in different cell types, and their effects in cell physiology and related targets as reported in the literature.

miRs	Regulation	Cell type & physiology	Origin	Targets	Reference
29b	Increased	MC3T3 preosteoblastic cells, osteogenesis	Mouse	Collagen 1A1	[13]
30b/30c	Decreased	Coronary artery smooth muscle cells, calcification	Human	Runx2	[18]
30a/30b/30c/30d/30e	Decreased (30b/30c) Increased (30a/30d/30e)	Mesenchymal stem cells/bone marrow stromal cells/MC3T3-E1, osteogenesis	Mouse	Runx2, Sox9, Osteopontin, and various others	[30]
125b	Decreased	Coronary artery smooth muscle cells	Human	Osterix (sp7)	[15]
133a/135a	Decreased	C2C12 cells, osteogenesis	Mouse	Runx2 (miR-133a)/Smad5 (miR-135a)	[11]
133a	Decreased	Aortic smooth muscle cells and isolated arteries, phenotypic switch, and vascular remodeling	Rat	Acta2, SM-Myosin Heavy Chain	[10]
143/145	Increased	Pulmonary artery smooth muscle cells, phenotype modulation	Human	Klf4/Myocardin	[9]
143/145	Decreased	Aortic smooth muscle cells, migration and phenotypic switch	Human	Klf4/Myocardin	[9]
204	Decreased	Aortic smooth muscle cells, calcification	Mouse	Runx2	[17]
223	Increased	Aortic smooth muscle cells, migration and phenotypic switch	Human	RhoB/Mef2c/Acta2	[27]

regulate the expression of multiple genes because of its ability to bind to its mRNA targets as either a perfect or imperfect complement. The human genome may encode more than 1 500 miRs that could target about 30% to 60% of the genes expressed in the various human cell types. Conversely, studies soon showed correlations between miRs expression and diseases and miRs have recently entered the cardiovascular field [8]. To focus more precisely on the mechanisms that cause VC, reports at first implicated miRs during the VSMC phenotypic switch and the vascular remodeling [9, 10]. Concomitantly, miR signatures were found during osteoblast and osteoclast differentiation [11–14], implying miRs which were later involved in VSMC-driven VC [15–18].

Magnesium (Mg^{2+}) has recently been introduced as a new player in the field of VC. Since an inverse relationship between serum Mg^{2+} concentrations and VC was reported [19], a limited number of clinical as well as *in vitro* studies assessed a potential beneficial effect of Mg^{2+} reviewed in [20]. During the last four years, several studies confirmed that Mg^{2+} supplementation alleviates VC in both rodent and bovine models [21, 22]. Two studies further detailed the mechanistic aspects involved in phosphate-induced VC of human aortic VSMC (HAVSMC) [23, 24]. The various studies showed that Mg^{2+} negatively regulates VC through transient receptor potential melastatin (TRPM)7 activity and modulates expression of calcification markers such as anticalcification proteins (Osteopontin, Matrix Gla Protein), osteogenic proteins (Osteocalcin, Bone Morphogenetic Proteins), and osteogenic and VC related transcription factors (Cbfa1/Runx2 and Osx).

In the present study, we investigated a panel of miRs during Mg^{2+} attenuated inorganic phosphate- (Pi-) induced VC (Table 1).

This selection of miRs was extrapolated from the literature, which was sparse at the initiation of the study, as well as miRNA databases. We chose miR-29b, -30b, -125b, -133a, -143, -204, and -223 because they were shown to play a key role in the course of VSMC phenotypic switch, osteogenesis, or VC. Additionally, after browsing miR databases, we selected miRs that could target key mediators of VC such as col1a1, cbfa1/Runx2, Smad1, and Osx. For the first time in a human model, we were able to show the role of several miRs in the course of VC and thus gain additional mechanistic insights into the mode of action and beneficial effects of Mg^{2+} during this process.

2. Materials and Methods

2.1. Chemicals. All chemicals were purchased from Sigma unless otherwise stated.

2.2. Cell Culture of HAVSMC. Primary human VASMC were isolated in our laboratory from explants of human aortic tissue obtained with appropriate ethical approval. The samples were obtained after aortic valve bypass surgery or other types of surgery on the aorta from patients with various cardiovascular diseases (Pr Caus, Pôle Coeur Thorax Vaisseaux, CHU Amiens, France). This protocol was approved by the French Ethics Committee "Comité de Protection des Personnes (CPP) Nord-Ouest II" under ID #2009/19. The investigations were performed according to the principles outlined in the Declaration of Helsinki for use of human tissue or subjects. The medial tissue was separated from segment of human aorta after removal of endothelium. Small pieces of tissue (1-2 mm^2) were placed in culture dish in Dulbecco's Modified

Table 2: Primers used for q-PCR were published previously or designed through the use of PrimerBank [30, 31, 42].

Gene name	Forward (5′-3′)	Reverse (5′-3′)	GenBank
GAPDH	ATGGAAATCCCATCACCATCTT	CGCCCCACTTGATTTTGG	NM_002046
β-actin	CCTCACCCTGAAGTACCCCA	TGCCAGATTTTCTCCATGTCC	NM_001101
Collagen 1A1	ACGAAGACATCCCACCAATCAC	TCATCGCACAACACCTTGC	NM_000088
Osterix	CCTCTGCGGGACTCAACAAC	AGCCCATTAGTGCTTGTAAAGG	NM_152860
Runx2	AGCTTCTGTCTGTGCCTTCTGG	GGAGTAGAGAGGCAAGAGTTT	NM_001024630
Smad1	TTCCATGCCTCCTCCACAAG	AGGCATTCGGCATACACCTC	NM_005900

Eagle's Medium (DMEM) supplemented with 15% of fetal bovine serum (FBS, Dominique Dutscher), 4.5 g of glucose, 1 mmol/L of pyruvate, 100 U/mL of penicillin, and 100 μg/mL of streptomycin in a 5% CO_2 incubator at 37°C. Cells that migrated from explants were collected when confluent. The cells were maintained in DMEM supplemented with 15% FBS, and the medium was replaced twice per week. HAVSMC were identified by their typical morphology, and purity of the primary cell culture was further checked by immunocytochemistry using a monoclonal antibody against the α-smooth muscle actin protein 1A4 (Acta2) (Santa Cruz Biotechnology) [25]. The cells were used between passages 6 and 12, during which time they were able to calcify. The cells isolated from 3 independent donors were used for the various experiments of the study.

For calcification assays, cells were seeded at 7 500 cells/well in 48-well plates and treated for the indicated times with various media conditions from 2 to 3 days after plating.

For RNA isolation, cells were seeded at 150 000 cells/well in 6-well plates. For the whole miR study, the experimental setup included conditions with or without 3 mM of Pi; magnesium was added to reach 1.5 or 2 mM. The samples were incubated and stopped at the time points 4 h, 24 h, day 3, day 7, and day 10. For all experiments, cells were treated as described in the calcification assays section.

2.3. Calcification Assays. DMEM medium initially contains 0.9 mM of Pi, 1.8 mM of Ca^{2+}, and 0.8 mM of Mg^{2+}. Calcification assays were conducted in 1% FBS DMEM, and Pi concentration was increased to reach 3 mM. The effect of Mg^{2+} on calcification was assessed at total concentrations of 1.5 or 2 mM. When indicated, the Pi and Mg^{2+} concentrations in the media were increased using NaH_2PO_4 and $MgCl_2$ supplementation, respectively.

For precise calcium (Ca^{2+}) measurements, cells were washed with PBS without Ca^{2+} and Mg^{2+} and then decalcified with 0.6 N HCl overnight. The calcium content was determined colorimetrically with the o-cresolphthalein complexone method (OCP). Briefly, the principle of this method is based on the purple colored complex formed by Ca^{2+} with o-cresolphthalein complexone in an alkaline medium. The optical density (OD) of the samples was measured with a spectrophotometer at 565 nm and compared to a curve calibrated with Ca^{2+} standards [26]. The protein content was measured using Bio-Rad protein assay reagent (Bio-Rad) according to the manufacturer's protocol. The Ca^{2+} content of the cell layer was normalized to protein content.

For alizarin red staining (AR), cells were washed with PBS and fixed with ethanol 95%. Then samples were exposed to alizarin red 40 mM (pH 4.2). After two washing steps, the wells were photographed to document mineralization.

For Von Kossa staining (VK), cells were fixed with ethanol 95% for 15 min and rinsed and incubated with 5% $AgNO_3$ for 30 min. Cells were further rinsed with water, incubated for 5 min in a photographic developer solution, washed with water, then incubated for 5 min with a 5% sodium thiosulfate solution to remove unreacted silver, washed, and finally dried. Following their acquisition with a Photometrics CH250 CCD camera, pictures of wells were processed using the ImageJ software (NIH).

2.4. Specific Controls. Media from the various experimental conditions were assessed for correct Ca^{2+}, Pi, and Mg^{2+} levels using an ADVIA 1800 Siemens autoanalyzer (Siemens Healthcare Diagnostics). No change in pH was observed at Pi 3 mM with or without addition of Mg^{2+}. This excludes a potential role of medium acidification in the observed decrease of mineralization.

In previous experiments [24], we checked whether the effects of $MgCl_2$ on calcification reduction were due to chloride ions in $MgCl_2$ salt, but addition of 2.4 mM NaCl (the maximum concentration of Cl^- used) did not inhibit calcification after 10 and 14 days of culture in presence of 3 mM Pi using AR, VK, and OCP methods (data not shown).

2.5. RNA Isolation and Quantitative PCR. Total RNA from HAVSMC was isolated with the mirVana Isolation Kit (Applied Biosystems, Life Technologies) as per the manufacturer's instructions. Total RNA was further subjected to DNAse-I digestion (Ambion, Life Technologies). For selected miRs, samples of 50 ng of RNA were reverse-transcribed in a final volume of 25 μL using the Applied Biosystems Taqman assay probes with the Taqman MicroRNA Reverse Transcription Kit according to the manufacturer's protocol. Real-time quantitative PCR (q-PCR) was run on a StepOnePlus system (Applied Biosystems) using Taqman assay probes. For selected mRNA targets (collagen 1A1, Runx2, Smad1, and Osx), samples of 50 ng of RNA were reverse-transcribed in a final volume of 25 μL using a High-Capacity cDNA Synthesis Kit (Applied Biosystems) according to the manufacturer's protocol. Real-time q-PCR was then run on StepOnePlus using 10 μM primers of the selected targets (Table 2) with the Power SYBR Green PCR Master Mix (Applied Biosystems).

The U6 small nuclear RNA and GAPDH mRNA were used as endogenous controls for miR and mRNA expression,

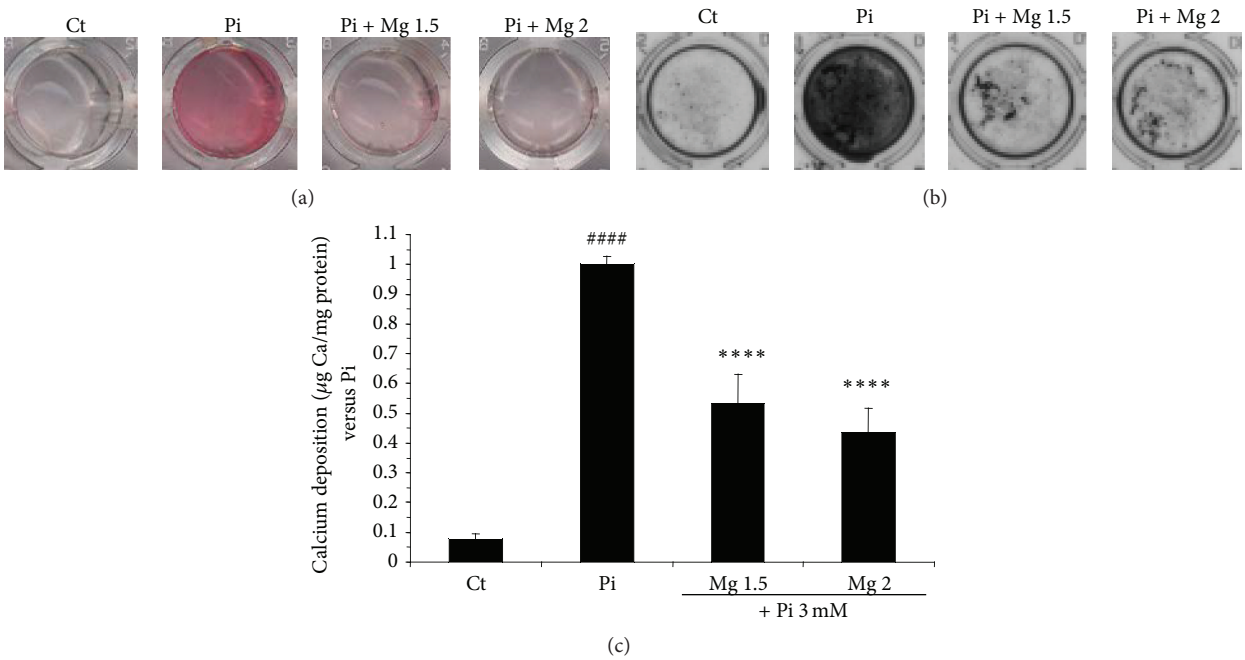

Figure 1: Magnesium prevents Pi-induced mineral deposition in HAVSMC. HAVSMC were cultured for 14 days at the indicated concentrations of Mg^{2+}, 1.5 and 2 mM (Mg 1.5 and Mg 2, resp.), and in the presence of 3 mM Pi (Pi). (a) Alizarin red staining pictures, to allow visualization of calcium deposition, are representative of the respective conditions. (b) Von Kossa staining pictures, to allow visualization of calcium deposition, are representative of the respective conditions. (c) Calcium deposition from HAVSMC of the three different donors was assessed using OCP with increasing concentration of Mg^{2+} and induction of calcification by Pi. Data are represented as a ratio of Pi and are expressed as mean ± SEM for each condition ($n = 12$). Pi 3 mM condition was set to 1. Statistics were realised with ANOVA multigroup analysis using Fisher PSLD posttest. The control condition (Ct) is significantly different from all conditions. $^{\#\#\#\#}p < 0.0001$ versus Ct and $^{****}p < 0.0001$ versus Pi.

respectively (for shown results). Alternatively, U48 small nuclear RNA and β-actin were also used as additional endogenous controls and yielded the same trend of results (data not shown).

2.6. miRNA Target Site Prediction. A search for predicted target miR was performed with the databases TargetScan (http://www.targetscan.org/), miRanda (http://www.miranda-im.org/), and PicTar (http://pictar.mdc-berlin.de/).

2.7. Statistical Analysis. All results are expressed as mean ± standard error of the mean (SEM). For calcification assays, statistical significance was determined by variance multigroup analysis (ANOVA) using the Fisher PLSD posttest. For Taqman and SYBR green q-PCR experiments, the nonparametric Mann-Whitney test was used because of the standard deviation observed between donors. A p value < 0.05 was considered to be significant.

3. Results

3.1. Mg^{2+} Reduced Pi-Induced Mineral Deposition in Tested Samples. As previously described [24], the deposition of calcified matrix occurs by rising Pi concentration up to 3 mM. As shown in Figure 1(c), Mg^{2+} significantly decreases the amount of Ca^{2+} quantified by the OCP method after 14 days of incubation with the indicated conditions. These samples were concomitantly assessed for miR and mRNA expression. Here, AR and VK staining were only performed as qualitative tests to confirm the occurrence of the calcium phosphate deposition in our samples (Figures 1(a) and 1(b)). Calcification was checked at day 14 because we previously found that this was the optimal time point to show the efficiency of Mg^{2+} to prevent VC [24].

3.2. miR-125b and miR-223 Are Not Significantly Regulated during the Course of Calcification. miR-125b expression was very stable and remained unchanged in all of the tested conditions over the time course (4 h, 24 h, day 3, day 7, and day 10) for all donors (data not shown). This result seems to be in accordance with a previous work showing that miR-125b was not significantly downregulated until day 21 under calcifying conditions [15]. In this work, the authors did not test calcification time points prior to 7 days for miR-125b regulation. Here, we demonstrate that miR-125b is regulated by Pi neither at earlier time points (4 h, 24 h, and day 3) nor at day 7 or 10 as already mentioned in [15]. Likewise, Mg^{2+} had no effect on miR-125b expression (data not shown). In a different way, miR-223 was not found to be significantly regulated in the tested conditions during the whole time course, partly due to a high variability of its expression among the donors. Anyway, Mg^{2+} did not seem to have a beneficial effect in restoring basal miR-223 expression. Thus, the data

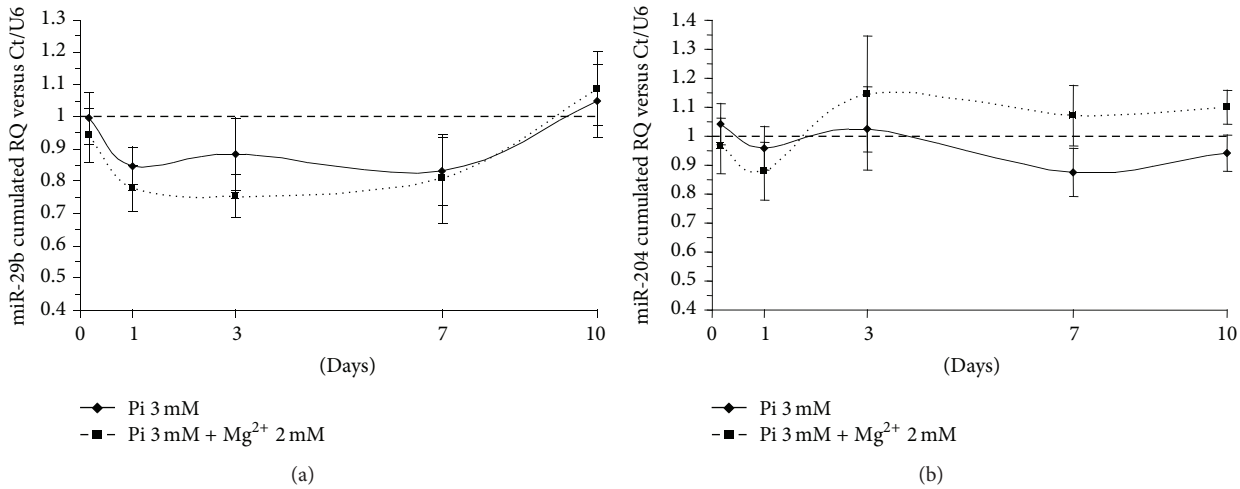

Figure 2: miR-29b and miR-204 expression is not significantly altered. Pi-induced HAVSMC were assessed at the time points 4 h, 24 h, day 3, day 7, and day 10. miR-29b and miR-204 were quantified using TaqMan microRNA assays (Applied Biosystem) on total RNA by q-PCR analysis and compared to control cells treated with basal medium. The control condition was set to 1 (dashed line). The cumulated relative quantity (RQ) of the three different donors of HAVSMC is represented as a ratio of the control condition and is expressed as mean ± SEM for each condition ($n = 6$). U6 small nuclear RNA was used as an internal reference gene. Statistics were realised with the Mann-Whitney test. No statistical significance was found.

suggest that the regulation of this miR cannot explain the beneficial effect of Mg^{2+} in attenuating VC (data not shown).

3.3. miR-29b and miR-204 Expression Is Markedly but Not Significantly Altered. The regulation profile of miR-29b shows a marked tendency to decrease in the presence of Pi (3 mM), particularly until day 7. However, the statistical significance was not reached due to the variability observed between the tested donors, meaning that the SEM is large compared to the observed variation. Adding Mg^{2+} (2 mM) in the calcifying condition (Pi 3 mM) was not effective to recover or tend to return to the basal expression of miR-29b. This is shown by the expression curves which progress tightly together (Figure 2(a)).

In Figure 2(b), the expression levels of miR-204 stay within the control range at days 1 and 3 and slightly decrease from day 7 onwards in the presence of Pi 3 mM. Conversely, miR-204 was upregulated from days 3 to 10 when Mg^{2+} (2 mM) was added to the calcifying condition. However, no significance was found between the conditions and time points because of the substantial interdonor biological variations.

3.4. Mg^{2+} Is Able to Reverse a Pi-Induced Decrease in miR-30b Expression. Data on miR-30b regulation show a significant, progressive decrease in its expression in the calcifying condition from day 3 onwards. This decrease is worsened throughout day 10, reaching higher statistical significance. Adding Mg^{2+} to the calcifying condition resulted in an initial decrease of miR-30b expression that quickly rose and then returned to the basal expression level from day 7 onwards, being nonsignificantly different from the control condition set to 1 (Figure 3(a)). The results are in line with previous publications suggesting that the miR-30b decrease was a procalcifying event [18] in human smooth muscle cells.

3.5. Mg^{2+} Potentiates miR-133a Expression and Prevents Its Pi-Induced Decrease. Regarding the time course of miR-133a regulation for the various conditions, HAVSMC cultured with 3 mM Pi led to a progressive decrease of miR-133a expression, whereas the addition of Mg^{2+} resulted in an upregulation of miR-133a (Figure 3(b)). Beginning at day 3, statistical significance is found between the Pi 3 mM and Pi 3 mM + Mg^{2+} 2 mM conditions as well as between control (set to 1, dashed line) and Pi 3 mM + Mg^{2+} 2 mM. Similar results were found at day 7. After 10 days of induced calcification, miR-133a expression levels were lowered in conditions containing Pi. However, only strong statistical significance was found between control and Pi 3 mM conditions. Thus, the addition of Mg^{2+} 2 mM only partially reverses the decrease of miR-133a. The downregulation of miR-133a is consistent with findings in [11] in which miR-133/135 were downregulated during osteogenic differentiation.

3.6. Mg^{2+} Prevents miR-143 Pi-Induced Decrease Expression. Figure 3(c) shows the time course of miR-143 regulation for the various conditions. At day 3, Pi 3 mM significantly lowered miR-143 expression, and this decrease remained stable until day 10, although not reaching statistical significance on days 7 and 10. Conversely, Mg^{2+} constantly increased miR-143 levels from the early time points until day 10 over the time course, resulting in a significant difference between the Pi 3 mM and Pi 3 mM + Mg^{2+} 2 mM conditions on day 7. A decrease of miR-143/145 is correlated with several important cardiovascular diseases; for review see [9]. Downregulation

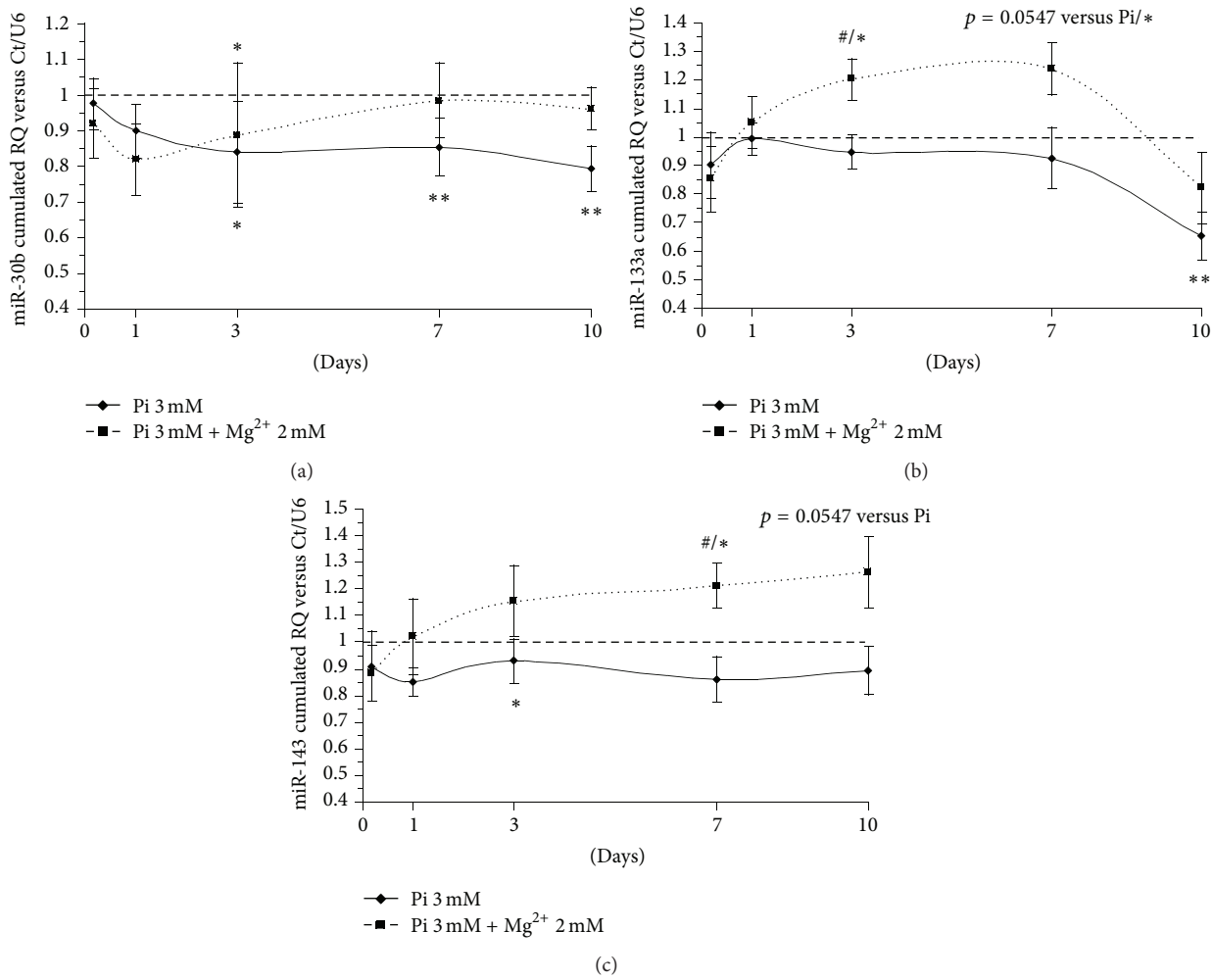

Figure 3: miR-30b, miR-133a, and miR-143 expression is deregulated by Pi and restored by Mg^{2+}. Pi-induced HAVSMC were assessed at the time points 4 h, 24 h, day 3, day 7, and day 10. miR-30b (a), miR-133a (b), and miR-143 (c) were quantified using TaqMan microRNA assays (Applied Biosystem) on total RNA by q-PCR analysis and compared to control cells treated with basal medium. The control condition was set to 1 (dashed line). The cumulated relative quantity (RQ) of the three different donors of HAVSMC is represented as a ratio of the control condition and expressed as mean ± SEM for each condition ($n = 6$). U6 small nuclear RNA was used as an internal reference gene. Statistics were realised with the Mann-Whitney test. $^{*}p < 0.05$ versus Ct, $^{**}p < 0.01$ versus Ct, and $^{\#}p < 0.05$ versus Pi.

of miR-143/145 was previously studied during HAVSMC Pi-induced calcification by our team [27], thus supporting the present results.

3.7. Expression of Collagen 1A1 and Runx2 Is Not Significantly Upregulated by Pi. We chose to present the data concerning miR specific targets at day 3 and day 10 for all the selected targets because the most marked miR modulations are observed between these two time points. As indicated in Table 1, miR-29b targets collagen 1A1 (col1A1) mRNA, a classic marker of calcification. The expression of collagen 1A1 was not significantly modulated in our experimental setup (Figure 4(a)). This result could be related to the lack of a significant change in miR-29b expression.

The expression of Runx2 mRNA is regulated by several miRs: miR-30b, miR-133a, and miR-204 (Table 1). We mentioned that miR-30b and -133a were found to be differentially expressed (Figures 3(a) and 3(b)). Corresponding Runx2 regulations were not observed. Surprisingly, Runx2 was found to be significantly downregulated at day 3, in both the presence and absence of Mg^{2+}, whereas at day 10 expression rose to lie nonsignificantly above control levels (Figure 4(b)). We could hypothesize that the rise in Runx2 expression might continue after day 10 for the following reasons. The Pi-induced downregulation of miR-30b and -133a is at its maximum at day 10. The effects of miR downregulation, however, are not seen concomitantly on the target expression. A further time point is needed at least 24 h to several days later to detect the maximum effects of miR expression on their relative mRNA targets. This suggests that the experimental setup was probably concluded too early and missed the time window that would detect a significant upregulation.

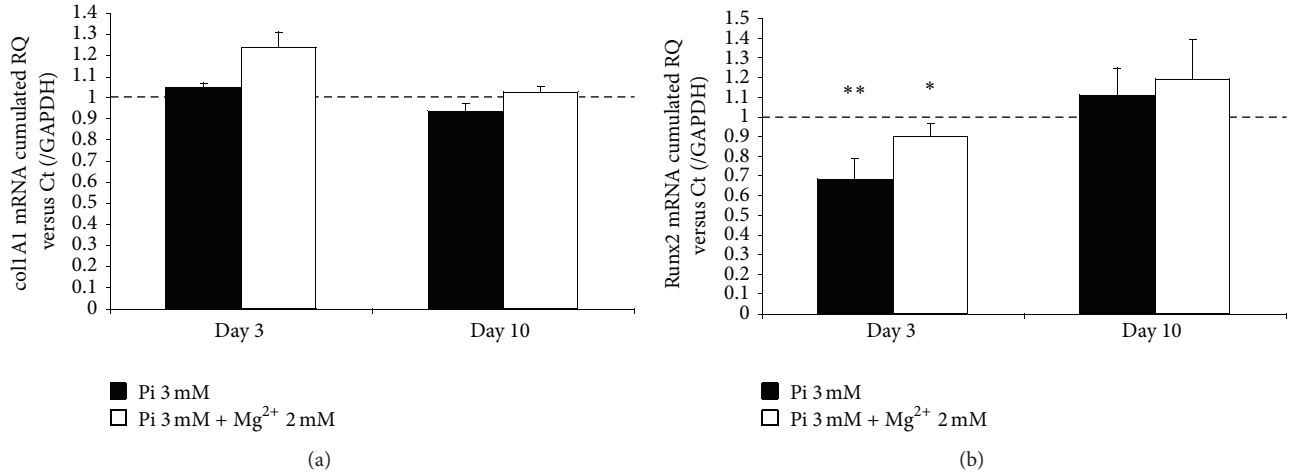

Figure 4: The expression of col1A1 and Runx2 mRNA is not markedly induced by Pi. Pi-induced HAVSMC were assessed at day 3 and day 10. Total RNA was extracted and reverse-transcribed, and expression of col1A1 (a) and Runx2 (b) was analyzed by q-PCR and compared to control cells treated with basal medium. The control condition was set to 1 (dashed line). The cumulated relative quantity (RQ) of the three different donors of HAVSMC is represented as a ratio of the control condition and expressed as mean ± SEM for each condition ($n = 6$). GAPDH was used as an internal reference gene. Statistics were realised with the Mann-Whitney test. $^{*}p < 0.05$ versus Ct and $^{**}p < 0.01$ versus Ct.

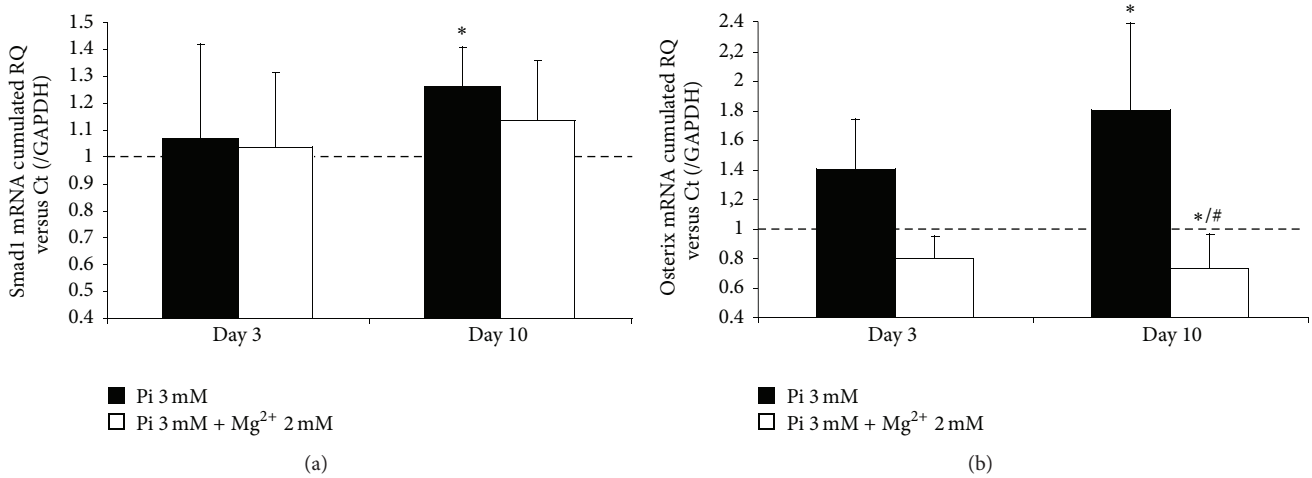

Figure 5: The expression of Smad1 and Osterix mRNA is increased by Pi induction whereas the presence of Mg^{2+} prevents an upregulation. Pi-induced HAVSMC were assessed at day 3 and day 10. Total RNA was extracted and reverse-transcribed, and expression of Smad1 (a) and Osterix (b) was analyzed by q-PCR and compared to control cells treated with basal medium. The control condition was set to 1 (dashed line). The cumulated relative quantity (RQ) of the three different donors of HAVSMC is represented as a ratio of the control condition and is expressed as mean ± SEM for each condition ($n = 6$). GAPDH was used as an internal reference gene. Statistics were realised with the Mann-Whitney test. $^{*}p < 0.05$ versus Ct and $^{\#}p < 0.05$ versus Pi.

3.8. Upregulation of Smad1 and Osx Expression Is Restored by Mg^{2+}. In a recently published study [28], a link was established between miR-30b regulation and mRNA expression of Smad1, Runx2, and caspase-3 in human primary aortic valve interstitial cells. These three mRNA were identified and confirmed as potential target genes of miR-30b (Table 1). Here, we assessed the Smad1 expression in cultured HAVSMC and found a marked and significant increase of Smad1 at day 10 in the calcifying condition (Figure 5). Conversely, in the presence of Mg^{2+}, Smad1 expression is decreased towards control level. This correlates with the observed miR-30b regulation.

A recent report established Osx as a target of miR-143 in *in vitro* osteogenesis [29]. In this work, the most prominent modulation was observed for Osx mRNA expression. As shown in Figure 5(b), Osx expression was markedly but not significantly upregulated at day 3 in the calcifying condition (by approx. 40%) and further and significantly upregulated at day 10 (by approx. 80%). The presence of Mg^{2+} abrogated the Osx upregulation, at both day 3 and day 10. Moreover,

the addition of Mg^{2+} resulted in a significant decrease of Osx expression at day 10 compared to the control and the calcifying condition (Pi 3 mM). As suggested in [29], these results are correlated and could be mirrored to miR-143 expression.

4. Discussion

The present study performed a limited miR screening based on available literature (implications in VSMC phenotypic switch, osteogenesis, or VC, Table 1) in HAVSMC primary cells. The aim was to detect miRs implicated in VC, regulated by Mg^{2+}, and subsequently to study the targets of the most relevant miRs. It reveals three main findings: (i) our screening showed a downregulation of key miRs such as miR-30b, miR-133a, and miR-143 during Pi-induced calcification of HAVSMC; (ii) osteogenesis and VC markers related to these miRs, such as Smad1 and Osterix, were found to be modulated accordingly; and (iii) Mg^{2+} had a protective effect by interfering with the Pi-induced VC process as the modulations of the affected miRs and their related targets were partially abrogated or even improved.

Based on literature and confirmed by miR databases, a selection of 7 miRs was assessed in our HAVSMC model during Pi-induced calcification at 4 h, 24 h, day 3, day 7, and day 10. Our results confirmed the implication of miR-30b in calcification and brought miR-133a as well as miR-143 from phenotypic switch and vascular remodeling into the field of VC. To briefly comment on the results of the other selected miRs, Goettsch et al. [15] found a statistically significant increase of miR-125b in human coronary artery smooth muscle cells after 21 days in calcifying conditions (10 mM β-glycerol phosphate/10 nM dexamethasone/100 μM ascorbate phosphate). In our model (HAVSMC), miR-125b expression was found to be stable for 10 days in calcifying conditions (3 mM Pi), confirming a late involvement of miR-125b in the VC process. Furthermore, the high variability among the tested donors might have prevented us from obtaining statistical significance in the Pi-induced regulation of miR-29b, miR-204, and miR-223. It seems that the rather limited number of samples could be a limitation of our study. Adding several other donors could be an option to improve the significance and clarify the involvement of the selected miRs in VC. For miR-29b, the Pi-induced expression profile resembles that presented in [13], where miR-29b was first decreased before being increased in MC3T3-E1 and rat calvaria osteoblast models of osteogenesis. For miR-204, the overall expression profile in the calcifying condition showed a constant but not significant decrease from day 3 until day 10. This decrease is similar to what was described in primary mouse aortic SMC in [17], where the expression of miR-204 constantly decreased from day 3 until day 14. For both miR-29b and miR-204, further investigations should be conducted in HAVSMC or in other human vascular models to clarify the role that these miRs as well as their putative targets play in the VC process.

Before the initiation of our study, Balderman et al. reported a decrease of miR-30b/c during BMP-2 (Bone Morphogenetic Protein-2) induced VC of human artery smooth muscle cells [18]. Knockdown of miR-30b/c established Runx2 as their preferential target during VC. Of note, Smad proteins (1/5/8) were activated by BMP-2 during the VC process. Moreover, a siRNA strategy to decrease Smad1 mRNA and consequently Smad1 protein levels prevented the decrease of miR-30b/c. To date, the whole miR-30 family has been implicated in the osteogenesis process [30]. In our experimental setup, miR-30b was found to be downregulated during Pi-induced VC, confirming previous findings. This decrease was abolished by the addition of Mg^{2+} to the calcifying medium. In the meantime, miR-30b was found to be a multifunctional regulator of aortic valve interstitial cells in calcified aortic valve disease [28]. Indeed, its levels were decreased during calcification, whereas its modulation altered Runx2, Smad1, phosphorylated Smad1/5/8, and caspase-3 protein levels; that is, target levels were increased by miR-30b inhibition and decreased by overexpression strategies.

The implication of miR-133a in a mineralization process was first described during BMP-2 induced osteoblastogenesis [11], where it was found to be downregulated. A sequence analysis indicated the presence of a putative miR-133a binding site located in the 3′ UTR of Runx2 mRNA. Inhibition and overexpression of Runx2 protein as well as other calcification markers were shown using ectopic miR-133a and anti-miR-133a, respectively. It is of note that, despite the use of ectopic anti-miR-133a, Runx2 mRNA levels remained unaffected. Lately, miR-133a was shown to be downregulated in primary murine vascular SMC calcified by the addition of 10 mM β-glycerol phosphate [31]. Thus, our data expand Liao et al. observations on miR-133a downregulation during VC to a human vascular model. Conversely, upregulation of miR-133a was found when Mg^{2+} was added to the calcifying condition.

Considering the previous studies, we decided to assess Runx2 and Smad1 mRNA expression to see the potential consequences of a miR-30b and miR-133a Pi-induced decrease. Unfortunately, we were not able to provide evidence of a significant rise in Runx2 mRNA levels, as de Oca et al. did, using a higher Pi concentration than us during similar incubation times in HAVSMC [23]. Indeed, convincingly showing significant upregulation of Runx2 mRNA expression appears to be a challenge in human VC while browsing at the literature. A main finding of our study was the demonstration of the significant Pi-induced mRNA upregulation of Smad1, another major player in the field of VC. It is now clearly established that cooperative interactions between Runx2 and BMP/Smad signaling pathways occur to induce the osteoblast phenotype [32]. Moreover, a physical interaction between Runx2 and Smad1/3/5 appears essential for osteogenic activity *in vitro* [33]. As similarly found in human valve interstitial cells [28], an increase of Smad1 mRNA expression is reported during VC in our study.

First described to contribute to the VSMC phenotypic switch [18, 27], miR-143 was recently involved in VC of uremic mice [34]. Indeed, vascular smooth muscle-specific miR-143 expression was decreased in aortas of $ApoE^{-/-}$ mice in states of atherosclerosis and/or CKD during VC progression.

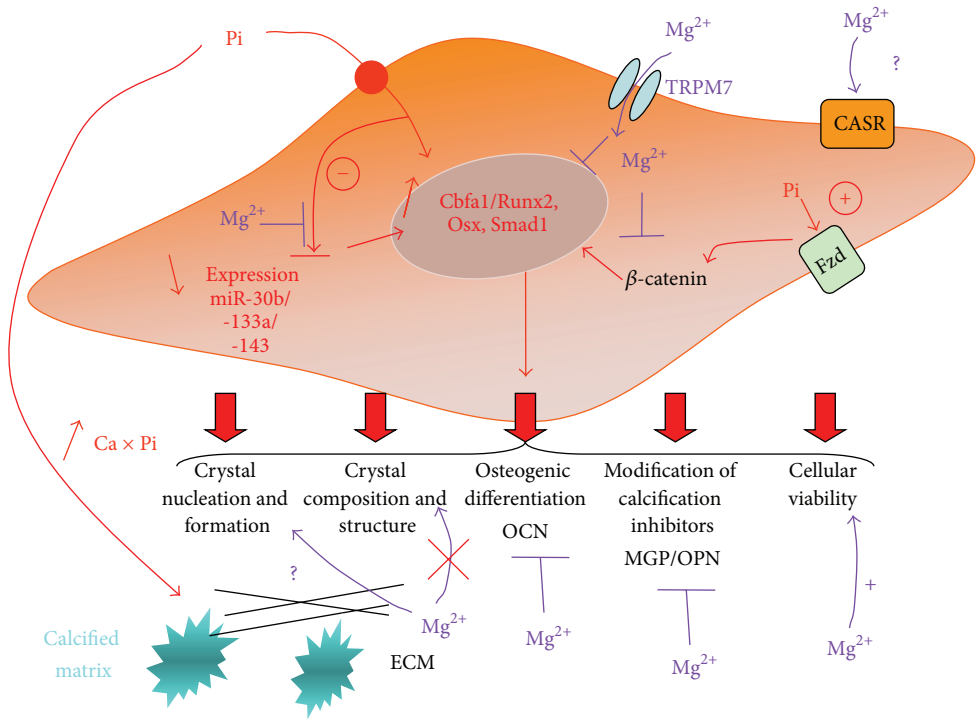

Figure 6: Multiple modes of action of Mg^{2+} in attenuating Pi-induced VC in HAVSMC. This figure summarizes all the knowledge from the previous [23, 24, 38] and the current report concerning Mg^{2+} from its entry into the cell through TRPM7 and its role in crystal composition and structure, modifications of calcification inhibitors, cell viability, osteogenic differentiation, inhibition of the Wnt/β-catenin pathway, and finally the influence of Mg^{2+} on osteogenes expression (Runx2, Osterix, and Smad1) through specific miR modulation (miR-30b, miR-133a, and miR-143). The question marks indicate undetermined mechanisms. ECM, extracellular matrix; OCN, osteocalcin; MGP, Matrix Gla Protein; OPN, Osteopontin; Ca × Pi, calcium by phosphate product; TRPM7, transient receptor potential melastatin-7; Fzd, Frizzled-3 receptor (Wnt signaling proteins); CASR, calcium sensing receptor.

During osteogenic differentiation of MC3T3-E1 cells, miR-143 had decreased levels of expression and had a role in osteogenesis inhibition. Moreover, Osx was identified to be a direct target of miR-143 [29]. Here, regarding miR-143 modulation, Osx was tested as its most important putative target in the field of VC. A prominent increase in the Osx mRNA level was found in HAVSMC after 10 days in calcifying conditions. Osx is characterized as a necessary and essential actor in mineralizing tissues [29, 32]. Parallel observations in animal models demonstrated that while Osx-knockout mice expressed wild-type Runx2 levels in osteoblasts, Osx is not expressed in Runx2-knockout mice, suggesting that Osx acts downstream of Runx2 [35]. Alternatively, additional Runx2-independent or synergistic pathways were involved [36, 37]. Recently, the functional and physical interactions between Osx and Runx2 through their respective phosphorylations by MAPK were shown to modulate the transcriptional osteogenic program [37]. Both Runx2 and Osx are able to bind to their responsive sequences on the promoters and interact with each other via regulatory regions that lead to stabilization of the transcriptional complex. In HAVSMC, de Oca et al. showed a slight and nonsignificant increase of Osx mRNA expression during VC [23]. Our results are now definitely correlating the upregulation of Osx mRNA to the Pi-induced VC in HAVSMC. Such an observation underlines the critical role of Osx in HAVSMC Pi-induced VC as part of the process in a Runx2-independent or synergistic manner.

Magnesium was tested in this study and results confirmed its beneficial role in preventing the VC process. Although previous studies already established the beneficial role of Mg^{2+} and demonstrated its ability to counteract the main calcification mediators [21–24, 38], the precise intracellular mode of action of Mg^{2+} remains elusive. Our data suggest that Mg^{2+} is able to antagonize the Pi-induced decrease of 3 miRs (miR-30b, miR-133a, and miR-143) involved in mineralization processes or SMC phenotypic switch. In HAVSMC, the decrease in these three miRs is a potential signature indicating that SMC are engaged in VC process. We hypothesize that the addition of Mg^{2+} abrogates the required inhibition of these miRs thus suppressing overexpression of two key factors of VC, Runx2 and Osx. These insights complete previous publications studying Pi-induced VC in HAVSMC [23, 24, 38]. The past and present findings are summarized in Figure 6, where the multiple modes of action of Mg^{2+} in Pi-induced VC are depicted: from its entry into the cell through TRPM7, its modulation of Ca/P crystal composition and structure, its modifications of calcification inhibitors, enhancement of cell viability, suppression of osteogenic differentiation, inhibition of Wnt/β-catenin pathway, and finally its active influence on osteogenesis (Runx2, Osx, and Smad1) through specific

miR modulation (miR-30b, miR-133a, and miR-143). *In vitro*, magnesium's influence in Pi-induced VC could be tested in additional works on initiation of the crystal formation or on a putative activation of the calcium sensing receptor (CaSR).

In summary, we found that Pi, the most prominent natural inducer of VC, was able to decrease the expression of miR-30b/miR-133a/miR-143. These modulations represent a phenotypical switch and could be seen as a signature indicating that VC is initiated in HAVSMC and, by extension, into the arterial wall. The respective mRNA targets, Osx and Smad1, were modified accordingly towards osteogenic induction. The addition of Mg^{2+} was able to restore basal levels or even to upregulate miR expression. We are now able to assert that Mg^{2+} is effective relatively early during Pi-induced VC by cancelling osteogenic gene expression through miR-30b/miR-133a/miR-143 expression reinforcement, resulting in a retention of the SMC phenotype. How Mg^{2+} selectively modulates the 3 miRs is not yet known and could be a matter of additional investigations. To date, only nonspecific roles could be attributed to Mg^{2+} in miR maturation [39–41]. Obviously, the relevance of our past and present findings on the influence of magnesium during VC needs to be confirmed in a cohort of CKD patients in a clinical setting in further works. The use of magnesium as a drug, to lower serum calcium and phosphorus, and its effect on outcomes in CKD patients was detailed in [20]. To our knowledge, magnesium-containing phosphate binders have not yet been investigated for quantitative VC reduction in a controlled, prospective clinical setting, and this step now appears necessary.

Disclosure

The funders had no role in data collection, analysis, preparation of paper, and decision to publish.

Competing Interests

Janine Büchel and Sonja Steppan are employees of Fresenius Medical Care Deutschland GmbH. Ziad A. Massy received research grants and honorarium from Fresenius Medical Care Deutschland GmbH. Loïc Louvet and Laurent Metzinger have no competing interests. This work was funded in part by a grant from the Picardie Regional Council (MIRNA).

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Research Article

Modulation of Circulating MicroRNAs Levels during the Switch from Clopidogrel to Ticagrelor

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Background. Circulating microRNAs are appealing biomarkers to monitor several processes underlying cardiovascular diseases. Platelets are a major source for circulating microRNAs. Interestingly, the levels of specific microRNAs were reported to correlate with the level of platelet activation. The aim of the present study was to test whether the treatment with the novel antiplatelet agent, ticagrelor, is associated with modulation in the levels of key platelet-derived microRNAs. *Methods and Results*. Patients were randomly selected from those participating in the SHIFT-OVER study, in which we had previously evaluated the effect of the therapeutic switch from clopidogrel to ticagrelor on platelet aggregation. Circulating levels of selected microRNAs were measured before and after the therapeutic switch from a dual antiplatelet therapy including acetylsalicylic acid (ASA) and clopidogrel to the more potent ticagrelor. Interestingly, the circulating levels of miR-126 ($p = 0.030$), miR-223 ($p = 0.044$), and miR-150 ($p = 0.048$) were significantly reduced, while the levels of miR-96 were increased ($p = 0.038$). No substantial differences were observed for the remaining microRNAs. *Conclusions*. Switching from a dual antiplatelet treatment with clopidogrel to ticagrelor is associated with significant modulation in the circulating levels of specific microRNAs. If confirmed in larger, independent cohorts, our results pave the way for the use of circulating microRNAs as biomarkers of platelets activity in response to specific pharmacological treatments.

1. Introduction

Despite the recent progress in the diagnosis and treatment, cardiovascular diseases are still the major source of morbidity and mortality worldwide. Furthermore, many pathophysiologic mechanisms still need to be disentangled, thus preventing the development of novel efficient and specific diagnostic and therapeutic strategies for a large number of patients. Platelets play an important role in the pathophysiology of cardiovascular diseases, especially in the development of their thrombotic complications, such as acute coronary syndromes (ACS) [1].

MicroRNAs (miRs) recently emerged as powerful regulators of biological processes, offering a further opportunity to better understand the biological mechanisms responsible for the development of cardiovascular diseases, including cellular function and cell-to-cell communication [2]. miRs are released into the bloodstream, offering the opportunity to monitor the biological status of the cardiovascular system through the measurement of the expression pattern of specific miRs in the blood [3, 4]. In particular, it became recently clear that platelets are a major source for circulating miRs [5]. Although muscle-enriched miRs (miR-499 and miR-133a) are released from the myocardium into the coronary circulation in ACS patients [6], miR-126, one of the most expressed platelet-related miRs [7], shows a negative concentration gradient across the coronary circulation, suggesting consumption by means of degradation, or tissue uptake or platelet adhesion/entrapment during the passage through the myocardium [6]. This latter finding is particularly interesting,

given that levels of specific circulating miRs are associated with the degree of platelet activation [7] and can be therefore potentially used as biomarkers to monitor the efficacy of antiplatelet therapy.

On the basis of these findings, the aim of the present study was to evaluate whether the treatment with the novel P2Y12 antagonist, ticagrelor, is associated with modulation in the levels of key miRs associated with platelet function.

2. Materials and Methods

2.1. Study Population. Selected microRNAs were measured from plasma samples obtained from 16 patients from the SHIFT-OVER study [8], which enrolled 50 patients that were randomly assigned in a 1 : 1 fashion to either the "no load" group (switch to ticagrelor (90 mg BID) without loading dose) or the "load" group (switch to ticagrelor (180 mg) with loading dose). In particular, 8 patients were randomly selected from each of the following study groups: "no load" (group 1), including patients that were switched from clopidogrel to the maintenance dose of ticagrelor (90 mg *bis in die*) with no administration of the initial loading dose, and "load" (group 2), including patients that received a 180 mg loading dose, plus 90 mg *bis in die* (maintenance dose). To minimize the risk for selection bias, random selection of patients was performed using the online random number generator "random.org". No statistical significant differences in clinically relevant characteristics were found between the randomly selected and nonselected patients. Patients from both groups received additional treatment with acetylsalicylic acid (ASA). Plasma samples were obtained at the following time points: (i) at baseline, when patients were on dual antiplatelet therapy including ASA 100 mg/die and clopidogrel sulphate 75 mg/die, and (ii) 24 hours after the patients had been shifted from clopidogrel to ticagrelor 90 mg *bis in die*. All patients provided written informed consent.

2.2. Blood Sampling. Blood samples were obtained through venous puncture. All blood samples were analyzed after a resting phase of 30 min. Plasma was obtained from blood samples harvested in 2.7 mL tubes containing 1.6 mg ethylenediaminetetraacetic acid/mL blood. We added 5 nmol/L *Caenorhabditis elegans* miR-39 (cel-miR-39) to the samples, during the extraction phase, to be used for normalization, as previously described [9]. Reverse transcription and quantitative (q)PCR were then performed with TaqMan microRNA assay kits, according to the instructions of the manufacturer. Values were normalized to cel-miR-39 and are expressed as $2^{-(\mathrm{CT[microRNA]}-\mathrm{CT[cel\text{-}miR\text{-}39]})}$. All measurements were run in duplicate and the mean CT values were calculated.

2.3. Platelet Aggregation Test. Whole blood platelet aggregometry was performed using a point-of-care Multiplate platelet analyzer, as previously described [8]. Briefly, 300 μL of saline was mixed with whole blood at 37°C. After 3-minute incubation, 20 μL of the agonist solution was added to obtain a final concentration of 6.4 μmol/L adenosine diphosphate (ADP). Aggregometry results are reported as aggregation units (U), representing the mean values of 2 independent measurements. ΔU indicates the difference between aggregation levels measured at T_0 (before the first administration of ticagrelor) and T_{24} (24 hours after the first ticagrelor administration).

2.4. Selection of Specific MicroRNAs. The specific microRNAs to be analyzed were selected among those that are known to be highly expressed in platelets or that had been previously associated with platelet activity. The following microRNAs were selected for the analysis: miR-233 and miR-126 are among the most highly expressed microRNAs in platelets and their levels are known to correlate with the level of platelet inhibition [5, 7, 10]; miR-150, miR-155, and miR-146a are key modulators for platelets production and activation [11, 12]; miR-96 is increased in subjects with hyporeactive platelets [13]; the levels of platelet miR-26b were found to be upregulated in patients with polycythemia [14].

2.5. Statistical Methods. Continuous variables are presented as the mean ± SD unless otherwise noted. Categorical variables were compared using the χ^2 test. Levels of circulating microRNAs between PRE and POST were compared using the Wilcoxon test (paired comparison) or the Mann-Whitney U test (unpaired comparisons). Statistical significance was assumed at $p < 0.05$. All statistical analyses were performed using SPSS software (version 20.0, Chicago, IL).

3. Results

Circulating microRNAs were measured in plasma samples obtained from 16 patients, selected from those included in the "no load" ($n = 8$) and the "load" ($n = 8$) groups from the previously published SHIFT-OVER study [8]. Baseline patients' characteristics are reported in Table 1. Levels of selected microRNAs measured in plasma samples obtained at baseline, when patients were still on dual antiplatelet therapy with ASA 100 mg/die and clopidogrel sulphate 75 mg/die (PRE), were compared to those measured 24 hours after the patients had been shifted from clopidogrel to ticagrelor 90 mg *bis in die* (POST).

Further significant inhibition of platelet aggregation was observed 24 h after the pharmacological switch from clopidogrel to ticagrelor (384 ± 154 U to 180 ± 64 U, $p < 0.001$). This effect was found both in the group of patients receiving the loading dose of ticagrelor (436 ± 148 U to 200 ± 70 U, $p = 0.008$) and in those receiving no loading dose during the switch from clopidogrel to ticagrelor (332 ± 173 U to 160 ± 54 U, $p = 0.008$). Results of platelet aggregation before and after the therapeutic switch are shown in Figure 1.

Interestingly, comparing circulating levels of microRNAs measured after the therapeutic shift (POST) to those obtained at baseline (PRE), we found a 1.8-fold reduction for miR-126 (from 0.21×10^{-7} to 0.12×10^{-7}, $p = 0.030$), a 2.1-fold reduction for miR-223 (from 0.49×10^{-6} to 0.29×10^{-6}, $p = 0.044$), and a 2.8-fold reduction for miR-150 (from 0.96×10^{-6} to 0.39×10^{-6}, $p = 0.048$). On the contrary, we observed a 2.6-fold increase (from 0.22×10^{-6} to 0.60×10^{-6}) in

Table 1: Baseline patients' characteristics.

Patients' characteristics	No load ($n = 8$)	Load ($n = 8$)
Age (mean ± SD)	61 ± 12	59 ± 7
Males/females	6/2	7/1
Family history of CVD	12.5%	12.5%
Acute coronary syndrome		
UA/NSTEMI (%)	37.5%	62.5%
STEMI (%)	62.5%	37.5%
Hypertension (%)	37.5%	37.5%
Multivessel disease	87.5%	100%
Diabetes mellitus (%)	25%	50%
Smokers (%)	62.5%	25%
Previous AMI (%)	12.5%	25%
Chronic kidney disease (%)	0%	0%

UA: unstable angina; NSTEMI: non-ST segment elevation acute myocardial infarction; STEMI: ST segment elevation acute myocardial infarction; AMI: acute myocardial infarction.

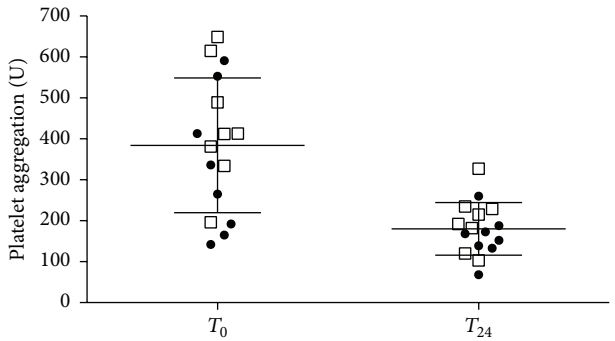

Figure 1: Results of platelet aggregation before (T_0) and 24 h after (T_{24}) the therapeutic switch from clopidogrel to ticagrelor. Rounded symbols indicate platelet aggregation levels in patients from the "no load" group, while squares represent patients from the "load" group.

the circulating levels of miR-96 ($p = 0.038$). Levels of these microRNAs at PRE and POST are displayed in Figure 2. On the other hand, no substantial differences in the circulating levels of miR-26b, miR-155, and miR-146a or the non-platelet-associated miR-190 were observed (all p = ns).

Of note, no significant differences were found in delta values (POST-PRE) between the two study groups (no load versus load) for miR-223 ($p = 0.234$), miR-150 (p = ns), miR-96 ($p = 0.202$), miR-155 ($p = 0.250$), or miR-26b ($p = 0.400$). On the contrary, a major decrease in miR-126 levels (3.6-fold versus 1.2-fold decrease; $p = 0.002$) was found in the group of patients randomized to receive a loading dose (load), as compared to group 1 (no load).

4. Discussion

The present study characterized the modulation in the circulating levels of specific microRNA, known to be associated with platelet function and/or activity. In fact, we found that plasma levels of some microRNAs were significantly decreased (miR-126, miR-223, and miR-150), while the levels of other molecules were increased (miR-96).

Very recently, a selective decrease in the plasma levels of specific microRNAs was reported in healthy volunteers and confirmed in patients on low-dose ASA treatment, after the administration of prasugrel [7]. Our results extend those observations to a hard clinical setting, with patients with acute coronary syndrome being already on dual antiplatelet therapy. In addition, while Willeit et al. evaluated the response to prasugrel [7], we demonstrated that a similar effect on circulating microRNAs can be observed with increasing intensity of platelet inhibition using ticagrelor (previous observation). This latter point makes our findings and previous ones more interesting, since they suggest that the same panel of microRNAs could be used to monitor the level of platelet inhibition, independently of the pharmacological agent administered to the patient.

Also interestingly, the same microRNAs that we found to be significantly decreased after treatment with ticagrelor in the present study had been associated with the risk of acute myocardial infarction in previous studies [15].

In addition to the potential use as biomarkers, the observed modulation in the levels of specific microRNAs in response to increasing platelet inhibition in patients with acute coronary syndrome represents a further confirmation of the pathophysiological role played by platelet-derived microRNAs in cardiovascular diseases. For example, we previously observed a negative concentration gradient across the coronary circulation of miR-126, suggesting platelet adhesion/entrapment in the myocardium. These results, together with the finding of high levels of miR-126 in human platelets, led to the hypothesis that it could play a role in the development of cardiovascular disease. According to this hypothesis, it was recently demonstrated that platelet-derived microparticles are able to deliver quanta of miR-126 to macrophages, with consequent shift of their functional profile towards a phagocytic phenotype [16]. Consequently, the reduction in the circulating levels of platelet-derived miR-126 could have a major impact on cardiovascular diseases. In this regard, our finding of a more pronounced reduction of miR-126 levels after the therapeutic switch to ticagrelor in the group of patients randomized to receive a loading dose (load) is particularly intriguing, since it suggests that measurement of microRNAs could provide additional information over platelet aggregation tests.

It was recently reported that platelet hyperreactivity is associated with an increased expression of VAMP8/endobrevin, a v-SNARE with a key role in platelet degranulation. Interestingly, VAMP8 is a known target of miR-96 [13]. Hence, the increase in miR-96 levels that we observed in POST samples, after the switch to the newer and more efficient P2Y12 inhibitor, is quite interesting and, together with previous evidence that miR-96 targets VAMP8, suggests the involvement of this specific microRNA in the modulation of platelet activation.

The complex but interesting relationship between the levels of platelet-derived miR-223 and the degree of platelet inhibition is well worth a comment. In fact, since miR-223

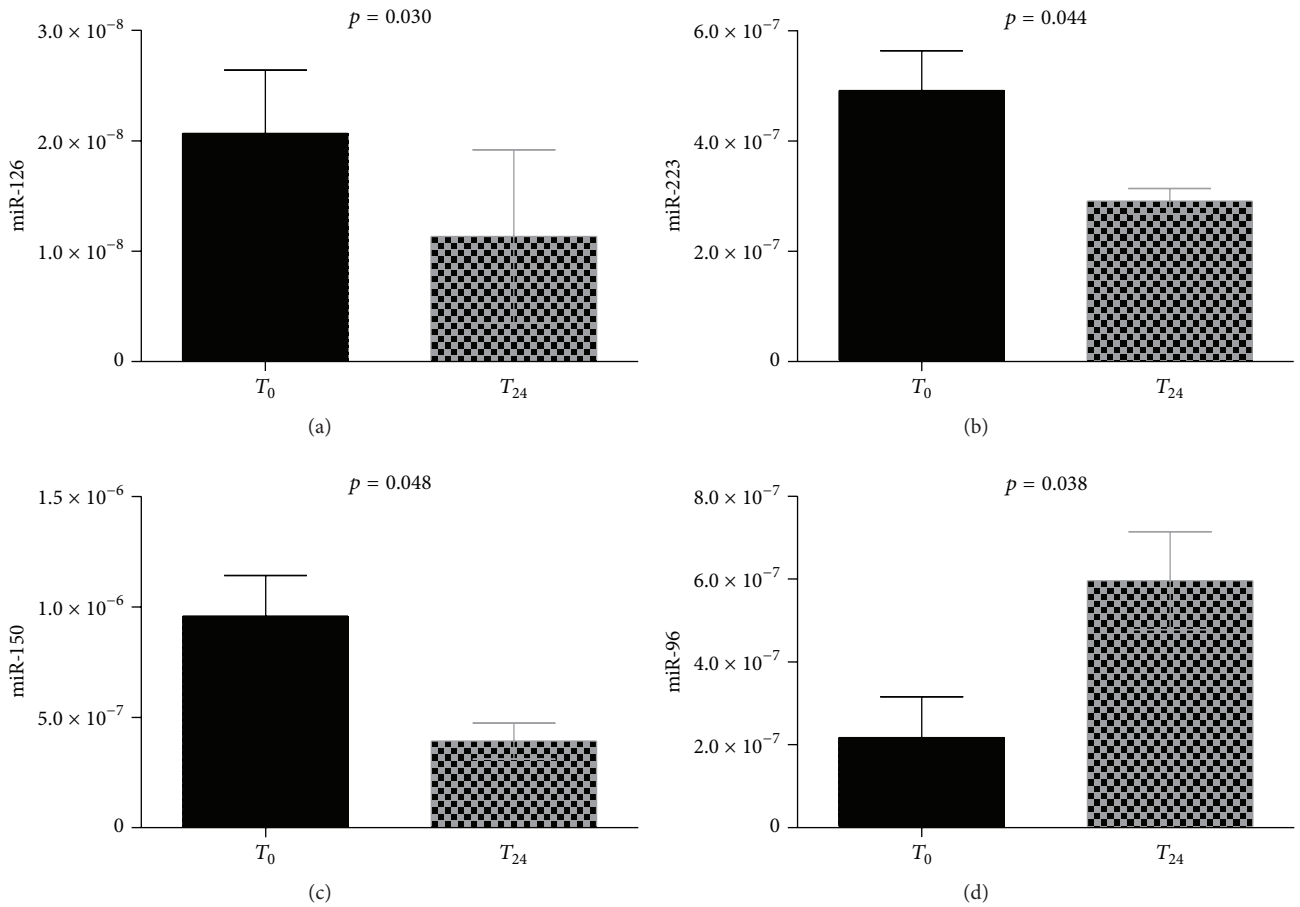

Figure 2: Levels of microRNAs. The bar graphs report mean and standard deviation values for miR-126 (a), miR-223 (b), miR-150 (c), and miR-96 (d) aggregation before (T_0) and 24 h after (T_{24}) the therapeutic switch from clopidogrel to ticagrelor.

targets the gene for the ADP receptor P2Y12, it plays a key role in platelet activation and responsiveness to therapy. In fact, miR-223 levels were associated with high on-treatment platelet reactivity [10, 17]. However, for the same reasons, miR-223 is seemingly not an optimal marker for platelet reactivity, since its levels not only depend on the degree of stimulation/inhibition of the P2Y12 ADP receptor, but also modulate its expression levels.

Though very appealing, some issues still limit the introduction of circulating microRNAs into clinical practice. Potential limitations of their use include the intrinsic complexity associated with the use of qPCR for the measurement of microRNA levels in plasma. In fact, the relatively low expression level for some microRNAs, the absence of a well-validated "housekeeping" microRNA for normalization, and the elevated costs and long analytical times currently hamper the use of circulating microRNAs as biomarkers in clinical practice. However, several alterative detection methods are being developed and tested with varying results. Among the others, the use of nanosensor in association with microfluidic filters could allow the reliable and fast label-free detection of specific microRNAs with substantially lower costs compared to currently used methods [18, 19]. It is known that platelet microRNAs have good intraindividual stability, with larger interindividual variability [20]. The results reported in the present study are based on paired analyses of two different measurements obtained from the plasma of the same patient at two different time points. Consequently, the effect of the switch to a more potent antiplatelet agent on circulating microRNAs in the present study was intrinsically normalized for individual baseline levels. In this respect, it is not predictable whether a single measurement could be actually helpful in individual patients. Furthermore, despite random selection of patients from the SHIFT-OVER population and application paired analysis that reduces the influence of moderator variables, the risk for selection bias cannot be excluded, given the limited sample size. Though intriguing, findings of the present study do not represent conclusive evidence on the most appropriate drug posology in the clinical setting. Future studies should be specifically designed to evaluate this aspect.

In conclusion, switching from a dual antiplatelet treatment with clopidogrel to ticagrelor is associated with significant modulation in the levels of specific platelet-associated circulating microRNAs. If confirmed in larger, independent cohorts, our results pave the way for the use of circulating

microRNAs as biomarkers of platelets activity in response to specific pharmacological treatments.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Annarita Carino and Salvatore De Rosa contributed equally to the present work.

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Review Article

The Discovery of Novel Genomic, Transcriptomic, and Proteomic Biomarkers in Cardiovascular and Peripheral Vascular Disease: The State of the Art

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Cardiovascular disease (CD) and peripheral vascular disease (PVD) are leading causes of mortality and morbidity in western countries and also responsible of a huge burden in terms of disability, functional decline, and healthcare costs. Biomarkers are measurable biological elements that reflect particular physiological or pathological states or predisposition towards diseases and they are currently widely studied in medicine and especially in CD. In this context, biomarkers can also be used to assess the severity or the evolution of several diseases, as well as the effectiveness of particular therapies. Genomics, transcriptomics, and proteomics have opened new windows on disease phenomena and may permit in the next future an effective development of novel diagnostic and prognostic medicine in order to better prevent or treat CD. This review will consider the current evidence of novel biomarkers with clear implications in the improvement of risk assessment, prevention strategies, and medical decision making in the field of CD.

1. Introduction

Cardiovascular disease (CD) and peripheral vascular disease (PVD) are leading causes of mortality and morbidity in western countries. CD and PVD impose also a huge burden in terms of disability, functional decline, and healthcare costs. CD includes ischemic heart disease, hypertension, heart failure, and cerebrovascular disease and PVD includes arterial and venous disease [1–3].

In the last decades great progresses have been made in the treatment of these diseases; nevertheless, mortality and morbidity remain high, and trusting only in cardiovascular risk factors knowledge, we are also unable to effectively predict which subjects are at real risk of getting these kinds of health problems [4].

For these reasons, great efforts have been made, in recent years, to identify several biomarkers with diagnostic and prognostic implications as well as for primary or secondary prevention purpose [5].

In the postgenomic era in biomedical research, the biomarker research focused on the role of genes (genomics) including the understanding of gene transcriptional regulation (transcriptomics), the biochemical functions of all the gene products and their interactions (proteomics), and learning how they influence cellular biochemistry, metabolism, and disease development [6, 7].

The aim of this review is to summarize the most frequently studied novel biomarkers with clear implications in the improvement of risk assessment, prevention strategies, medical decision making, and clinical outcomes and also in their ease of use in daily clinical practice.

2. Literature Search

Based on the large amount of evidences existing in the field of biomarkers, we decided to search for relevant articles in three main areas of interest: genomics, proteomics, and

transcriptomics in CD and PVD. PubMed, Scopus, and ScienceDirect databases were used for the search strategy.

3. Genomic Biomarkers

Recent genome-wide association studies (GWAS) identified several well-replicated single-nucleotide polymorphisms (SNPs) associated with coronary artery disease (CAD), but only modest improvements in CAD risk prediction were actually seen through the use of genetic risk scores based on multiple SNPs. Therefore, it has been suggested that many more SNPs are needed to effectively identify a substantial genetic risk for CAD [8–10].

Heritability of hypertension ranges from 31% to 68% and there is also an evident difficulty in identifying genes for this condition as a large number of candidate genes have been tested for association with hypertension without convincing results, with a reported 50% survival four years after diagnosis [11].

Heart failure (HF) has important genetic implications. Actually, while HF is often caused by CAD, hypertension, diabetes, and valvular heart disease, several studies suggested that the risk of HF depends also on genetic predisposition but due to the highly variable expressivity and penetrance of genetic alterations, this field needs further investigations [12].

Some studies investigated carotid artery atherosclerosis (CAA) from a genetic point of view: Lan et al. [13] studied the relationship between polymorphisms in the macrophage migration inhibitory factor (MIF) gene and the severity of CAA in a cohort of Taiwanese patients with ischemic stroke. MIF is a cytokine that was originally isolated from T lymphocytes and identified as an inhibitor of the random migration of macrophages and it is also constitutively expressed by smooth muscle cells and endothelial cells in normal blood vessels. The study demonstrated that polymorphisms in the MIF gene promoter were associated with CAA severity in ischemic stroke patients and these genetic variants may serve, in the next future, as markers for an individual's susceptibility to CAA.

Biscetti et al. [14] investigated the distribution and the interaction between gene polymorphisms encoding proinflammatory molecules in a population with internal carotid artery stenosis (ICAS). They found that the following genes and their variants, interleukin- (IL-) 6 (IL-6), IL-1β, monocyte chemoattractant protein-1 (CCL2), macrophage inflammatory protein-1α (MIP-1α/CCL3), E-selectin (SELE), intercellular adhesion molecule 1 (ICAM1), and Matrix Metalloproteinase-3 (MMP-3) and MMP-9, were independently and significantly associated with ICAS.

Family studies show that heritability of peripheral arterial disease (PAD) ranges from approximately 20% to 45% after adjusting for atherosclerotic risk factors but to date no definitive genetic markers have been identified for PAD. A relatively small number of case-control studies have assessed the associations of specific gene polymorphisms with the presence of PAD. However, results coming from these studies have not established a consistent genetic marker for PAD [15, 16].

Genetic influences may play a crucial role in the onset of arterial aneurysm, and the most genetically studied region of aneurysm formation is the thoracic aorta where several genes have been identified as predisposing factor for aneurysm and also dissection.

For thoracic aneurysms and dissection, there is some evidence that, despite predicting the risk for the onset, routine genetic screening may also provide information that enables genetically personalized care for affected patients. Genetic alterations of Transforming Growth Factor (TGFB), Transforming Growth Factor Beta Receptor 1 (TGFBR1), TGFBR2, small mother against decapentaplegic 2 (SMAD2), and Transforming Growth Factor Beta 2 (TGFB2) genes seem to be pivotal, as they may be responsible for both syndromic and nonsyndromic ascending thoracic aortic aneurysm and dissection [17].

For abdominal aortic aneurysm, a recent systematic review and meta-analysis [18] evidenced how in the last years, due to uncorrected multiple testing and flexible study design, several apparently false associations have been reported. This article showed several supported associations for lipoprotein metabolism genes polymorphisms such as low-density lipoprotein receptor-related protein 1 (LRP1), low-density lipoprotein receptor (LDLR), Sortilin 1 (SORT1), and lipoprotein(a) (LPA). Other gene polymorphisms, such as the ones found for interleukin-6 receptor (IL6R), Matrix Metalloproteinase-3 (MMP-3), Angiotensin II Receptor, and Type 1 (AGTR1), seem to be also involved.

While several genetic models have been studied and proposed to explain the heritability of intracranial aneurysms (IAs), at the moment, no diagnostic test based on genetic knowledge is currently available to effectively identify patients who are at higher risk for developing IAs [19].

On the venous side, recent evidences showed that the gene polymorphisms prothrombin (factor II) G20210A and factor V Leiden not only increase the already well-known risk for venous thromboembolism (VTE) deep vein thrombosis (DVT) and pulmonary embolism (PE) but also raise the risk for cerebral venous thrombosis, a stroke subtype that is particularly more common in women, especially of reproductive age [10, 20].

Chronic Venous Disease (CVD), Varicose Veins (VV) and its more severe manifestation, Chronic Venous Insufficiency (CVI), with its main complication, and Chronic Venous Ulceration (CVU) were widely studied: Serra et al. showed that a functional variant of FOXC2 gene may account for a predisposition for VV [21].

A recent study demonstrated that regulatory genes of arachidonic acid metabolism and mediators of the inflammatory reaction, hydroxyprostaglandin dehydrogenase-15 (HPGD), are overexpressed in CVI patients. The study also showed that regulatory genes of collagen production (collagen type 13α1 and collagen type 27α1 genes) were downregulated in veins affected by superficial reflux disease [22].

Jin et al. showed the association between an insertion and a deletion polymorphism (rs3917) within the 3′ untranslated region of COL1A2 (alpha-2 type I collagen gene) causing

a genetic variation in the COL1A2 gene that seems to influence the possibility of developing CVI [23].

A further candidate gene which was studied is thrombomodulin, a marker of endothelial injury [24, 25]. The hemochromatosis C282Y gene mutation has been associated with the onset of CVU by Zamboni et al.; in fact this group showed how this mutation was more frequently associated with skin changes in patients with CVI [25, 26]. While these studies are promising, the samples sizes were small, the studies were unpowered, and they lack also replication and, therefore, the area of the genetic basis of venous disease needs further investigation [25].

4. Transcriptomic Biomarkers

Since several decades, the clinician has access to numerous tools for diagnosis and therapeutic follow-up of most known pathologies. In today's medicine, progress in molecular biology techniques allows studying now in one single experimentation a given family of molecules. Transcriptomics is the technique that allows the study of RNA. The most studied RNA group is represented by the messenger RNAs (mRNAs) which are defined as ribonucleotide sequences complementary of the coding strand of the DNA genes. In this instance, the RNA group is called the transcriptome. However, there exists other RNA types in the cell, such as ribosomal RNAs which constitute 80% of total RNAs and are instrumental for translation of mRNA sequence into proteins. A whole wealth of other noncoding RNAs has also appeared in the last 15 years and we will discuss their use later in this chapter.

The cDNA microarrays allow simultaneous measurement of several tens of thousands of genes in a given sample using hybridization of retrotranscribed RNA. The resulting cDNAs are labelled and amplified on specific probes fixed to the array (also known as chip or biochip). These studies are usually performed by comparing the transcriptome of two populations (typically, a control, healthy group is compared to a diseased group) to detect a signature of genes specific for a given disease. Cyanide dyes, such as Cy3 and Cy5, are used to give to each sample a specific color. Differential expression is analyzed using different statistical analysis processes.

Early transcriptome analyses have established catalogs of genes expressed preferentially in specific cells or tissues. To cite one of the most ancient studies, Piétu et al. have published the existence of genes expressed only in skeletal muscle [27]. Usual applications include also the measurement of kinetic response to a specific effector/drug and the clustering analysis of genes responding with a similar pattern. Transcriptomic studies can also permit the characterization of regulatory pathways in given tissues, at various stages of development. These expression profiles can, in turn, help to establish a "gene signature," characteristic for a pathological sample, such as a tumor. Transcriptomics can also be useful to provide prognostic predictor values. Expression profiles, for this instance, are derived from the comparison of two groups of patients sharing several criteria but different in one specific clinical feature, such as, for example, hypertensive and normotensive state. In fact, comparing the two groups will help identify genes differentially expressed between the two groups to establish a prognostic signature.

In the first effort to obtain a global portrait of gene expression in the failing heart, Barrans and colleagues [28] constructed a human cardiovascular cDNA microarray containing approximately 10 000 genes and selected 38 genes showing a minimum of twofold differential expression, in the diseased tissue. Gaertner et al. [29] performed an elegant transcriptomic study that compared the transcriptomes of myocardial samples taken from the left ventricle (LV) and right ventricle (RV) of 13 terminal cardiomyopathy patients from their heart-transplantation program. Of these, six had arrhythmogenic right ventricular cardiomyopathy, whereas seven had idiopathic dilated cardiomyopathy, without signs of familial etiology or coronary artery disease. Furthermore, they compared the data with six nonfailing donor hearts. They used the Affymetrix HG-U133 Plus 2.0 arrays to provide coverage of the whole human genome with 47,000 transcripts and variants of 38,500 characterized human genes and 54,000 probe-sets. They found that only ~15-16% of the genes was commonly regulated in the failing myocardium compared to nonfailing samples. In addition, arrhythmogenic right ventricular cardiomyopathy and idiopathic dilated cardiomyopathies were clearly distinct on the transcriptome level. Comparison of the expression patterns between the failing RV and LV using a paired t-test revealed a lack of major differences in ARVC (arrhythmogenic right ventricular cardiomyopathy) hearts. Note that this transcriptomic study on myocardial samples may provide important information on the changes occuring in diseased hearts but is not useful in predicting susceptible individuals but may still give clues to predict more precisely susceptible individuals. Huan et al. [30] performed a meta-analysis of results deriving from six studies of transcriptomic profiles of blood pressure (BP)/hypertension in the blood of 7017 individuals not receiving antihypertensive drug treatment. Thirty-four genes, mostly involved in inflammatory response and apoptosis pathways, were found to be differentially expressed in relation to BP, including FOS, a leucine-zipper transcription factor, and PTGS2, implicated in prostaglandin biosynthesis, that were already involved in hypertensive processes. Authors estimated that their analysis could explain 5%–9% of interindividual variance in BP.

Transcriptomics can also help to investigate the gene regulation involved in therapeutic approaches. Lam et al. [31] looked at the effect of six-month calorie restriction (CR) and/or structured exercise on the transcriptome of abdominal subcutaneous fat in a cohort of 24 overweight patients. They found that CR elicited more regulatory changes (88 genes impacted, including 27 transcription/translation regulators interestingly) than structured exercise (39 genes impacted, including only one transcription/translation regulator). This may explain at least in part the preponderant effect of CR on delaying primary aging. Others have looked at the roles of differentially expressed mRNAs in venous diseases. Zhou et al. [32] have used microarray approaches to explore the molecular mechanisms and potential biomarkers of single venous thromboembolism (SVT) and recurrent venous thromboembolism (RVT) in the blood. They found

that at least 22 mRNAs were deregulated. They were implicated in translational elongation, cell proliferation, ribosomal pathways, negative regulation of heart contraction, and protein export. The authors concluded that all these genes are potential biomarkers of SVT and RVT. Once relevant genes are identified using systemic approaches, one can look at polymorphisms of their sequences and study the relevant differences between patients and healthy individuals. For instance, Deser et al. [33] have studied polymorphisms of IL-18 in peripheral blood mononuclear cells from peripheral artery disease patients. They found that IL-18 mRNA levels may be an important marker for peripheral artery occlusive diseases, as they are correlated with several biochemical markers such as triglyceride and cholesterol levels.

These are, of course, only representative examples of the numerous studies using microarrays in the cardiovascular field (a PubMed search using the terms "cardiovascular + microarray + transcriptome" brings 249 hits). However, it is important to note that the main current flaw in the microarray technique is that it is difficult to compare data between studies due to the lack of standardization in platform fabrication, assay protocols, and analysis methods. Also, since most of the findings are recent, the various mRNAs described in this review have not yet been reproduced by multiple researchers and further work will be clearly needed to establish if they may function as effective biomarkers in the CD field.

A growing number of publications show that microRNAs (miRNAs) are new solutions to identify biomarkers in the serum and the pathological tissues. A vast number of them use RNA-based microarray approaches specifically developed for these small RNAs, with the generic term of RNomics. The miRNAs are short noncoding RNA of 18–24 nt. This class of small RNA is involved in regulating gene expression at the posttranscriptional level by translational repression or degradation of the target mRNA. The miRNAs regulate several biological processes and then are implicated in several pathologies. As an example, Li et al. [34] have shown that a signature of seven miRNAs is sufficient to predict overall survival and survival without relapse in a Chinese population suffering from stomach cancer. Numerous studies demonstrate an alteration of miRNA expression in CD [35], particularly in the serum. Indeed, Mitchell et al. [36] demonstrated, for the first time, the presence of circulating miRNA in human plasma. This discovery was surprising as plasma is known to have high RNase activity [37]. These circulating miRNAs have many origins and way of transport; they could be released from cells by passive leakage due to injury or chronic inflammation (in this case the way of transport is unknown); the miRNAs could be also released by active secretion via cell-derived membrane vesicles or released by a protein miRNA complex like argonaute 2, nucleophosmin-1 (NMP1), or lipoprotein (HDL). Cell-derived membrane vesicles and ribonucleoproteic complex confer the stability and the protection against RNase activity to these circulating miRNAs. Many studies have demonstrated a correlation between plasma miRNA and diseases, establishing them as potential noninvasive biomarkers of several pathologies mainly in cancer and cardiovascular diseases [38]. Concerning CD, an early increase of miR-208a, miR-133, and miR-1 is observed in plasma of patients suffering of myocardial infarction [39]; in essential hypertension miR-let-7e is increased and miR-296-5p is decreased in patients' plasma [40].

Patients with coronary artery disease or diabetes display reduced levels of endothelial-enriched miRNAs, such as miR-126 [41]. Embolization of carotid stenotic plaques is the direct cause of stroke in nearly 20% of cases. The genetic mechanisms and especially the roles played by miRNAs in the regulation of plaque destabilization and rupture are mostly unknown. We compared the expression of seven miRNAs allegedly involved in plaque growth and instability between symptomatic and asymptomatic human carotid plaques. Six miRNAs were significantly overexpressed in symptomatic versus asymptomatic plaques, and one (miR-125a) expression was significantly inversely correlated with the circulating level of low-density lipoprotein cholesterol in the symptomatic group. This suggests a potential regulatory role for these miRNAs in evolution of the plaque towards growth, instability, and rupture. Studies based on larger sample sizes are required to determine the potential use of miRNAs as biomarkers or therapeutic targets for stroke [42]. AF is a complex arrhythmic atrial disease with important cardiovascular and systemic consequences. Sardu et al. [43] have studied the potential involvement for miRNAs to be biomarkers for their changes after atrial fibrillation catheter ablation and have shown that several miRNAs are deregulated, opening new perspectives for clinical evaluations and therapeutic management strategies in the context of an arrhythmic disease. The miRNAs are also important as novel biomarkers for arterial aneurysms and peripheral artery diseases. Zhang et al. have measured miRNAs in plasma samples from 10 abdominal aortic aneurysm (AAA) patients and 10 healthy controls by microarray and confirmed the most differentially expressed miRNAs in a training cohort of 120 subjects, including 60 AAA patients and 60 normal controls [44]. They concluded that miR-191-3p, miR-455-3p, and miR-1281 are the most useful diagnosis biomarkers for AAA. On the other hand, Stather et al. [45] used whole-blood from 15 AAA male patients and ten healthy volunteers and confirmed the results using RT-q-PCR in peripheral blood and plasma samples from a cohort of 120 patients. They found that the most deregulated miRNAs in their hand were let-7e, miR-15a, miR-196b, and miR-411. These results and others are summarized in a recent review, focusing on miRNA expression modulation during AAA initiation and propagation [46]. Various efforts have been done to study the roles of differentially expressed miRNAs in venous diseases. We have shown that several miRNAs are dysregulated in the cerebral microvasculature in the CKD context [47], highlighting a possible role for these miRNAs in the appearance of cerebral strokes common in CKD patients. Very recently, a pilot study involving 20 patients suffering from unprovoked venous thromboembolism has shown that plasma levels of miR-103a-3p, miR-191-5p, miR-301a-3p, and miR-199b-3p were downregulated in plasma of patients, while the levels of miR-10b-5p, miR-320a, miR-320b, miR-424-5p, and miR-423-5p were upregulated.

Another category of players, a heterogeneous group of long transcripts that regulate gene expression at transcriptional and posttranscriptional levels called long noncoding RNAs, has now entered the field [48]. As is usually the case, lncRNA biomarkers were first described in studies performed in various cancers [49, 50]. However, such clinical studies were until recent times never performed in cardiovascular diseases. A recent study [51] indicated that the myocardial transcriptome is dynamically regulated in advanced HF and the expression profiles of lncRNAs can discriminate failing hearts of different pathologies. Using a microarray (arraystar lncRNA array) focused on 33,045 human lncRNAs, Kumarswamy et al. [52] studied the plasma RNA from patients with or without left ventricular remodeling after myocardial infarction. They identified the mitochondrial lncRNA LIPCAR as a potential biomarker of development in myocardial infarction patients, with additional association with cardiovascular death, independent of other predictors. It is now increasingly clear that lncRNAs have an important regulatory role in cardiac physiopathology and might be innovative biomarkers of cardiovascular disease development and prognosis [53].

5. Proteomic Biomarkers

Proteomics is the study of proteins expressed by a genome of an organism that are involved in both physiologic and pathologic conditions. Proteomic analysis may provide the opportunity to understand the pathophysiology of disease and it also may permit us to evaluate candidate biomarkers in tissues of human beings as well as to identify therapeutic targets and, thus, individualized therapies. Although recent research evaluated several systematic changes in protein expression in response to intrinsic or extrinsic perturbations in the field of cardiovascular disease, yet there is no enough validation for the majority of these candidates to effectively serve as sensitive and specific biomarkers for clinical application [54].

Based on the amount of evidences existing in the current and updated literature we selected those biomarkers dealing with the extracellular matrix (ECM) in cardiovascular tissues as the ECM constitutes more than half of the wall mass and vascular wall. Moreover, matrix protein synthesis and metabolism are strictly related to blood vessel structure, homeostasis, and functions. In fact, ECM is a highly dynamic structure that continuously undergoes remodeling mediated by several matrix-degrading enzymes, and, thus, variations in the composition and structure of ECM may affect the overall structure, biomechanical properties, and cell response of several organs and biological system. In this context, Matrix Metalloproteinases (MMPs) are a family of proteolytic enzymes that are regulated by inflammatory signals to mediate changes in ECM [55, 56].

Friese et al. identified several patterns of plasma MMPs in hypertension with overexpression of MMP-9 in the early manifestations of the disease and then elevation of MMP-2 and MMP-10 linked to target organ damage [57].

The progression of ventricular remodeling, after a myocardial infarction or other conditions, such as viral injury, is also mediated through MMP activation that subsequently causes the breakdown of the collagen and the elastin framework. This process leads to ventricular hypertrophy and dilation with subsequent congestive heart failure [58].

There is increasing evidence that alterations in the MMPs regulation and expression may be directly implicated in arterial and venous disease. Significant progress has been made in identifying the changes in the levels and activity of MMPs in several physiological and pathological conditions [59].

Increased activity and/or loss of control of MMPs have been related to vascular remodeling [60]. In fact MMPs play also a role in vascular smooth muscle (VSM) cell migration and neointima formation after localized vascular injury. MMPs may also promote aneurysm formation and aneurysm rupture. Varicose Veins development may be mechanistically similar to aneurysm development and MMPs may, in this way, contribute to vein wall weakness and subsequent vein dilation. The specific role of MMPs in venous ulcers pathogenesis remains unclear; however, MMPs hyperactivity and dysregulation seem to inhibit wound healing via excessive basement membrane degradation leading to loss of epidermal integrity [60–62]. In this context, MMP-1 and MMP-8 seem to be primarily involved with chronic or irreversible complications of vascular disease (such as difficult-to-heal or infected venous ulcers and postthrombotic syndrome) as some recent studies performed on plasma, wound fluid, and tissue sample concentrations demonstrated [63–65].

Recent studies have shown that MMP-9, an endopeptidase capable of degrading the extracellular matrix, is thought to be particularly associated with atherosclerosis and plaque rupture. Therefore, MMP-9 is considered to be an important mediator of vascular remodeling and plaque instability. The MMP-9 action is also enhanced by neutrophil gelatinase-associated lipocalin (NGAL), also known as lipocalin-2, a 25 kDa glycoprotein, that is, found in the granules of human neutrophils. In fact NGAL was shown to be able to bind and stabilize MMP-9 [66].

The formation of a complex with NGAL and MMP-9 seems to be crucial for atherosclerotic plaque erosion and thrombus formation and recent studies have postulated that this complex may be implicated in aneurysms rupture and in delayed wound healing of chronic venous ulcers as some recent studies performed on plasma and affected tissues levels demonstrated [67, 68]. A further recent experience showed that NGAL plasma levels were independently associated with increased risk of CVD mortality, all-cause mortality, and cardiovascular disease during long-term follow-up [69].

Increased plasma levels of MMP-9 and NGAL expression have been also related to deep vein thrombosis and pulmonary embolism [70].

MMP-9 and NGAL seem also to increase together the systemic inflammation which participates in atherosclerosis evolution from the early development of endothelial dysfunction, to formation of mature atheromatic plaques, to the ultimate endpoint, rupture, and thrombotic complications [66].

Thus, several studies into this field have shown that MMPs and NGAL may be released into the blood in sufficient

Table 1: Main biomarkers associated with CD and PVD with a medium/high level of evidence validated by multiple research and detectable through blood samples.

Main disease and subclass	Genomic biomarkers (genes)	Transcriptomic biomarkers	Proteomic biomarkers
CD			
HYT	—	—	MMP-2; MMP-9; MMP-10 (disease development and prognosis)
CAD	—	lncRNA LIPCAR (disease development and prognosis)	—
Arterial PVD			
AAA	LRP1; LDR; SORT1; IL6R MMP-3; AGTR1 (susceptibility)	—	MMP-9; NGAL (disease development and prognosis)
CAA	—	—	—
PAD	—	—	—
Venous PVD			
VV	FOXC2 (susceptibility)	—	—
CVU	—	—	MMP-9; NGAL (disease development, prognosis, response to treatment)
VTE	G20210A Factor V Leiden (susceptibility)	—	MMP-9; NGAL (disease development and prognosis)

quantities to act as diagnostic or prognostic markers for various clinical conditions [58].

Substrate specificity for the MMPs and for NGAL is not yet fully characterized. Additionally, several studies showed how MMPs may act cooperatively both in degrading ECM and in the various pathological processes. Therefore, more research is needed to identify specific MMPs patterns for each kind of related diseases.

There are also two other families of metalloproteinases, ADAMs (a disintegrin and metalloproteinases) and ADAMTSs (a disintegrin and metalloproteinases with thrombospondin motifs), which can be detected in the blood, and they seem to be involved in several mechanisms of cardiovascular and peripheral vascular disease [71, 72], especially in CVD evolution toward CVU where a recent study [71] identified high serum levels of ADAM-10, ADAM-17, and ADAMTS-4 to be responsible of the maintaining of chronic inflammation in patients with CVD, but their exact role remains to be further clarified.

There are also naturally occurring inhibitors of metalloproteinases, called Tissue Inhibitors of Metalloproteinases (TIMPs), which can partially or completely neutralize metalloproteinases function in activated states. In fact, when there is imbalance in the metalloproteinases and TIMPs activity ratio in favour of metalloproteinases activation, there is increased vascular remodeling and also increased atherosclerotic plaque formation for the arterial side [58].

So, understanding the regulation and the role of metalloproteinases in vascular wall imbalance and in endothelial dysfunction genesis and progression is fundamental for the development of therapeutic agents that may prevent or treat vascular diseases [73].

6. Expert Commentary

In order to assess the potential usefulness of the biomarkers in the field of CD and PVD in a way that can be useful for clinicians we can affirm that currently genomic biomarkers can only give information of low to medium risk of developing clinical conditions related to CD and PVD as in this area the participation in the disease is multigenic and it is often difficult to enucleate the contribution of each individual gene, with the exception of the risk of VTE where the genetic alterations identified offer a high level of risk profile.

Transcriptome biomarkers studies are still recent and they have not been reproduced by multiple researchers and the level of risk cannot be assessed at this time yet. Transcriptome biomarkers have the potentiality to measure the kinetic response to a specific effector/drug, to characterize the various stages of development of a disease, but further experiences need to prove their reproducibility.

Proteome biomarkers are currently at a good point of development and are coming to be used in the clinical practice in the near future for both disease predisposition and follow-up tracking during treatment. Some of these such as MMP-9 and NGAL plasma levels have been validated by more than one research group for their effective role

in describing response to treatment and their predictive role for some acute complications such as arterial aneurysm rupture.

Table 1 resumes the main biomarkers with a medium/high level of evidence, among those discussed in this paper, whose detection may be turned into clinical practice in the near future.

7. Future Perspective and Conclusions

In the next future, it is desirable to develop and validate stratification risk software which may integrate data coming from genetic, transcriptomic, and proteomic biomarkers in order to provide integrated information which could determine effective disease susceptibility, early diagnosis, and staging and tracking of CD and also to provide treatment information at a personalized level.

Thus, a significant proportion of morbidity and mortality related to CD and PVD could be prevented through biomarkers-based strategies, thus making cost-effective interventions accessible and affordable, both for people with established disease and for those at high risk of developing the disease.

Abbreviations

AAA: Abdominal aortic aneurysm
ADAM: A disintegrin and metalloproteinase
ADAMTS: A disintegrin and metalloproteinase with thrombospondin motifs
AF: Atrial fibrillation
AGTR1: Angiotensin II Receptor, Type 1
ARVC: Arrhythmogenic right ventricular cardiomyopathy
BP: Bblood pressure
CAA: Carotid artery atherosclerosis
CAD: Coronary artery disease
CCL2: Monocyte chemoattractant protein-1
CD: Cardiovascular disease
cDNA: Complementary DNA
COL1A2: Alpha-2 type I collagen gene
CR: Calorie restriction
CVD: Chronic Venous Disease
CVI: Chronic Venous Insufficiency
CVU: Chronic Venous Ulceration
DVT: Deep vein thrombosis
ECM: Extracellular matrix
GWAS: Genome-wide association studies
HDL: High density lipoprotein
HF: Heart failure
HYT: Hypertension
IA: Intracranial aneurysm
ICAM1: Intercellular adhesion molecule 1
ICAS: Internal carotid artery stenosis
IL: Interleukin
IL6R: Interleukin-6 receptor
LDLR: Low density lipoprotein receptor
lncRNA: Long noncoding RNA
LPA: Lipoprotein(a) (LPA)
LRP1: Lipoprotein receptor-related protein 1
LV: Left ventricle
MIF: Migration inhibitory factor
MIP-1α/CCL3: Macrophage inflammatory protein-1α/CCL3
miRNA: MicroRNA
MMP: Matrix Metalloproteinase
NGAL: Neutrophil gelatinase-associated lipocalin
NMP1: Nucleophosmin-1
PAD: Peripheral arterial disease
PE: Pulmonary embolism
PVD: Peripheral vascular disease
RV: Right ventricle
RVT: Recurrent venous thromboembolism
SELE: E-selectin
SMAD2: Small mother against decapentaplegic 2
SNPs: Coronary artery disease
SORT1: Sortilin 1
SVT: Single venous thromboembolism
TGFB: Transforming Growth Factor
TGFB2: Transforming Growth Factor Beta 2
TGFBR1: Transforming Growth Factor Beta Receptor 1
TGFBR2: Transforming Growth Factor Beta Receptor 2
TIMPS: Tissue Inhibitors of Metalloproteinases
VTE: Venous thromboembolism
VV: Varicose Veins.

Competing Interests

The authors declare that they have no competing interests.

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Review Article

Roles and Clinical Applications of OPG and TRAIL as Biomarkers in Cardiovascular Disease

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Cardiovascular diseases (CVD) remain the major cause of death and premature disability in Western societies. Assessing the risk of CVD is an important aspect in clinical decision-making. Among the growing number of molecules that are studied for their potential utility as CVD biomarkers, a lot of attention has been focused on osteoprotegerin (OPG) and its ligands, which are receptor activator of nuclear factor κB ligand (RANKL) and TNF-related apoptosis-inducing ligand. Based on the existing literature and on our experience in this field, here we review what the possible roles of OPG and TRAIL in CVD are and their potential utility as CVD biomarkers.

1. Introduction

Cardiovascular diseases (CVD) remain the major cause of death and premature disability in Western societies. In 2013 there were more than 54 million deaths globally and 32% of them (17 million) were attributable to CVD [1]. Moreover, current predictions estimate that by the year 2020 cardiovascular diseases, notably atherosclerosis, will become the leading global cause of total disease burden [2]. These figures reinforce the need for diagnostic-prognostic tools that could help identify the subset of patients with the highest risk of morbidity and mortality from CVD and, therefore, that could help better tailor/focus our interventions.

Among the growing number of molecules that are studied for their potential utility as CVD biomarkers, much attention has been focused on osteoprotegerin (OPG) and its ligands, which are receptor activator of nuclear factor kB ligand (RANKL) and TNF-related apoptosis-inducing ligand (TRAIL), as reviewed in [3–6]. OPG is in fact a circulating glycoprotein, which was first characterized for its ability to block RANKL and inhibit bone reabsorption, hence its name. Subsequently, it has been demonstrated that OPG can inhibit TRAIL peripheral actions, which are related to cellular life and death, and that it can also have direct (ligand-independent) effects on the bone, the vasculature, and the immune system.

While the significance of OPG for vascular biology has gained epidemiological support [7], with a range of studies reporting associations between circulating OPG and incident CVD [8–10], the role and significance of RANKL and TRAIL are less clear. Recently, Secchiero and colleagues reported that patients with coronary artery disease displayed an increased OPG/TRAIL ratio, which was even higher in the subgroup of patients who developed heart failure, thus suggesting that the OPG/TRAIL ratio plays a significant role in the pathophysiology of CVD [11]. Here we review what the possible roles of OPG and TRAIL in CVD are and their potential utility as CVD biomarkers.

2. Overview on OPG and TRAIL Biology

2.1. OPG Biology. Osteoprotegerin (OPG) is a protein that belongs to the tumor necrosis factor (TNF) superfamily, which was identified by three independent groups [12–14]. Following the observation that when this molecule was injected into mice it increased their bone mass [15], the American Society of Bone and Mineral Research Committee called it osteoprotegerin [16] because it described its bone protective actions. In humans, OPG is expressed in health and disease states in a wide variety of tissues [3]. These include not only the bone [17–19], but also the heart, vessels,

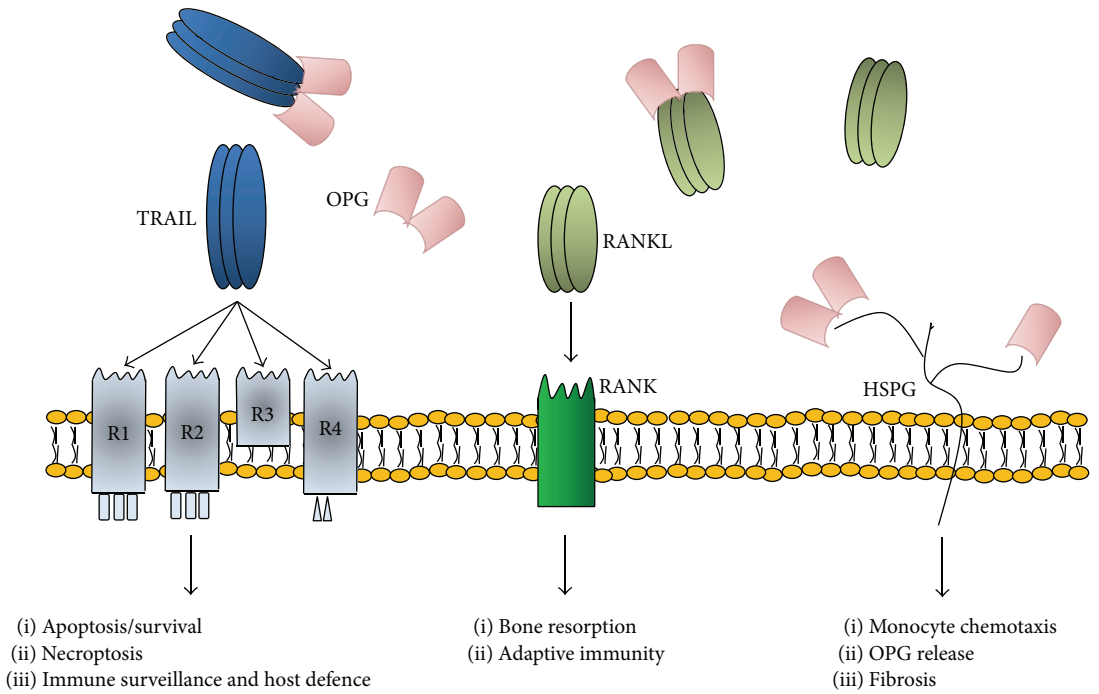

Figure 1: Representation of the TRAIL/OPG/RANKL system. Osteoprotegerin (OPG) is a secreted glycoprotein, whose predominant and more bioactive extracellular form is a disulphide-linked dimer. By acting as a decoy receptor for TRAIL and RANKL, OPG regulates many processes, such as cell apoptosis/survival and necroptosis, immune surveillance and host defence, and bone resorption. Moreover, OPG binds glycosaminoglycans such as heparin sulfate proteoglycans (HSPG), whereby it regulates monocyte chemotaxis, OPG release, and fibrosis. As for TRAIL, it is expressed as a transmembrane protein, which can be cleaved and released as a soluble molecule, which combines with two other molecules of TRAIL to form a trimeric ligand. TRAIL homotrimers bind to their specific receptors, which include two death receptors, TRAIL-R1 and TRAIL-R2, and three decoy receptors, TRAIL-R3, TRAIL-R4, and osteoprotegerin (OPG). Likewise, RANKL can be found in both membrane-bound and soluble forms. When it is released as a soluble molecule, RANKL combines with two other molecules of RANKL to form a trimeric ligand, which binds to its receptor RANK. HSPG is heparin sulfate proteoglycans; OPG is osteoprotegerin; R is receptor; RANK is receptor activator of nuclear factor kappa-B, RANKL is receptor activator of nuclear factor kappa-B ligand; TRAIL is TNF-related apoptosis-inducing ligand.

kidney, liver, spleen, thymus, lymph nodes [20], as well as the adipose tissue, and pancreas [21–23]. In the vessels, OPG is expressed by endothelial [24] and smooth muscle [25] cells. The gene encoding for OPG is located on chromosome 8 at position 8q24 [12], in a region that seems to harbor a gene cluster involved in the regulation of bone development and metabolism [12]. OPG gene locus spans approximately 29 kb and it has five exonic segments. OPG is expressed as a circulating glycoprotein of 401 amino acids with seven structural domains. Among them, domain 7 contains a heparin-binding region as well as the free cysteine residue that is required for disulphide bond formation and allows OPG to interact and combine with another molecule of OPG to form a dimeric ligand [12]. Therefore, circulating OPG can be found either as a free monomer of 60 kD or as a disulphide bond-linked homodimer form of 120 kD, which is usually biologically more active than the monomeric one [12]. Moreover, OPG can also circulate while bound to its ligands, which are RANKL and TRAIL, as represented in Figure 1.

RANKL and TRAIL are also two members of the TNFR superfamily of proteins that, in the absence of OPG, usually bind to specific transmembrane receptors and activate downstream signaling. On the one hand, by blocking RANKL [26], which stimulates osteoclast formation and activation [27], OPG prevents bone loss; this represents the rationale for its current use in patients with osteoporosis [28, 29]. On the other hand, by blocking TRAIL, OPG prevents TRAIL-induced apoptosis of tumor cells [30]. However, given that TRAIL induces apoptosis in transformed cells such as malignant, virally infected, and overactivated cells, while it spares the normal ones, the actions of TRAIL (and therefore of OPG-TRAIL) are less well characterized in nontransformed cells. Moreover, OPG may also have direct (ligand-independent) actions in the vasculature, bone, and immune system, mediated by its heparin-binding domain [31–33], which interacts with cellular heparin sulfate proteoglycans that usually take part in cell-surface signaling [34].

It has to be noted that current enzyme-linked immunosorbent assays (ELISA) measuring circulating OPG do not differentiate between its form (monomer rather than disulphide-linked dimer) and site of origin [6]. Moreover, OPG can be quantified by different ELISA (R&D Duoset, BioVendor, and Biomedica) [6], which use different forms of the molecule as the reference standards (Figure 2). This results in differences in the lower detection limits (being 65 pg/mL for R&D Duoset, 115 pg/mL for

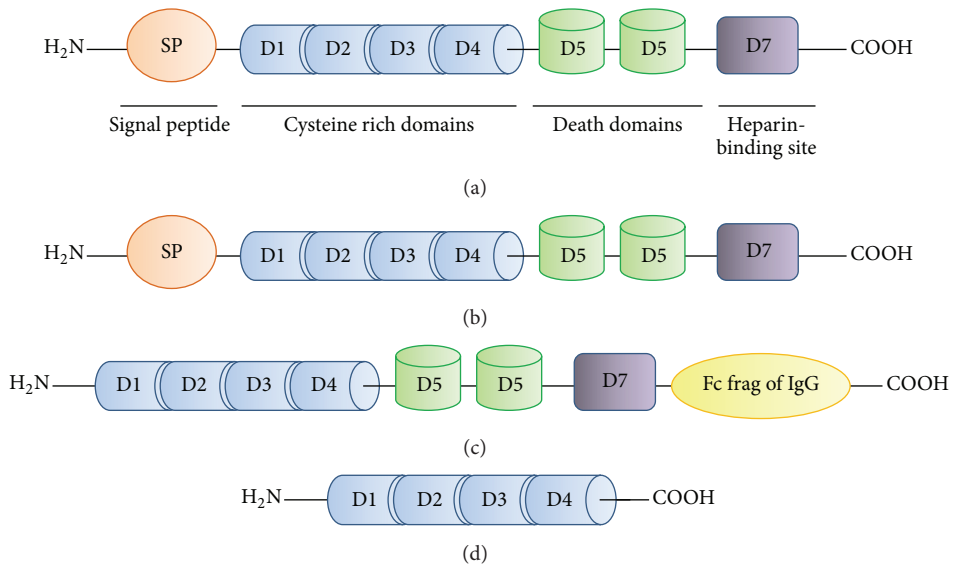

Figure 2: Schematic representation of OPG structural domains as compared to the standards of the available ELISA kits. (a) OPG structural domains; (b) R&D Duoset ELISA standard; (c) BioVendor ELISA standard; (d) Biomedica ELISA standard. ELISA is for enzyme-linked immunosorbent assays; OPG is for osteoprotegerin.

BioVendor, and 1.4 pg/mL for Biomedica) as well as in the final concentrations [35]. Clancy and colleagues [36] demonstrated that OPG concentrations for the same samples were significantly different when they were measured by different assays, while concordance correlation coefficients for intra- and interassay reproducibility were good.

2.2. TRAIL Biology. As mentioned earlier, TRAIL is also a protein that belongs to the TNF superfamily and was cloned on the basis of its high homology to other TNF family members, such as FasL/CD95L and TNF-α [37]. The percentage of identity with FasL/CD95L and TNF-α is in fact 28% and 23%, respectively. In humans, TRAIL is expressed in health and disease states in a wide variety of tissues, including the vessels, where it is expressed in vascular smooth muscle cells (VSMC) [38]. The gene encoding for TRAIL is located on chromosome 3 at position 3q26. TRAIL gene locus spans approximately 20 kb and it has five exonic segments. In humans, TRAIL is expressed as a type II transmembrane protein of 281 amino acids. Like TNF-α, TRAIL can be cleaved at the stalk domain, and by combining with other two molecules of TRAIL, it forms a circulating homotrimer with biological activity [39]. As represented in Figure 1, the human receptors for TRAIL include not only death receptors (DR) but also decoy receptors (DcR) [40, 41]. TRAIL DR comprise TRAIL-R1 [42] and TRAIL-R2 [43], which are both type I transmembrane proteins containing an intracellular death domain (DD) that classically stimulates apoptosis upon TRAIL binding and are both expressed in the vessels. Compared to TRAIL, which is normally expressed by VSMC, TRAIL-R1 and TRAIL-R2 are also expressed by endothelial cells (EC) [44–46]. As for TRAIL DcR, they include TRAIL-R3 [47], TRAIL-R4 [48, 49], and OPG [50]. DcR1 and DcR2 are transmembrane receptors that differ from DR in that their cytoplasmatic domain lacks an intact DD, while OPG is a soluble decoy receptor that is lacking both transmembrane and cytoplasmatic residues.

In the absence of OPG, TRAIL homotrimers bind TRAIL-R1 and TRAIL-R2 on the surface of target cells (Figure 1). Through such binding, TRAIL is able to trigger cellular apoptosis in malignant, virally infected, and overactivated immune cells, hence its acronym. Recently, it has been shown that TRAIL can also induce necroptosis, which is a regulated and programmed form of necrosis that takes place after TRAIL binding to its specific death receptors and which can be useful to the body when apoptosis has been blocked [51, 52]. With respect to TRAIL's ability to induce apoptosis in tumor cells, studies on TRAIL-knockout mice have in fact demonstrated that mice without TRAIL are viable and fertile but more susceptible to tumor metastases, indicating that TRAIL regulates immune surveillance and host defence against tumor initiation and progression [53, 54]. In particular, TRAIL seems to mediate the ability of natural killer cells and cytotoxic T lymphocytes to block tumor growth and metastasis development [55]. Interestingly, one of the unique aspects of TRAIL, as compared to other proapoptotic ligands [56, 57], is that TRAIL has the ability to induce apoptosis preferentially in transformed cells, such as tumor or infected cells, while it spares the normal ones [58]. In particular Ashkenazi and colleagues demonstrated that the exposure of cynomolgus monkeys to recombinant human- (rh-) TRAIL at 0.1-10 mg/Kg/day over 7 days did not induce detectable toxicity, whereas, by comparison, TNF-α induced severe toxicity at much lower doses such as 0.003 mg/Kg/day [59]. This is the rationale for its use in clinical settings as an antitumor drug [39].

While it has been clearly demonstrated that TRAIL induces apoptosis in transformed cells, in nontransformed

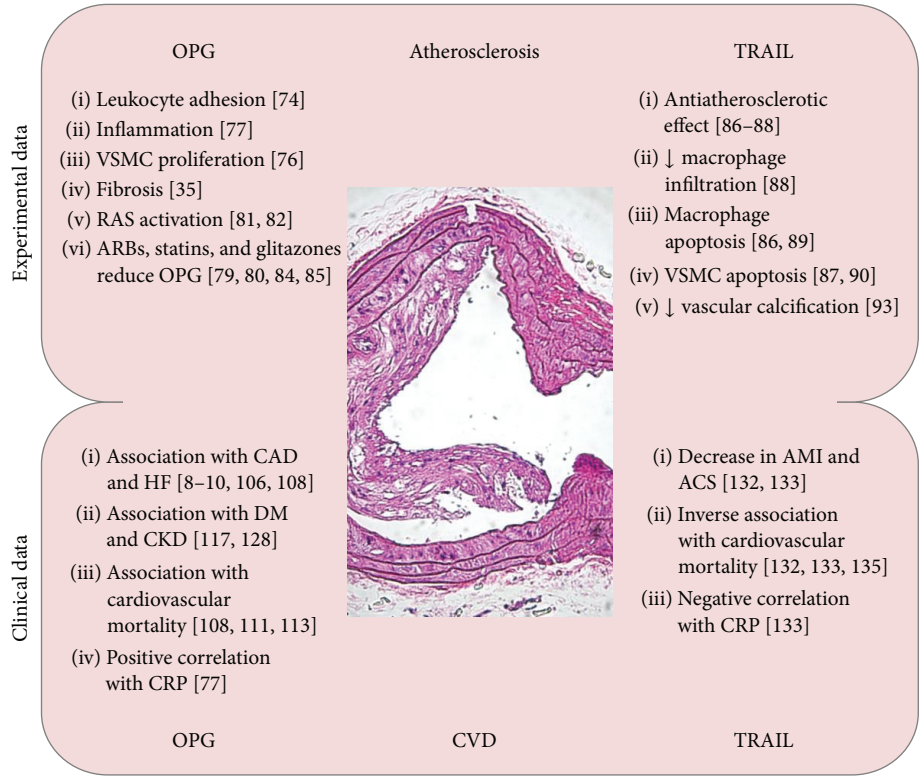

Figure 3: Roles of OPG and TRAIL in atherosclerosis and CVD. In the upper part of the image, summary of the main experimental data supporting OPG and TRAIL involvement in atherosclerosis. In the lower part of the image, summary of the main clinical data showing OPG and TRAIL associations with CVD. In the middle, representative image of an aortic atherosclerotic plaque stained by hematoxylin and eosin (10x original magnification). ACS is acute coronary syndromes; AMI is acute myocardial infarction, and ARBs are angiotensin II type 1 receptor blockers; CAD is coronary artery disease; CKD is chronic kidney disease; CRP is C-reactive protein; DM is diabetes mellitus; OPG is osteoprotegerin; RAS is renin-angiotensin system; TRAIL is TNF-related apoptosis-inducing ligand; VSMC is vascular smooth muscle cell.

cells, the actions of TRAIL are less well characterized. For example, this molecule could actually mediate nonapoptotic signaling. It has in fact been shown that when TRAIL-R1 and TRAIL-R2 are activated they not only stimulate the extrinsic apoptotic pathway, but also may activate survival/proliferation pathways, such as nuclear factor κB (NF-κB), ERK1/ERK2, and Akt [44, 60] (Figure 1). Consistent with the concept that TRAIL triggers nonapoptotic signals in normal cells, we have also shown that systemic TRAIL delivery significantly reduced cardiac fibrosis and apoptosis in a mouse model of diabetic cardiomyopathy [61]. Potential mechanisms underlying the ability of TRAIL to activate such opposed pathways include the redistribution of TRAIL receptors [62, 63] and the intracellular inhibition of the apoptotic cascade [64].

3. Role of OPG and TRAIL on Atherosclerosis

3.1. OPG and Atherosclerosis. The current view of atherosclerosis is that it is an inflammatory disease of the vessels [65], mediated by leukocyte vascular recruitment and migration. In particular, once different stimuli/forms of injury increase endothelium adhesiveness to circulating cells, leukocytes migrate into the subendothelial space promoting lesion initiation, which is usually followed by macrophage recruitment, VSMC migration and proliferation, fibrous cap formation, and atherosclerotic plaque development [65]. This process is generally stimulated by a combination of factors such as dyslipidemia, hyperglycemia, and shear stress that activate common pathways, promoting all the events leading to the development of atherosclerotic plaques. Interestingly, both OPG and TRAIL are found in atherosclerotic plaques [66], where they seem to participate in this process by exerting opposite actions (Figure 3).

As for OPG, the first studies evaluating its effects on the vasculature indicated that it could protect the vessels against calcification, given that OPG deficiency resulted in early-onset severe osteoporosis as well as significant medial calcification of the aorta and the arteries [67]. Similarly, OPG inactivation in ApoE-knockout mice resulted in augmented vascular calcification and increased size of atherosclerotic plaques, as compared to their controls [68]. However, in another study where LDLr-knockout mice were fed with an atherogenic diet and treated with fc-OPG, fc-OPG reduced plaque calcification but did not affect the number and size of the lesions, suggesting that although OPG protected against vascular calcification, it did not affect atherosclerosis progression and severity [69]. By contrast, our group has shown that

human full-length OPG induced the proliferation of rodent vascular smooth muscle cells and increased atherosclerosis extension in diabetic ApoE-knockout mice, suggesting that this molecule could actually promote atherosclerosis [70]. Moreover, an infusion of full-length recombinant OPG in ApoE-knockout mice every 3 weeks for 3 months also resulted in increased vascular collagen content in the media [35].

To reconcile these results, it is possible that OPG, initially secreted to protect the vasculature against calcification, would actually damage it by promoting inflammation and fibrosis. The concept that OPG can actually promote atherosclerosis development is supported by several *in vitro* studies demonstrating that OPG has proinflammatory and profibrotic effects on the vasculature. As for inflammation, it has been demonstrated that when leukocyte-endothelial cell adhesion takes place, it increases the leukocyte production of proinflammatory cytokines such as TNF-α and interferon-γ, which would upregulate OPG expression in EC and VSMC [71–73]. Moreover, in line with the *in vitro* observation that OPG stimulates EC expression of adhesion molecules [73], we have recently shown that OPG increases leukocyte adhesion to endothelial cells [74] both *in vivo* and *in vitro*, contributing to atherosclerotic plaque formation. As for vascular fibrosis, consistent with our earlier finding that human full-length OPG induced the proliferation of rodent VSMC, we have found that VSMC treatment with full-length recombinant OPG induced fibrogenesis with increased expression of fibronectin, collagen I, collagen III, and collagen IV, as well as MMP-2 and MMP-9, and TGF-β [35]. Pretreatment with the specific TGF-β receptor inhibitor, prior to treatment with OPG, attenuated OPG-induced fibrogenesis and proliferation in VSMC. These results suggest that OPG is a potent inducer of fibrogenesis, growth factor synthesis, and proliferation in VSMC, both *in vitro* and *in vivo*, and that its actions are largely dependent on the autocrine induction of TGF-β, which itself stimulates OPG in a vicious cycle that results in the autoinduction of both OPG and TGF-β [35].

Nevertheless, OPG could also promote atherosclerosis by stimulating systemic inflammation and the renin-angiotensin system (RAS) activation, which is one of the most important pathways leading to atherosclerosis [75, 76]. As for systemic inflammation, we have recently shown that OPG delivery increases IL-6, MCP-1, and TNF-α circulating levels [77], which is consistent with the view that it takes part in the pathogenesis of atherosclerosis and CVD by amplifying inflammation [5]. Consistent with this claim, we have also reported a positive correlation between OPG and CRP [77]. With respect to the interplay with the RAS, experimental evidence suggests that there is a mutual stimulatory effect between OPG and the RAS [35, 78–82]. It has in fact been demonstrated that angiotensin II (Ang II) increases OPG expression in human aortic smooth muscle cells [78] as well as in murine VSMC [35]. Not surprisingly, treatment with the Ang II type 1 receptor (AT1R) blocker Irbesartan reduced OPG secretion from human abdominal aortic aneurysm explants [79]. Consistent with this finding, a recent study has demonstrated that AT1R blockade with Irbesartan significantly reduced OPG expression in human primary vascular cells and carotid atheromas [80]. Interestingly, if Ang II stimulates vascular OPG expression in a dose-dependent manner, OPG reciprocally stimulates vascular AT1R protein expression in a dose-dependent manner [81]. Consistent with this observation, we have observed that OPG delivery significantly increased ACE and AT1R gene and protein expression in the pancreas [82], where we hypothesized that OPG might control their transcription by activating the mitogen-activated protein kinase signaling [31] that regulates ACE and AT1R expression.

Interestingly, in addition to RAS blockers, there are other antiatherosclerotic drugs [83], such as statins and glitazones, which have exhibited the ability to reduce OPG in the vessels. As for statins, they reduced TNF-α and IL-1α-induced OPG expression in EC and VSMC [84]. As for glitazones, on the other hand, which are pharmacological PPAR-γ ligands, they significantly decreased the expression of OPG in human aortic smooth muscle cells [85].

3.2. TRAIL and Atherosclerosis. Contrary to OPG, animal studies [86–88] suggest that TRAIL protects against atherosclerosis. In the first of these studies, TRAIL treatment, delivered either as soluble recombinant TRAIL by intraperitoneal injection or in an adenoviral-vector, significantly reduced the accumulation and complexity of atherosclerotic plaques in diabetic ApoE-knockout mice [86]. Here, we speculated that TRAIL effects were mediated by its ability to induce apoptosis of infiltrating macrophages within the plaque, which had been previously observed *in vitro* by a different group [89]. The second study was conducted in TRAIL ApoE-double-knockout mice and demonstrated that TRAIL deficiency worsened atheromatous lesion formation, possibly by increasing VSMC content within the plaque [87]. In the mice lacking TRAIL, there was a reduction in VSMC apoptosis, indicating that TRAIL would induce VSMC apoptosis [90] rather than their survival [91] and that this could be the mechanism protecting against plaque enlargement. Consistent with our previous findings, Di Bartolo and colleagues reported a significant increase in atherosclerotic plaque formation and progression in ApoE- and TRAIL-double-knockout mice [88]. Here, TRAIL deficiency significantly influenced plaque stability, as it increased the extension of the necrotic core and macrophage infiltration, while reducing VSMC and collagen content [88]. This work is of particular interest not only because it confirms TRAIL antiatherosclerotic effects but also because it sheds light onto a possible role for TRAIL in glucose metabolism regulation [92]. Recently, it has also been shown that TRAIL inhibits vascular calcification [93], as TRAIL deficient mice exhibited a significant increase in tissue RANKL, which leads to vascular calcification. Consistent with this finding, VSMC exposed to calcium and TRAIL displayed significantly lower alizarin red staining (used to quantify vascular calcification) as compared to those exposed to calcium alone, indicating that TRAIL protects against calcium-induced VSMC calcification *in vitro* [93].

Overall, it is very difficult to draw conclusions on the mechanisms underlying the antiatherogenic effects of TRAIL

by simply looking at *in vitro* data. Potentially, TRAIL is a molecule with two faces [94], the first that can induce apoptosis [95] and stimulate inflammation [45, 97] and the second that can promote cell survival [44, 96] and inhibit inflammation, depending on its dose and cell responsiveness. Nevertheless, animal studies show that TRAIL protects against atherosclerosis, possibly by inducing apoptosis of macrophages and VSMC [86–90]. Other potential mechanisms underlying TRAIL antiatherogenic effects include protection of normal vascular cells and anti-inflammatory actions [44, 92, 98, 99]. As mentioned earlier, both EC and VSMC express TRAIL receptors and Secchiero and colleagues have shown that recombinant TRAIL is able to promote their survival/proliferation by activating intracellular signaling pathways, such as ERK/MAPK, Akt, and NF-κB, which are known to promote survival and proliferation [44]. Moreover, the same authors showed that TRAIL upregulates the production and release of prostanoids, including PGE2 and PGI2, and increases NO production and eNOS activity in endothelial cells, without activating NF-κB, which are all involved in the maintenance of vascular homeostasis [98]. It has also been shown that TRAIL counteracts leukocyte adhesion induced by TNF-α or IL1-β by downregulation of CCL8 and CXCL10 chemokine expression [99]. This is consistent with the observation that TRAIL can significantly reduce systemic and tissue inflammation, as assessed by measuring IL-6, MCP-1, and TNF-α expression [92], which on the contrary were found elevated in TRAIL-knockout mice [88]. Recently, it has also been shown that administration of human recombinant TRAIL reduced allergic airway inflammation in a mouse model of asthma [100].

4. Clinical Applications of OPG and TRAIL as Biomarkers of CVD

4.1. OPG and CVD. Keeping in line with the dichotomy between the role of OPG and TRAIL in atherosclerosis (Figure 3), while TRAIL appears to be antiatherosclerotic, OPG has been shown to be associated with CVD onset and progression. OPG levels are in fact positively correlated with markers of vascular damage such as endothelial dysfunction [101–103], vascular stiffness [104], and coronary calcification [105], as well as with the presence of coronary artery disease (CAD) [106, 107]. Consistent with this, OPG has been found associated with the risk of future CAD in apparently healthy men and women, independent of established cardiovascular risk factors [8, 9]. In patients with acute coronary syndromes, OPG has been linked to the incidence of death, heart failure (HF) hospitalizations, myocardial infarction (MI), and stroke [108], which has been successively observed in the general population as well [109]. Moreover, although initially it appeared that OPG was an independent risk factor for incident CVD and vascular mortality but not for mortality due to nonvascular causes [8, 110], it has been recently demonstrated that high levels of OPG can also predict nonvascular mortality [111].

Left ventricular dysfunction is one of the key prognostic indicators of cardiovascular morbidity and mortality [112]. Interestingly, OPG has been found to be elevated in both clinical and experimental HF [10]. Moreover, different studies have evaluated the prognostic utility of OPG in patients with HF. In the first one, Ueland and colleagues showed that, in patients with history of myocardial infarction and left ventricular dysfunction, baseline OPG was significantly higher in those who died from vascular and nonvascular causes as compared to those who survived [113]. In a subsequent study, Omland and colleagues showed that in patients with acute coronary syndrome the baseline levels of OPG correlated significantly with the incidence of heart failure [108]. More recently it has been shown that OPG is predictive of hospitalization for HF in patients with advanced systolic HF and ischemic heart disease independently of conventional risk markers [114].

It is well known that diabetes mellitus and chronic kidney disease (CKD) are associated with an increased risk of CVD and vascular mortality [115, 116]. Interestingly, in both conditions OPG levels are elevated and predict CVD onset. Several groups have reported that OPG levels are elevated in patients with type 1 and type 2 DM, as reviewed in [6]. Nevertheless, beside the positive relationship between OPG and type 2 DM, which has been known since 2001 [117], in diabetic patients there is also a strong association between circulating levels of OPG and micro- and macrovascular complications [118, 119]. Here, OPG is associated with cardiovascular events [119, 120] and the presence and severity of silent myocardial ischemia [121–124], as well as with the risk of developing end-stage renal disease [125]. Consistent with the experimental data showing an inhibitory effect of glitazones on vascular OPG [85], in type 2 DM patients, pioglitazone was found to decrease OPG levels [126, 127], which showed correlation with glucose control [126].

As for CKD, on the other hand, OPG is increased in patients with nondiabetic [128, 129] and diabetic [119, 125, 130] CKD, where it predicts kidney function deterioration and vascular events and cardiovascular and all-cause mortality [130]. Consistent with implications in CKD, it has been recently reported that elevated OPG is associated with increased 5- and 10-year risk of rapid renal decline, renal disease hospitalization, and/or deaths in elderly women [131].

4.2. TRAIL and CVD. Contrary to OPG, the serum levels of TRAIL have been found significantly decreased in patients affected by or predisposed to CVD. In regard to this issue, it is notable that serum levels of TRAIL are significantly decreased in patients with acute myocardial infarction within 24 hours of admission, compared to healthy controls [132]. Relatedly, also Michowitz and colleagues found that circulating TRAIL was significantly lower in patients with acute coronary syndrome as compared to those with stable angina or normal coronary arteries and that it was negatively correlated with the level of C-reactive protein, which is an independent predictor of acute vascular events and adverse outcomes in patients with HF [133]. Given that the same authors found that TRAIL expression was increased in vulnerable plaques, where it localized with T cells and oxidized low-density lipoprotein, they argued that TRAIL decrease in

patients with CVD might be due to its consumption into the plaques. Other reasons underlying TRAIL decrease in patients with acute cardiovascular events might include the parallel increase in circulating OPG, as well as the increase of metalloproteinase-2 (MMP-2). While OPG acts as a decoy receptor for TRAIL, whereby its binding may interfere with TRAIL dosage explaining TRAIL decrease, the increase in MMP2 could explain TRAIL decrease as it has been shown that MMP-2 can induce TRAIL cleavage [134].

Consistent with these findings, circulating TRAIL levels are inversely associated with an increased risk of CVD and cardiac mortality [132, 135]. In the work by Secchiero and colleagues the patients with myocardial infarction who developed in-hospital adverse clinical outcomes displayed the lowest levels of TRAIL, indicating that the lower the level of TRAIL, the higher the risk of HF or death after myocardial infarction [132]. In the work by Michowitz and colleagues low TRAIL levels at discharge were associated with an increased incidence of cardiac death and heart failure in the 1-year follow-up [133]. Similarly, an inverse association of TRAIL levels with mortality was observed in patients with advanced heart failure [136], as well as in patients with CKD [137]. Moreover, in older patients (i.e., aged on average 68 years) with cardiovascular diseases, low levels of TRAIL were associated with increased risk of death over a period of 6 years [135].

5. Conclusions

Experimental studies suggest that there is some dichotomy in OPG and TRAIL actions, the first being proatherogenic and the second being antiatherogenic. However, the role of OPG and TRAIL in atherosclerosis has not been fully understood yet. It remains unclear whether OPG increase and TRAIL decrease should be regarded as risk factors rather than risk markers of CVD; therefore, further studies are needed to clarify what the pathogenic importance of OPG and TRAIL is in the process of atherosclerosis. On the other hand, clinical studies reinforce the view that OPG and TRAIL could be promising biomarkers of CVD onset and progression. More evidence (possibly gained after measurement standardization) is needed to evaluate the predictive and diagnostic value of OPG and TRAIL for clinical use.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Analyzing Dynamic Changes of Laboratory Indexes in Patients with Acute Heart Failure Based on Retrospective Study

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Background. Changes of N-terminal probrain natriuretic peptide (NT-proBNP) have been studied whether in the long term or the short term in patients of acute heart failure (AHF); however, changes of NT-proBNP in the first five days and their association with other factors have not been investigated. *Aims*. To describe the dynamic changes of relevant laboratory indexes in the first five days between different outcomes of AHF patients and their associations. *Methods and Results*. 284 AHF with dynamic values recorded were analyzed. Changes of NT-proBNP, troponin T, and C-reactive protein were different between patients with different outcomes, with higher values in adverse group than in control group at the same time points ($p < 0.05$). Then, prognostic use and risk stratification of NT-proBNP were assessed by receiver-operating characteristic curve and logistic regression. NT-proBNP levels at day 3 showed the best prognostic power (area under the curve = 0.730, 95% confidence interval (CI): 0.657 to 0.794) and was an independent risk factor for adverse outcome (odds ratio, OR: 2.185, 95% CI: 1.584–3.015). Classified changes of NT-proBNP may be predictive for adverse outcomes in AHF patients. *Conclusions*. Sequential monitoring of laboratory indexes within the first 5 days may be helpful for management of AHF patients.

1. Background

Acute heart failure (AHF) is the most common reason of hospitalization in patients aged 65 and older, with mortality rate up to 30–40% within one year [1, 2]. Prognosis is poor in hospitalized AHF patients [3, 4]. Accurate prognostic approach and risk stratification may be useful for management of AHF patients.

Surveillance of brain natriuretic peptide (BNP) and N-terminal- (NT-) proBNP in plasma has been reported to be prognostic, offering powerful risk stratification for monitoring the whole HF stages [5]. Kwan et al. concentrated on the prognostic significance of serial measurements of NT-proBNP within first 24 hours at admission and again at discharge for patients with chest pain. The results showed that dynamic surveillance of NT-proBNP in the short term did not improve its prognostic ability [6]. Lindahl et al. investigated the prognostic ability of NT-proBNP changes within six months in 1,216 patients with acute coronary syndromes [7] and found NT-proBNP level was stable from 2 days to 6 months and can be more predictive for two-year mortality. Then, Masson and his colleagues analyzed the predictive power of NT-proBNP changes at 4 months from baseline in 1,742 chronic heart failure (CHF) patients [8]. The results revealed that NT-proBNP measurements and classification into four categories of trends may be a superior approach for risk stratification for CHF patients. Myocardial injury, chronic kidney disease (CKD), and pulmonary infection are shown to be high risk factors for mortality of AHF patients, which were well characterized by troponin T (TNT),

glomerular filtration rate (GFR), and C-reactive protein (CRP) [9, 10]. In 2013, a report by Metra et al. focused on the short-term (within 2 weeks) dynamics of markers for cardiac, renal, and hepatic damage and cardiac congestion in AHF patients after taking serelaxin [11]. However, sequential dynamics of NT-proBNP and other indexes during the first 5 days and relation among them have not been studied in AHF patients.

The purpose of this study was to observe the changes of NT-proBNP and other indexes within the first 5 days after AHF between patients with different outcomes and their relation, eventually to find the best prognostic approach for AHF patients.

2. Materials and Methods

2.1. Study Design and Data Collection. The present study was performed in Chinese PLA General Hospital, Beijing, China. From November 2008 to March 2015, AHF patients presenting to emergency room or intensive care unit were screened. NT-proBNP and other indexes values within the first 5 days were reviewed. Finally, 284 AHF of 251 patients with at least 3 of 5 time points were enrolled, which means that some patients have been enrolled into the study more than once. Then we recorded the baseline information, including sex, age, basic medical history, and vital signs. All data was retrieved from the electronic medical record system of the hospital. The study complied with the guidelines approved by Chinese PLA General Hospital's Ethics Committee. All subjects involved in this study provided informed consents.

The inclusion criteria were established similar to the previous study by Bian et al. [12]. Patients, aged over 18, were enrolled into the study based on the discharge diagnosis of AHF (International Classification of Diseases, Ninth/Tenth Revision coding) or combination of patients' complaint (dyspnea or orthopnea), physical examination (lung rales or oxygen desaturation), and rescue measures (oxygen, mechanical ventilation, diuretics, and cardiac stimulants) or tracheal intubation. The cases without any of the above were excluded.

Patients recovered at discharge were divided into the control group, while patients with all-cause mortality in hospital were divided into the adverse outcome group.

2.2. Definition. Analytical methods used in hospital laboratory were summarized in Figure 1. Categorization of plasma TNT, CRP, and eGFR changes was performed according to the cutoff values defined on the basic of previous researches and guidelines. In accordance with the universal definition of myocardial infarction, TNT level was defined as elevated when its value exceeded 0.01 ng/mL (the 99th percentile of the upper reference in healthy individuals) [13]. CRP was defined as elevated at the value of 1.00 mg/dL and less than 90 mL/min was defined as eGFR decline [6, 7]. The eGFR was calculated based on formula MDRD with adjustment for gender [14].

2.3. Statistical Analysis. Statistical analysis was performed using SPSS version 19.0. Continuous variables were presented as the mean ± standard deviation or median (25th–75th percentiles) according to normality. For differences comparison between groups, independent t-test was used for variables normally distributed. Mann-Whitney U test was applied for variables abnormal distribution. Chi-square test was used for categorical variables. Comparison for changes of NT-proBNP, CRP, TNT, and eGFR over time at the same group was assessed by the Wilcoxon signed rank test (or Friedman test). Differences in levels of NT-proBNP, CRP, TNT, and eGFR at the same time point between different groups were analyzed through the Mann-Whitney U test. Log-transformed NT-proBNP, TNT, and CRP were also used.

NT-proBNP changes were analyzed in 3 different ways: absolute change from basic level, relative percentage change from basic level, and tendency changes [8]. Tendency changes were defined as four categories according to a cutoff value which was calculated as NT-proBNP concentration at day 3 with the best prediction accuracy for adverse outcome: low to low (cases with NT-proBNP both below the cutoff value); high to low (cases with NT-proBNP above the value at baseline and below at day 3); low to high (cases with NT-proBNP below the value at baseline and above at day 3); and high to high (cases with NT-proBNP both both above the value).

Receiver-operating characteristic (ROC) curve was applied to assess the prognostic usage of NT-proBNP at different time points. Logistic regression was used to assess the odds ratios of single measurement of NT-proBNP and the four categories of changes for adverse outcomes. The latter one was not adjusted for other demographic or clinical indicators. In the study, two-tailed p value < 0.05 was defined as statistically significant.

3. Results

3.1. Clinical Baseline Characteristics. This study included 143 men and 108 women with 284 AHF recorded in total, meaning that some patients are enrolling into the study more than once. Patient baseline characteristics between different clinical outcomes are shown in Table 1. Patients with adverse outcome had more adverse baseline characteristics, such as older age (median 74 years; Q1–Q3, 61–80 years), higher prevalence of CKD (55.2%) and stroke (29.2%), and higher level of NT-proBNP, TNT, and CRP in plasma, while in controls, NYHA classes III to IV (80.9%) and a history of CAD (52.1%) were more prevalent.

3.2. Dynamic Changes of Laboratory Indexes. Significant differences were observed in baseline of NT-proBNP, TNT, CRP, and eGFR between groups with different outcomes (Table 1). Then we analyzed the dynamic changes of the four indexes. Firstly, in 85 AHF with NT-proBNP measurements at all five time points, dynamics of NT-proBNP had a significant decrease over time in control group ($p = 0.031$) but an observable elevation in adverse outcome group (Figure 2(a), $p = 0.003$). Relatively consistent results were obtained in all AHF with NT-proBNP examinations not less than 3 of 5 time points (Figure 2(b), control: $p = 0.008$; adverse: $p = 0.018$). In control group, NT-proBNP baseline was 7024 pg/mL (2748–13923 pg/mL) and decreased to 4438 pg/mL (1725–9460 pg/mL) at day 5; however, in adverse group, NT-proBNP

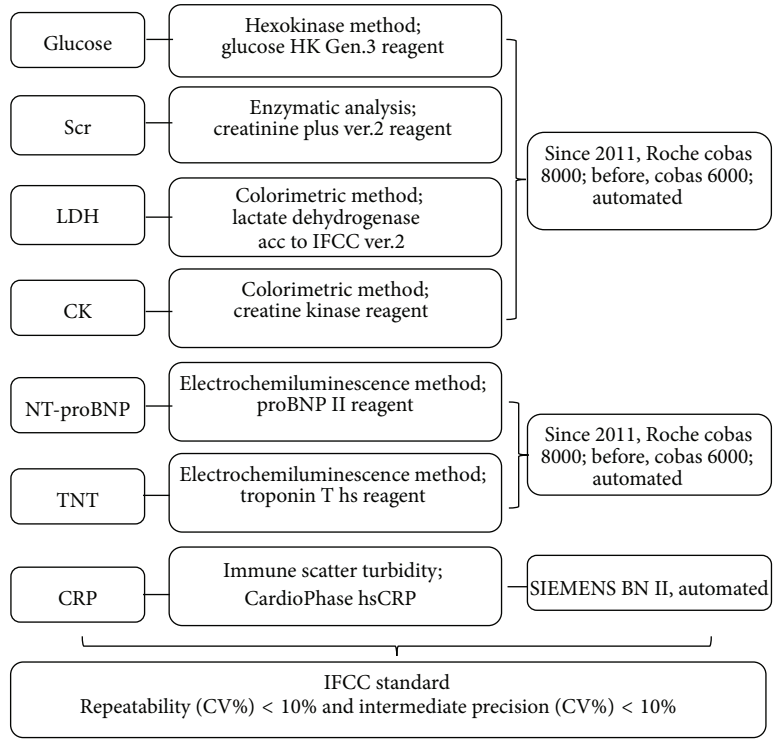

Figure 1: Flow chart of analytical methods of all indexes in hospital laboratory.

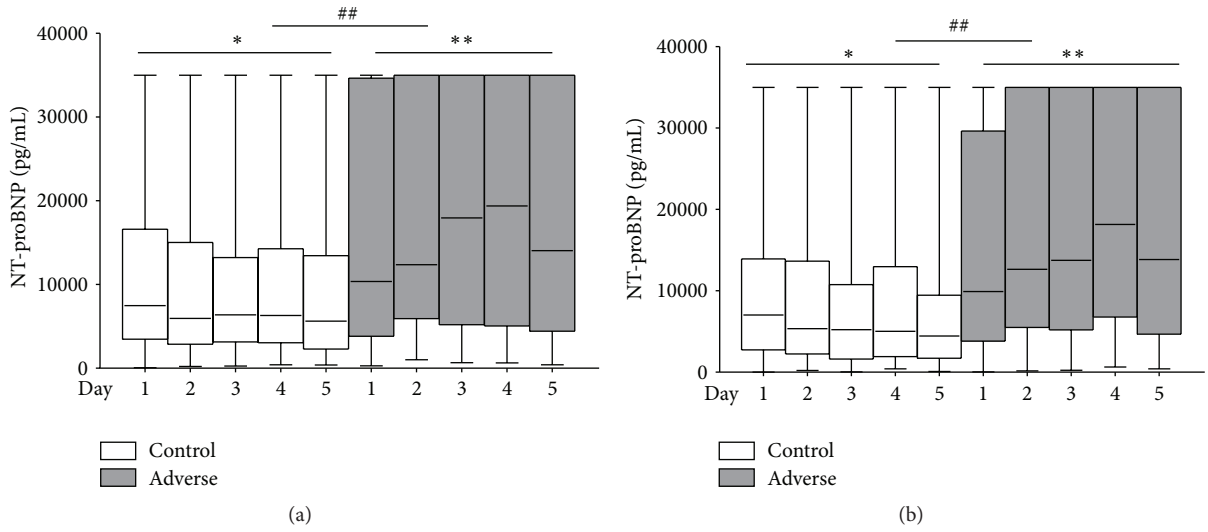

Figure 2: Plasma NT-proBNP changes over time in AHF patients. (a) showed NT-proBNP levels at days 1 (baseline), 2, 3, 4, and 5 in 85 AHF with measurements at all time points (control, $p = 0.031$ and adverse, $p = 0.003$). (b) NT-proBNP levels over time in all 284 AHF with measurements of 284, 237, 211, 185, and 180 at days 1, 2, 3, 4, and 5, respectively (control, $p = 0.018$, and adverse, $p = 0.008$). $^{*}p < 0.05$, $^{**}p < 0.01$ (significance in the same group); $^{\#\#}p < 0.01$ (significance between different groups).

concentration was 9922 pg/mL (3825–29632 pg/mL) at baseline and increased to 13855 pg/mL (4670–35000 pg/mL) at day 5. Then changes of other indexes in all patients with at least 3 time points were described as below.

For TNT, in the first tertile, the baseline in control group was 0.021 ng/mL (0.018–0.028 ng/mL) and 0.020 ng/mL (0.016–0.026 ng/mL) at day 5 ($p > 0.05$); however, in adverse group, it was 0.030 ng/mL (0.017–0.040 ng/mL) at baseline and increased to 0.046 ng/mL (0.030–0.075 ng/mL) at day 5 (Figure 3(a), $p = 0.007$). Then in the upper two tertiles, TNT median (Q1–Q3) level at baseline was 0.117 ng/mL (0.056–0.293 ng/mL) and 0.120 ng/mL (0.054–0.442 ng/mL) in control group, but in adverse group, TNT baseline was 0.196 ng/mL (0.104–0.503 ng/mL) and elevated

Table 1: Comparisons of baseline characteristics between AHF patients with different clinical outcomes.

	$N = 284$		
	Control ($n = 188$)	Adverse event ($n = 96$)	p value
Age (years), median (Q1–Q3)	67 (52–77)	74 (61–80)	0.006
Male, n (%)	109 (58.0)	55 (57.3)	0.912
NYHA III to IV, n (%)	152 (80.9)	67 (69.8)	0.036
History			
CAD, n (%)	98 (52.1)	67 (35.6)	0.004
MI, n (%)	77 (41.0)	39 (40.6)	0.957
Hypertension, n (%)	133 (70.7)	68 (70.8)	0.988
Diabetes, n (%)	72 (38.3)	40 (41.7)	0.583
Arrhythmia, n (%)	64 (34.0)	36 (37.5)	0.564
COPD, n (%)	37 (19.7)	20 (20.8)	0.819
CKD, n (%)	64 (34.0)	53 (55.2)	0.001
Stroke, n (%)	24 (12.8)	28 (29.2)	0.001
Vital signs			
RR (bpm), median (Q1–Q3)	18 (18–20)	18 (18–21)	0.357
Pulse (bpm), mean ± SD	94 ± 21	95 ± 23	0.656
SBP (mmHg), mean ± SD	132 ± 27	126 ± 23	0.076
DBP (mmHg), mean ± SD	78 ± 17	72 ± 14	0.005
Lab indexes			
LVEF (%), mean ± SD	43 ± 11	47 ± 13	0.114
NT-proBNP (pg/mL), median (Q1–Q3)	7024 (2729–13923)	9922 (3825–29632)	0.017
TNT (ng/mL), median (Q1–Q3)	0.052 (0.026–0.194)	0.104 (0.042–0.447)	0.002
CRP (mg/dL), median (Q1–Q3)	1.69 (0.40–5.77)	4.20 (1.40–14.00)	<0.001
eGFR (mL/min), median (Q1–Q3)	73 (32–98)	44 (21–80)	0.004
CK (U/L), median (Q1–Q3)	98.9 (53.8–171.1)	87 (46.8–169.5)	0.251
LDH (U/L), median (Q1–Q3)	259.5 (209.6–334.2)	286.8 (220.1–443.5)	0.077
Glucose (mmol/L), median (Q1–Q3)	8.88 (6.25–12.23)	8.06 (6.13–10.54)	0.179

AHF: acute heart failure; CAD: coronary artery disease; CK: creatine kinase; CKD: chronic kidney disease; CK-MB: creatine kinase isoenzyme; COPD: chronic obstructive pulmonary disease; CRP: C-reactive protein; DBP: diastolic blood pressure; eGFR: estimated glomerular filtration rate; LDH: lactic dehydrogenase; LVEF: left ventricular ejection fraction; MI: myocardial infarction; NT-proBNP: N-terminal probrain natriuretic peptide; NYHA: New York Heart Association; Q: quartile; RR: respiratory rate; SBP: systolic blood pressure; and TNT: troponin T.

to 0.265 ng/mL (0.126–1.058 ng/mL) at day 5 (Figure 3(b), $p = 0.005$). As shown, TNT remained at low level over time in control group but continued to rise in adverse group ($p < 0.01$).

For patients in control group, CRP median (Q1–Q3) level was 1.80 mg/dL (0.40–6.80 mg/dL) at admission and increased to 3.90 mg/dL (0.88–10.33 mg/dL) at day 3 and then dropped to 2.70 mg/dL (1.18–5.83 mg/dL) at day 5; however, in adverse group, CRP baseline was 5.35 mg/dL (1.73–14.48 mg/dL) and 5.45 mg/dL (3.30–10.73 mg/dL) at day 3 and 4.65 mg/dL (2.13–8.08 mg/dL) at day 5. So, CRP underwent a process of first rose ($p < 0.001$) and then descended ($p = 0.003$) but kept at high level in adverse group without a significant decrease (Figure 3(c)).

In adverse group, eGFR maintained a lower level than that in control group (Figure 3(d)). Baseline of eGFR was 70 mL/min (27–97 mL/min) without a significant decrease over time in control group; however, in adverse group, it was 41 mL/min (17–76 mL/min) at admission and decreased to 29 mL/min (15–62 mL/min) at day 5. Besides, median levels of the four indexes were significantly different between different groups at the same time points ($p < 0.01$) except that CRP level at day 3.

3.3. Baseline and Changes of NT-proBNP Related to Other Indexes. Univariate analysis showed that NT-proBNP baseline was related to age, TNT, CRP, and eGFR and not significantly associated with history of CAD and stroke, NYHA functional classification, and DBP (Table 2). TNT and CRP baseline level positively correlated with NT-proBNP levels at any time points (Figure 4(a), $p < 0.001$). And NT-proBNP concentration was elevated at all time points when baseline eGFR declined below 90 mL/min (Figure 4(b)).

3.4. NT-proBNP Changes for Predicting the Adverse Outcome of AHF Patients. Because NT-proBNP changes within the first 3 days were stable and more predictive for adverse outcomes, we compared the predictive power of single measurement of NT-proBNP (at days 1, 2, and 3) versus dynamic changes, indicated as absolute or relative changes. ROC curve was used to evaluate the adverse outcome and AUC (95% CI) increased over time (at admission, days 2 and 3),

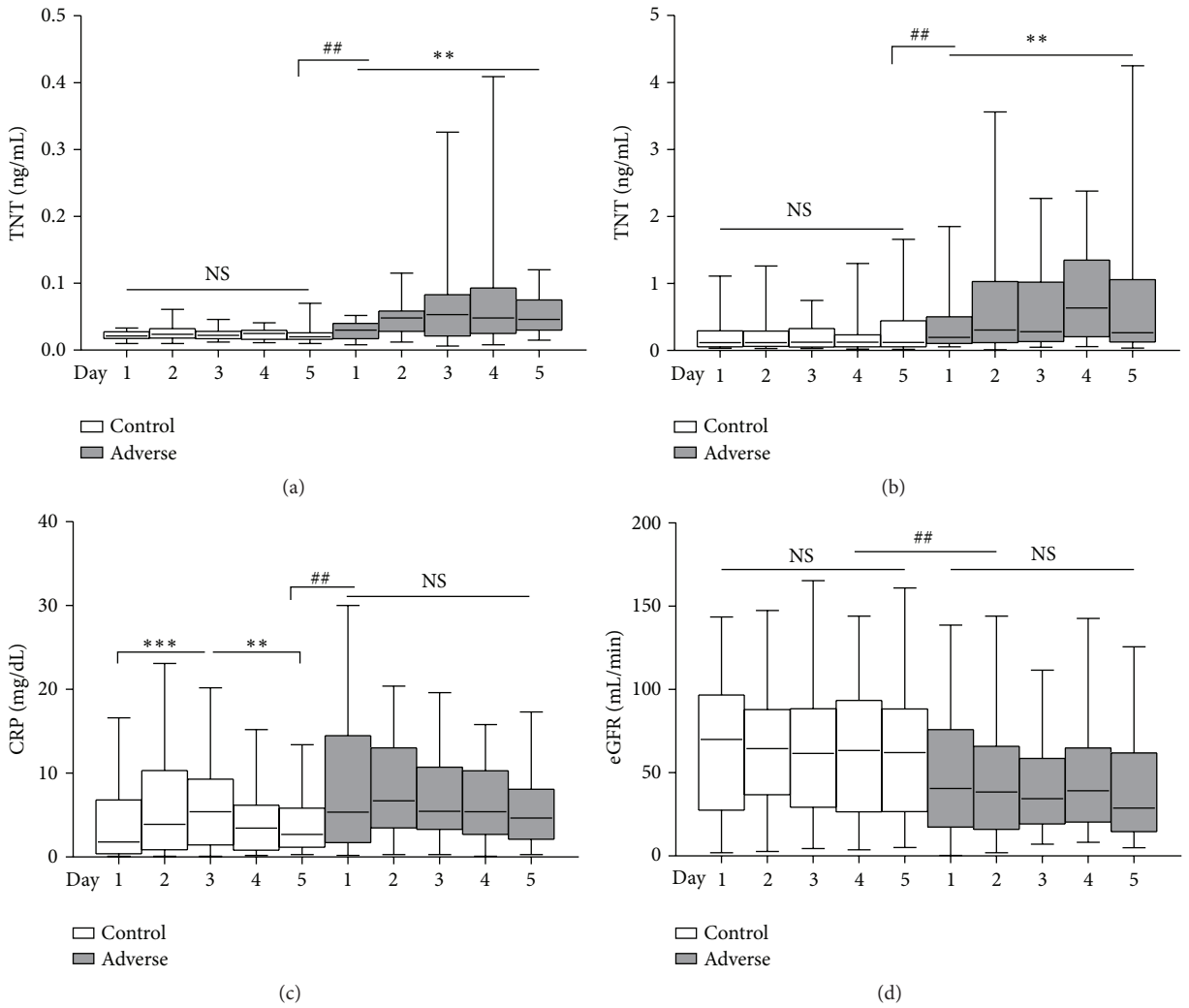

Figure 3: Changes of TNT, CRP, and eGFR over time between AHF patients with different outcomes. TNT changes categorized by percentile at baseline: (a) the first tertile: control, $p > 0.05$, and adverse, $p = 0.007$; (b) the upper two tertiles: control, $p > 0.05$, and adverse, $p = 0.005$; (c) changes of CRP over time: control, days 1 to 3, $p < 0.001$, days 3 to 5, $p = 0.003$, and adverse, $p > 0.05$; and (d) changes of eGFR over time: control, $p > 0.05$, and adverse $p > 0.05$. $^{***}p < 0.001$, $^{**}p < 0.01$ (significance in the same group); $^{\#\#}p < 0.01$ (significance between different groups). NS: not significant.

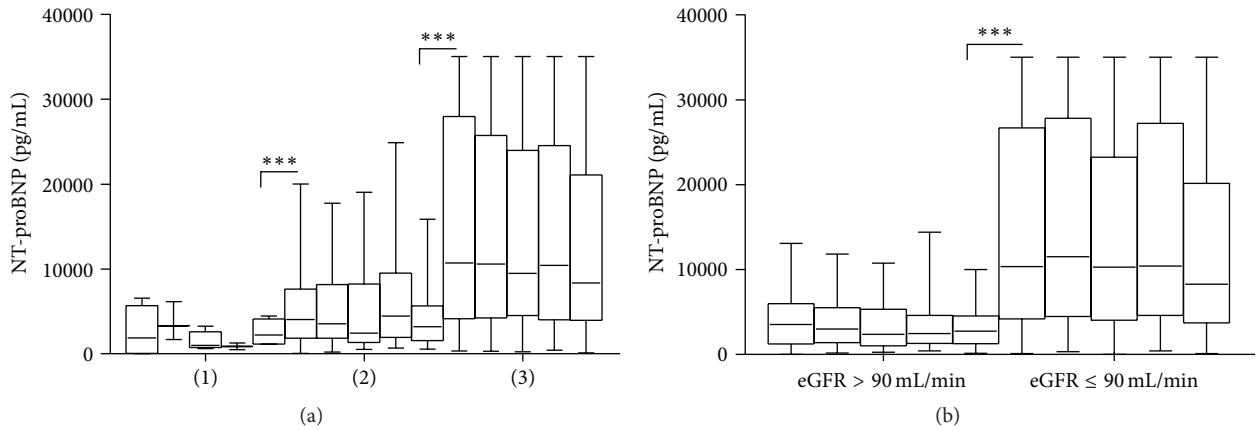

Figure 4: Changes of NT-proBNP level at admission, days 2, 3, 4, and 5 in patients: (a) (1) without TNT elevation: TNT < 0.01 ng/mL; (2) with TNT elevation, but without elevated CRP: TNT ⩾ 0.01 ng/mL and CRP < 1.00 mg/dL; (3) with elevation of both: TNT ⩾ 0.01 ng/mL and CRP ⩾ 1.00 mg/dL. (b) With eGFR decline and without. $^{***}p < 0.001$.

Table 2: NT-proBNP baseline related to baseline factors.

	Median pg/mL (Q1–Q3)	p value
Age		
<65 years	7994 (3320–19961)	0.011
<75 years	10800 (4160–28062)	
<85 years	5319 (2238–10091)	
≥85 years	7689 (3588–14203)	
NYHA		
I to II	7689 (2323–16930)	0.578
III to IV	7733 (3375–18439)	
CAD		
No	8684 (3889–19139)	0.094
Yes	7253 (2580–13827)	
Stroke		
No	7602 (3074–17651)	0.710
Yes	8263 (3319–19665)	
DBP		
<78 mmHg	7295 (2932–17520)	0.164
≥78 mmHg	7948 (3320–19791)	
TNT		
<0.01 ng/mL	1270 (31–4474)	0.003
≥0.01 ng/mL	7744 (3214–17818)	
CRP		
<1.00 mg/dL	5105 (2007–9551)	<0.001
≥1.00 mg/dL	10437 (3921–27245)	
eGFR		
≥90 (mL/min)	4075 (1505–7492)	<0.001
<90 (mL/min)	10158 (4170–26902)	

Abbreviations are the same as shown in Table 1.

Table 3: Significant predictors for adverse outcome among AHF patients.

	OR	95% CI	p value
Age* (65 years, +10 years)	2.032	1.147–3.601	0.015
History of CAD	4.448	1.524–12.979	0.006
Baseline CRP (1 increment on log scale)	1.856	1.420–2.425	<0.001
NT-proBNP at day 3 (1 increment on log scale)	2.905	1.583–5.331	0.001

*Age: centered at 65 years and 1 increment on 10-year scale.

which was 0.608 (0.532 to 0.681), 0.695 (0.621 to 0.762), and 0.730 (0.657 to 0.794), respectively. And the cutoff value of NT-proBNP was 8459 pg/mL at day 3. The absolute or relative changes have not improved the prognostic power with 0.680 (0.605 to 0.748) and 0.686 (0.612 to 0.754). In logistic regression model, NT-proBNP concentration at day 3 was an independent predictor for the adverse outcome (OR 2.185, 95% CI: 1.584–3.015), along with advanced age and history of CAD and CRP (Table 3). Variables associated with the adverse outcome with $p < 0.05$ entered as confounding factors in multivariate analysis.

3.5. Classified Changes of NT-proBNP. Adverse outcome rates for AHF patients classified by categorical changes of NT-proBNP were presented in Figure 5(a). Adverse outcome rate of patients in high to low group (threshold of 8459 pg/mL) was close to those of low to low group (25.0% versus 25.6%) and higher in patients of low to high group than those in group of high to high (70.6% versus 58.2%).

Patients with decreased level of NT-proBNP (high to low) had a risk for adverse outcome with OR of 0.967, 95% CI: 0.238–3.928, similar to those in the reference group (Figure 5(b)). In contrast, patients with worsened level of NT-proBNP had an obviously increased risk of adverse outcome (low to high, OR: 6.960, 95% CI: 2.181–22.213, $p < 0.001$), higher than those remaining at high level (high to high, OR: 4.039, 95% CI: 2.000–8.157, $p = 0.001$). The percentage of patients taking ACEI/ARB and beta-blockers was higher in control group than that in adverse group (45.2% versus 17.9%; 55.9% versus 38.9%). And the proportion of patients taking vasoactive drugs and morphine was lower in control group than that in adverse group (33.5% versus 61.1%; 13.8% versus 34.7%). It suggests that ACEI/ARB and beta-blockers would have protective activity in short-term management of AHF patients (Table 4).

4. Discussion

Acute heart failure is characterized by the acute episodes of signs and symptoms because of decompensation of cardiac function, needing emergency treatment. Mortality rate in hospital reaches up to 10% [15]. NT-proBNP is the well-established marker for HF, and its baseline level is predictive for patients with adverse outcomes in short-term [16, 17]. Lindahl and his colleagues found that serial measurements of NT-proBNP could improve its prognostic utility with increasing odds ratio over time [7]. Although the changes in laboratory parameters are important within hours of AHF, it is also necessary to analyze them within the first few days. The purpose of our study is to describe dynamic trends of several main indexes related to outcome of AHF, to warn us to pay close attention on clinical management in the future, and finally to decrease the mortality. In the present study, NT-proBNP level at day 3 had the best predictive accuracy within the first 3 days, suggesting that dynamic monitoring NT-proBNP is instructive for AHF management. Moreover, classified changes of NT-proBNP at day 3 from baseline would improve the predictive value of its single determination in patients. And this stratification strategy is also used in a different setting of 116 AHF patients, in which NT-proBNP was tested at different time points with intervals from the beginning of treatment to discharge [18]. And their results showed that absolute changes at discharge from baseline had the best predictive power, similar to conclusions from other investigations [19]. Our analysis showed that the usage of stratification strategy in NT-proBNP was more helpful for management of AHF patients.

As shown, higher level of TNT at baseline was associated with higher baseline and elevation of NT-proBNP over time. We know that plasma TNT level is well established for MI [20]. Elevated TNT level may be due to ongoing of the

Table 4: Management of AHF patients between different clinical outcomes.

Emergency treatment at admission	Control	Adverse outcome	p value
Diuretics, n (%)	169 (89.9)	87 (91.6)	0.649
ACEI/ARB, n (%)	85 (45.2)	17 (17.9)	<0.001
Beta-blockers, n (%)	105 (55.9)	37 (38.9)	0.007
Vasoactive drugs, n (%)	63 (33.5)	58 (61.1)	<0.001
Morphine, n (%)	26 (13.8)	33 (34.7)	<0.001
Digoxin, n (%)	78 (41.5)	39 (41.1)	0.944
Nitrates, n (%)	120 (63.8)	55 (57.9)	0.332
Oxygen supply, n (%)	168 (89.4)	86 (90.5)	0.760
Antiasthmatic, n (%)	111 (59.0)	58 (61.1)	0.745

ACEi: angiotensin-converting enzyme inhibitor; ARB: angiotensin receptor blocker.

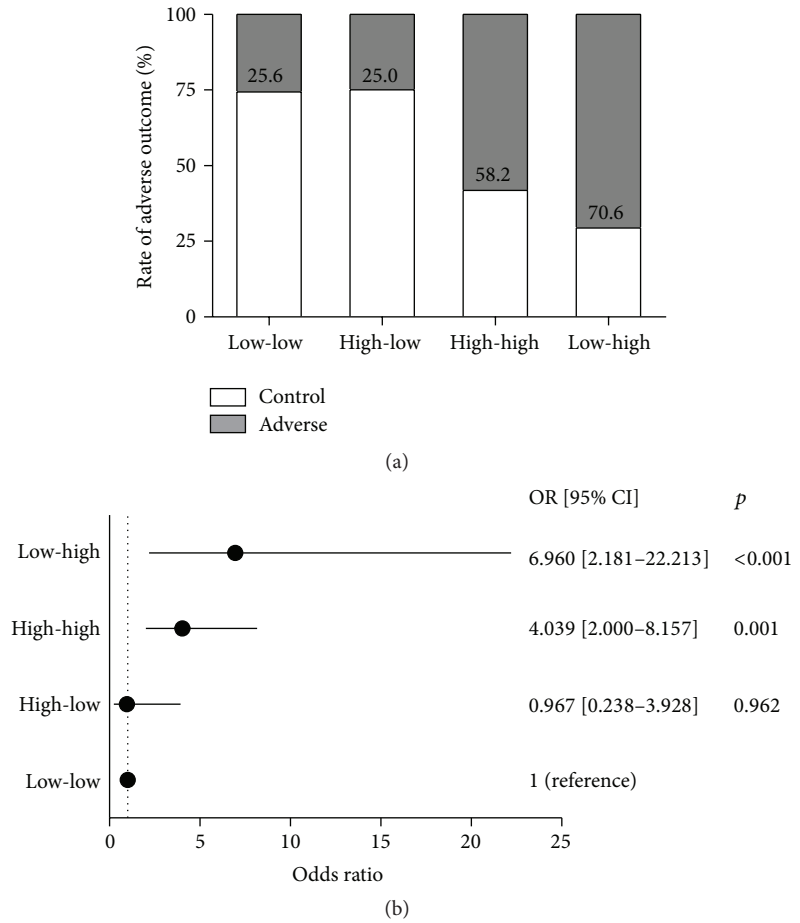

Figure 5: Classified changes of NT-proBNP. (a) Percentage and (b) odds ratio of classified changes of NT-proBNP for the adverse outcome of AHF patients.

established myocardial necrosis or large infarction area [21, 22]. Both of them could result in the dysfunction of left ventricular and then the subsequent NT-proBNP elevation at baseline. In the present study, TNT remained at low level (baseline median = 0.052 ng/mL) in control group; however, it worsened over time (baseline median = 0.104 ng/mL) in patients with adverse outcome. In addition, NT-proBNP and TNT sharing the similar trends in adverse group may illustrate the reason above.

More and more researches showed that CRP may be involved in HF development through activating complement system, stimulating cytokine generation, and then resulting in myocyte loss and functional deterioration [23–25]. Our results showed that CRP in control group rose at first and

then descended and the level overall was lower than that in adverse group. Moreover, CRP level had an influence on NT-proBNP because elevation of baseline CRP caused elevation of NT-proBNP at baseline and all the time points. Tumor necrosis factor-alpha and interleukin-6 were reported to associate with myocardial dysfunction, then mediating the expression of BNP and NT-proBNP [26, 27]. CRP can also be upregulated by interleukin-6. Therefore, it could explain the association between CRP level and NT-proBNP changes.

The mechanism of ACEIs and ARBs on renin angiotensin system (RAS) is different. ACEI inhibits the angiotensin-converting enzyme and ARBs inhibits Ang II activation through competitively binding to AT1 receptor. Consequently, vascular resistance is reduced, aldosterone is released, and salt and water retention is prevented, causing reduced cardiac preload and afterload. As reported, ARB drugs gave limited endpoints benefits to patients with myocardial infarction and cardiovascular disease, compared with placebo [28, 29]. However, other investigations showed that ACEI/ARB and beta-blockers drugs can significantly reduce all-cause mortality and cardiovascular mortality [30–33]. Consistent with them, our results showed that in control group the proportion of patients who received ACEI/ARB drugs and beta-blockers was significantly higher than that in adverse group, further determining their protective roles in management of AHF patients. Morphine use was considered to be associated with increased in-hospital death of AHF patients [34, 35]. Besides, a review from Ruiz-Laiglesia and Camafort-Babkowski suggested that vasoactive drugs may give benefits in the short term but cause mortality in the long term [36]. Combined with our results, morphine and vasoactive drugs should be used with caution in AHF.

5. Study Limitations

This study is a single-center and retrospective control study. Collecting complete continuous data was difficult. Only 85 AHF had complete data sets at all 5 time points, 176 AHF with all first 3 time points, and 284 AHF with at least 3 of 5 time points. Many patients were excluded from this research for without baseline or with only one measurement or died within 24 hours. Second, as a retrospective investigation, we are not sure whether specific treatment influences the outcomes. Finally, sequential monitoring of lab indexes for AHF needs to be assessed and validated in multicenter and large sample prospective research.

6. Conclusions

In this single-site study with relative small samples, the prognostic value was better in dynamic assessment of NT-proBNP than baseline level. Sequential monitoring of laboratory indexes within the first 3 days of AHF may be helpful for guiding clinical management of AHF patients.

Abbreviations

ACEi: Angiotensin-converting enzyme inhibitor
AHF: Acute heart failure
ARB: Angiotensin receptor blocker
CAD: Coronary artery disease
CHF: Chronic heart failure
CI: Confidential interval
CK: Creatine kinase
CKD: Chronic kidney disease
CK-MB: Creatine kinase isoenzyme
COPD: Chronic obstructive pulmonary disease
CRP: C-Reactive protein
DBP: Diastolic blood pressure
eGFR: Estimated glomerular filtration rate
LDH: Lactic dehydrogenase
LVEF: Left ventricular ejection fraction
MI: Myocardial infarction
NT-proBNP: N-Terminal probrain natriuretic peptide
OR: Odds ratio
NYHA: New York Heart Association
Q: Quartile
ROC: Receiver-operating characteristic curve
RR: Respiratory rate
SBP: Systolic blood pressure
TNT: Troponin T.

Competing Interests

The authors declare no conflict of interests related to financial issues or contribution.

Authors' Contributions

Yaping Tian was chiefly responsible for the investigation. Yaping Tian, Kunlun He, Yurong Wang contributed to the conception and designation of the study. Yurong Wang, Lei Fu, Qian Jia, Hao Yu, and Xueliang Huang collected the data for this analysis. Yurong Wang analyzed the data, prepared figures and tables, and wrote the paper. Pengjun Zhang and Chunyan Zhang helped performing the analysis and carried out constructive discussions. All authors reviewed and approved the final version of this paper.

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Research Article

A Nested Case-Control Study of Association between Metabolome and Hypertension Risk

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We aimed to explore novel small metabolites that associated with hypertension risk in a population-based nested case-control study. Among 460 individuals with optimal blood pressure (<120/80 mmHg) at baseline, 55 progressed to hypertension during 5 years of follow-up. Twenty-nine cases of incident hypertension and 29 controls, matched for age, sex, and baseline systolic blood pressure, were included in this study. Serum metabolites were measured by gas chromatography-tandem mass spectrometry. *t*-test and logistic regression analysis were applied to investigate the association between metabolites and incident hypertension. Among the 241 metabolites identified in this study, baseline levels of 26 metabolites were significantly different between hypertension and control groups. After adjusting for body mass index, smoking, and drinking, 16 out of the 26 metabolites were still associated with hypertension risk including four amino acids. Amino acids were negatively associated with risk of future hypertension, with odds ratio (OR) ranging from 0.33 to 0.53. Two of these amino acids were essential amino acids including threonine and phenylalanine. Higher level of lyxose, a fermentation product of gut microbes, was associated with higher risk of hypertension. Our study identified multiple metabolites that associated with hypertension risk. These findings implied that low amino acid levels and gut microbiome might play an important role in the pathogenesis of hypertension.

1. Introduction

Hypertension is a key risk factor for cardiovascular diseases. It is estimated that 2.33 million cardiovascular deaths were attributable to increased blood pressure (BP) in China [1]. With the economic development and lifestyle changes in recent years, there has been a dramatic increase in the prevalence of hypertension in China [2]. Prevention of hypertension can help to reduce the public health burden of cardiovascular diseases [3]. It is critical to understand the mechanisms underlying elevated BP and develop reliable prevention strategies for populations at high risk of developing hypertension.

Hypertension is a metabolic disease and its pathophysiology is still unclear. Recent advances in metabolomic technologies have enhanced the feasibility of acquiring high-throughput profiles of a whole organism's metabolic status [4]. As these techniques allow assessment of large numbers of small metabolites that are substrates and products in metabolic pathways, metabolomics can particularly increase the understanding of the pathophysiology of metabolic diseases such as hypertension [5]. Although some studies have evaluated metabolomic differences in participants with and without hypertension [6–11], few have sought to identify baseline metabolites that predict future hypertension [12]. Metabolites identified in these studies that associated with hypertension were not well replicated. There are great metabolic differences between populations [8] and no study has explored the relationship between metabolites and risk of hypertension in Chinese population, which has the largest number of hypertensive patients [13]. Therefore, we aimed to explore small metabolites that independently associated with incident hypertension in a cohort based nested case-control study. In addition, we used pathway analysis to identify possible metabolic pathways implicated in the development of hypertension.

2. Materials and Methods

2.1. Study Participants. Participants were recruited from the Chinese Multiprovincial Cohort Study- (CMCS-) Beijing Project [14], part of the nationwide population-based CMCS study investigating the risk factors related to the incidence of cardiovascular diseases [15]. The flow chart of participant selection was presented in Fig. S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/7646979. Of the 1478 participants who were free of hypertension and completed the baseline examination in 2002, 1133 took part in follow-up examination in 2007, with a follow-up rate of 76.7%. Among them, 460 were with optimal BP (<120/80 mmHg) at baseline. Fifty-five of these 460 participants developed hypertension and 208 participants were still with optimal BP in 2007. In this nested case-control study, 34 cases were randomly selected from the 55 participants who had optimal BP in 2002 and developed incident hypertension during follow-up. For each hypertensive case, we selected a control with optimal BP in both 2002 and 2007 and matched for gender, age (±3 years), and baseline systolic BP (±5 mmHg). Hypertension was defined as BP ≥ 140/90 mmHg and/or on antihypertensive therapy [16]. After excluding participants with more than 80% of metabolites below the detection limit or missing (five cases and five controls), 58 participants (29 cases and 29 controls) were eligible for final analysis. There was no significant difference in baseline characteristics between included participants and those excluded (Table S1).

All participants signed informed consent, and the Ethics Committee of Beijing An Zhen Hospital, Capital Medical University, reviewed and approved the protocols of this study.

2.2. Risk Factor Survey. The surveys in 2002 and 2007 were both conducted based on the WHO-MONICA (Monitoring of Trends and Determinants in Cardiovascular Disease) protocol for risk factor surveys. A standard questionnaire was designed to collect information on demographic characteristics, status of smoking and alcohol drinking, and personal medical history. Current smoking was defined as having smoked at least one cigarette per day in the past year. Drinking was defined as drinking at least once a week. Anthropometric measurements were recorded during physical examination. Body mass index (BMI) was calculated as weight in kilograms divided by height squared in meters. BP was measured in the right arm at a sitting position with a regular mercury sphygmomanometer after resting for at least 5 min. The mean value of two consecutive BP readings was used.

2.3. Laboratory Assays. Fasting total cholesterol (TC), triglyceride (TG), and fasting blood glucose (FBG) were determined by enzymatic methods. Low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) were measured by homogeneous assay (Daiichi, Tokyo, Japan). Overnight fasting venous blood samples were collected for laboratory measurements. To avoid the introduction of any analytical bias due to sample preparation, all samples were kept at room temperature to clot for 30 minutes and then put in ice box. Blood samples were centrifugated for 10 min (4000 ×g, 25°C) within three hours after blood collection. Serum was frozen at −80°C until execution of metabolomic analyses.

Metabolite profiles were measured using the gas chromatography-tandem mass spectrometry (GC/MS) method. Serum samples were thawed at room temperature for 15 min and vortex-mixed for 5 s. For GC/MS measurement, 300 μL pure ethanol (HPLC grade: Sigma-Aldrich, St. Louis, MO, USA) was added to a 100 μL serum sample in an Eppendorf tube. The mixture was vortex-mixed for 30 s and allowed to stand for 20 min at 4°C. After 80 μL pure methanol (HPLC grade: Sigma-Aldrich) was added, the mixture was vortex-mixed for another 30 s and centrifuged at 4°C at 12000 rpm for 15 min. Then, 150 μL of the supernatant was transferred to a screw vial and 10 μL dichlorophen (0.02 mg/mL: Sigma-Aldrich) was added as the internal standard. The mixture was evaporated to dry under a stream of nitrogen gas (4°C). After 30 μL methoxyamine pyridine hydrochloride (20 mg/mL) was added into the screw vial, the mixture was vortex-mixed for 30 s and oximated at 37°C for 90 min. We added 30 μL *N,O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (Sigma-Aldrich) to each vial and left the mixture to react at 70°C for 60 min. The samples were allowed to stand for 15 min at room temperature before GC/MS analysis.

A 1 μL aliquot of derivatized sample was injected in splitless mode into an Agilent 7890A GC system equipped with a 30 m × 0.25 mm × 0.25 μm capillary column (Agilent J&W Scientific, Folsom, CA, USA). The injector temperature was set at 280°C and helium was used as carrier gas. The column temperature was initially kept at 80°C for 2 min, then increased from 80°C to 320°C at 10°C min^{-1}, and held for 6 min. The column effluent was introduced into ion source of an Agilent 5975C mass detector. The ion source temperature was set at 230°C and the MS quadrupole temperature at 150°C [17]. Masses were acquired from 50 to 550 *m/z*. For quality control, we performed metabolite profiling in the same quality control serum sample, enabling detection of temporal drift in instrument performance. Each of these injections into the mass spectrometer was generated from a distinct 10 μL aliquot of pooled serum, extracted, and processed individually. The coefficients of variations (CVs) for each metabolite across a total of six replicates of quality control serum samples were calculated. Seventy-eight percent of the metabolites had CVs of less than or equal to 20%.

GC/MSD ChemStation Software (Agilent, Shanghai, China) was used for autoacquisition of GC total ion chromatograms and fragmentation patterns. Each compound had a fragmentation pattern comprising a series of split molecular ions; the mass charge ratios and their abundance were compared with a standard mass chromatogram in the National Institute of Standards and Technology (NIST) mass spectra library by the ChemStation Software. For each peak, the software generated a list of similarities comparing with every substance within the NIST library. Peaks with similarity index more than 70% were assigned compound names.

2.4. Statistical Analyses. Natural logarithm transformation was performed for continuous variables to minimize the

skewedness of distribution if necessary. Metabolites with <50% BDL/missing observations were treated as continuous variables in the analyses; and metabolites with 50%–80% BDL/missing observations were treated as ordinal variables. A two-sample t-test or Wilcoxon rank-sum test was used for comparison of metabolite levels between hypertension and control groups. We also performed logistic regression analysis to investigate the association between metabolites and incident hypertension, adjusting for BMI, smoking, and drinking. A sample size of 58 (29 in hypertension group and 29 in control group) has a power of 0.88 to detect the difference between two groups, assuming the mean concentrations of 11.6 ± 2.57 and 9.65 ± 2.15 μmol/L in hypertension and control groups, respectively [18], with a significance level of 0.05. A Bonferroni procedure was used to correct for multiple comparisons and a significance level of 2.07×10^{-4} (2-tailed) was considered for each individual test. Statistical analysis was performed with SAS 9.2 (SAS Institute Inc., Cary, NC, USA).

Metabolite set enrichment analysis was performed by MetaboAnalyst 2.0, a comprehensive, web-based tool designed for processing, analyzing, and interpreting metabolomic data [19]. Overrepresentation analysis was implemented using the hypergeometric test to evaluate whether a particular metabolite set was represented more than that expected by chance within a given compound list [19]. Metabolites with significant P values for hypertension risk were given as input in our study.

3. Results

3.1. Baseline Characteristics. Of the 1133 participants who were free of hypertension and were followed up for 5 years, 460 were with optimal BP at baseline. Among them, 55 developed hypertension during follow-up. Baseline characteristics of 29 new hypertensive cases and 29 controls included in this study are shown in Table 1. Age, gender, and systolic BP were matched variables. There were no significant differences in BMI, diastolic BP, FBG, TC, LDL-C, HDL-C, CRP, and creatinine at baseline between cases and controls.

3.2. Baseline Metabolite Levels and Risk of Hypertension. A total of 241 metabolites were identified in this study and the associations between baseline metabolite levels and risk of new onset hypertension were evaluated. Baseline levels of 26 metabolites were different between hypertension and control groups, including eight amino acids, seven carbohydrates, four carboxylic acids, three phenols, and four metabolites of other classes (Table 2). Five of these metabolites remained significant after Bonferroni correction including threonine ($P = 1.78 \times 10^{-4}$), talose ($P = 9.01 \times 10^{-5}$), lyxose ($P = 4.26 \times 10^{-5}$), methylmalonic acid ($P = 2.37 \times 10^{-5}$), and malonic acid ($P = 1.24 \times 10^{-5}$), and one with marginal significance (galactose, $P = 5.71 \times 10^{-4}$). Hypertension cases had lower baseline levels of amino acids than controls. After adjusting for BMI, smoking, and drinking, 16 out of the 26 metabolites were still significantly associated with hypertension risk and two of them were essential amino acids including threonine (odd ratio (OR): 0.33, 95% CI: 0.16–0.70, $P = 1.78 \times 10^{-4}$) and phenylalanine (OR: 0.49, 95% CI: 0.26–0.91, $P = 1.12 \times 10^{-2}$). A higher baseline level of lyxose, a fermentation product of gut microbes, was associated with higher risk of hypertension (OR: 2.88, 95% CI: 1.44–5.73, $P = 4.26 \times 10^{-5}$) (Table 2).

3.3. Relationship between Baseline Levels of Metabolites. We assessed correlations between baseline levels of 26 metabolites associated with hypertension risk. Amino acids were positively correlated with phenols and negatively correlated with carbohydrates and carboxylic acids (Figure 1). Phenylalanine was positively correlated with downstream metabolites within the phenylalanine and tyrosine metabolism pathway including tyrosine ($r = 0.73$) and norepinephrine ($r = 0.39$). Talose level was positively correlated with methylmalonic acid ($r = 0.57$) and malonic acid ($r = 0.64$) and negatively correlated with threonine ($r = -0.52$).

Pathway enrichment analysis was performed using the 26 metabolites associated with hypertension risk to identify novel pathways implicated in hypertension. Three metabolic pathways were identified, including metabolism of phenylalanine, tyrosine, and tryptophan biosynthesis ($P = 1.92 \times 10^{-3}$), aminoacyl-tRNA biosynthesis ($P = 4.91 \times 10^{-3}$), and nitrogen metabolism ($P = 5.57 \times 10^{-3}$) (Table 3). Of note, four out of five metabolites involved in these pathways were amino acids.

4. Discussion

Our study aimed to investigate human serum metabolites and hypertension risk in a well-defined nested case-control setting in a Chinese population. This nested case-control study examined metabolite profiles associated with the risk of hypertension. We identified a panel of metabolites whose baseline levels were associated with future development of hypertension. We found that participants who developed hypertension had reduced serum levels of amino acids, which implied that amino acids play an important role in development of hypertension. In addition, a gut microbial metabolite, lyxose, was associated with an elevated risk of hypertension.

It is worth noting that low levels of amino acids were associated with higher risk of hypertension in our study and two of them are essential amino acids (threonine and phenylalanine). Essential amino acids cannot be synthesized by the body, and levels of these amino acids in the body may mainly be decided by dietary protein intake. Recent studies suggest that inadequate intake of protein may lead to a shortage of essential amino acids and a subsequent elevation in BP [20, 21]. Evidence from randomized clinical trials indicates that administration of amino acids can improve endothelial function and decrease peripheral vascular resistance, resulting in decreased BP [22]. Amino acid metabolism may also regulate BP through insulin signaling, as it is essential to normal pancreatic β-cell function and insulin secretion [23]. In recent decades, the dietary pattern has changed rapidly in China. Data from the China Health and Nutrition Survey showed that the percentage of energy consumed from fat and protein (especially animal protein) had

Table 1: Baseline characteristic of study participants.

Characteristics	Individuals who developed hypertension ($n = 29$)	Individuals with optimal blood pressure ($n = 29$)	P^*
Age (years)	52.1 ± 4.2	51.9 ± 4.1	0.84
Men (%)	44.8	41.4	0.79
BMI (kg/m^2)	23.2 ± 2.6	24.0 ± 2.8	0.31
SBP (mmHg)	110.8 ± 6.6	110.2 ± 6.4	0.72
DBP (mmHg)	72.9 ± 4.1	72.4 ± 4.8	0.79
FBG (mg/dL)	83.9 ± 12.7	82.4 ± 7.9	0.60
TG (mg/dL)	84 (65, 106)	96 (64, 111)	0.64
TC (mg/dL)	202.9 ± 35.1	199.9 ± 30.0	0.73
LDL-C (mg/dL)	121.1 ± 30.3	116.5 ± 23.1	0.52
HDL-C (mg/dL)	56.6 ± 10.7	60.7 ± 15.9	0.26
CRP (mg/dL)	0.60 (0.30, 0.81)	0.68 (0.25, 1.17)	0.30
Creatinine (mg/dL)	0.99 ± 0.17	0.96 ± 0.18	0.54
Smoking (%)	20.7	10.3	0.47
Drinking (%)	13.8	6.9	0.67

BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; FBG: fasting blood glucose; TG: triglycerides; TC: total cholesterol; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; CRP: C reactive protein. Data are expressed as a percent for categorical variables, as mean ± standard deviation for continuous variables in cases of normally distributed data, and as medians (interquartile ranges) otherwise. $^*P$ values for the difference between two groups.

Table 2: Association of metabolites with incident hypertension.

Class	Metabolite	RT	Fold change	P	OR	P_{adj}
Amino acids and derivatives	Threonine	21.87	0.12	1.46×10^{-4}	0.33 (0.16–0.70)	1.78×10^{-4}
	Nicotinoyl glycine	8.28	0.49	2.31×10^{-2}	0.52 (0.28–0.96)	1.21×10^{-2}
	Glycine	8.58	0.36	2.71×10^{-2}	0.78 (0.44–1.38)	1.31×10^{-1}
	Phenylalanine	13.02	0.70	3.44×10^{-2}	0.49 (0.26–0.91)	1.12×10^{-2}
	S-Carboxymethylcysteine	24.78	0.49	4.03×10^{-2}	0.58 (0.32–1.05)	6.56×10^{-2}
	Tyrosine	16.70	0.58	4.76×10^{-2}	0.73 (0.42–1.29)	3.79×10^{-1}
	Aspartic acid	21.22	0.55	4.92×10^{-2}	0.53 (0.28–0.99)	2.38×10^{-2}
	Gly-Pro	13.38	0.40	4.99×10^{-2}	0.71 (0.4–1.23)	1.02×10^{-2}
Carbohydrate	Talose	16.88	10.88	1.69×10^{-6}	11.64 (3.39–39.96)	9.01×10^{-5}
	Lyxose	14.47	2.29	1.24×10^{-4}	2.88 (1.44–5.73)	4.26×10^{-5}
	Galactose	16.49	0.22	2.81×10^{-4}	0.34 (0.17–0.7)	5.71×10^{-4}
	Glucose-1-phosphate	16.41	1.04×10^{-6}	1.06×10^{-3}	0.34 (0.16–0.71)	2.40×10^{-3}
	Methyl-beta-D-galactopyranoside	14.32	0.37	6.73×10^{-3}	0.28 (0.13–0.6)	1.20×10^{-3}
	Dihydroxyacetone	7.06	0.50	3.12×10^{-2}	0.62 (0.34–1.11)	9.84×10^{-2}
	Melezitose	23.25	0.48	3.81×10^{-2}	0.54 (0.29–1.01)	8.38×10^{-2}
Carboxylic acids and derivatives	Methylmalonic acid	8.13	3.04	1.47×10^{-7}	5.24 (2.1–13.03)	2.37×10^{-5}
	Malonic acid	11.16	12.47	3.29×10^{-5}	12.55 (3.4–46.39)	1.24×10^{-4}
	Shikimic acid	14.33	5.56	5.85×10^{-3}	5.06 (2.07–12.33)	3.00×10^{-4}
	Oxalic acid	6.19	0.56	3.29×10^{-2}	0.57 (0.31–1.02)	7.11×10^{-2}
Phenols and derivatives	Thymol	14.17	0.31	4.14×10^{-3}	0.43 (0.23–0.83)	6.00×10^{-3}
	Noradrenaline	8.45	0.49	7.05×10^{-3}	0.46 (0.24–0.87)	5.49×10^{-3}
	2-Aminophenol	9.19	0.34	1.02×10^{-2}	0.55 (0.29–1.01)	5.44×10^{-2}
Others	2-Methoxyestrone	19.65	0.57	1.74×10^{-2}	0.39 (0.2–0.76)	8.71×10^{-3}
	Alpha-tocopherol	27.36	0.60	3.23×10^{-2}	0.47 (0.25–0.89)	3.82×10^{-2}
	Octadecanol	19.08	0.56	3.98×10^{-2}	0.64 (0.36–1.14)	2.47×10^{-1}
	2-Aminoethanethiol	6.08	0.59	4.26×10^{-2}	0.59 (0.32–1.06)	9.38×10^{-2}

RT: retention time; fold change: ratio of metabolite levels between case and control groups; OR: odds ratio; P_{adj}: P value adjusted for BMI, smoking, drinking, creatinine and C reactive protein, and postmenopausal status.

Table 3: Pathways associated with risk of hypertension.

Pathway	Total	Expected	Hit	Metabolites	P^*
Phenylalanine, tyrosine, and tryptophan biosynthesis	27	0.26	3	Phenylalanine, tyrosine, shikimic acid	1.92×10^{-3}
Aminoacyl-tRNA biosynthesis	75	0.72	4	Phenylalanine, glycine, threonine, tyrosine	4.91×10^{-3}
Nitrogen metabolism	39	0.37	3	Phenylalanine, tyrosine, glycine	5.57×10^{-3}

Total: the total number of compounds in the pathway. Expected: the expected matched number from the 26 metabolites associated with hypertension. Hit: the actual matched number from the 26 metabolites associated with hypertension. $^*P$: P value calculated from enrichment analysis.

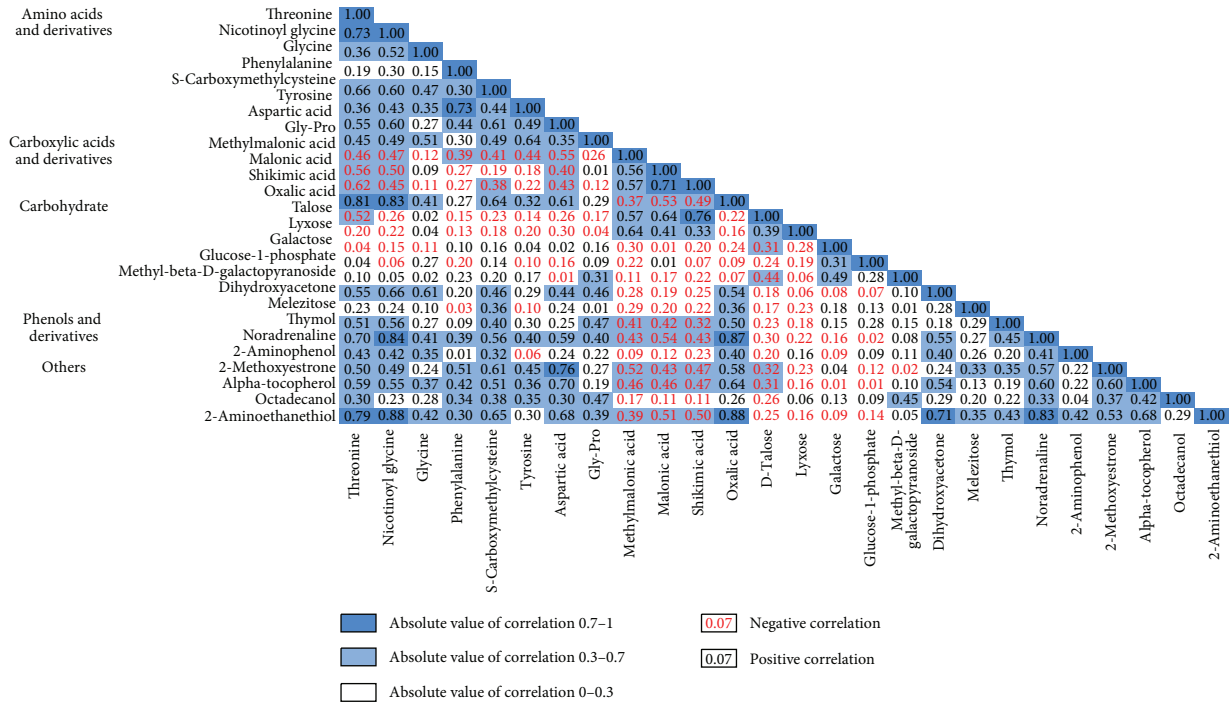

Figure 1: Correlation matrix for serum metabolite levels. This figure shows Spearman correlation coefficients for baseline levels of 26 metabolites that were different between hypertension and control groups. Correlation coefficients in red represent a negative correlation and in black represent a positive correlation.

been increased in the past decades, while energy consumed from carbohydrate had decreased in China [24, 25]. However, protein intakes of majority of population were still below the amount recommended by dietary guidelines.

The effects of threonine and phenylalanine on hypertension are supported by findings from experimental studies. In Sprague-Dawley rats, threonine-deficient diets can induce a specific uncoupling of mitochondria [26] and reduce mitochondrial ATP production. Attenuated intracellular ATP content results in elevated BP by increased sympathetic nervous system activation, whereas augmented reactive oxygen production following mitochondrial dysfunction lowers the capacity of nitric oxide-dependent vascular relaxation [27, 28]. Phenylalanine intervention could exert an antihypertension effect on spontaneously hypertensive rats (SHR) [29]. The antihypertensive action of phenylalanine observed in SHR could be explained by its direct and specific antiproliferative effect on vascular smooth muscle cells [30]. Our study demonstrated that in humans depletion of phenylalanine can also influence metabolites in its downstream pathway. In our study, baseline phenylalanine was significantly depleted in patients with hypertension, and along with its depletion there was downregulation of many metabolites within the phenylalanine and tyrosine metabolic pathway (such as tyrosine and norepinephrine).

Our study illustrates the power of untargeted metabolic profiling to identify new metabolic pathways implicated in the development of hypertension. Three metabolic pathways were identified including phenylalanine, tyrosine, and tryptophan biosynthesis, aminoacyl-tRNA biosynthesis, and nitrogen metabolism pathways, which again emphasizes the importance of amino acid metabolism in the development of hypertension. The phenylalanine, tyrosine, and tryptophan biosynthesis pathway may take part in BP regulation through the antiproliferative effect of phenylalanine on vascular smooth muscle cells [30]. The nitrogen metabolism pathway can affect production of nitrogen compounds including nitric oxide which is evolved in BP regulation. A more

comprehensive assessment of metabolites involved in these pathways in a larger sample is warranted and could shed further light on their mechanisms in BP regulation.

A novel finding of our study was that a higher baseline lyxose level was associated with a higher risk of hypertension. Lyxose is an aldopentose, a monosaccharide containing five carbon atoms including an aldehyde functional group. Lyxose is a key component of the bacterial cell wall [31] and is also a fermentation product of gut microbes which was reported as a potential biomarker of type 2 diabetes mellitus [32]. Human gut microbes can affect the amount of energy extracted from the diet and the risk of obesity, which in turn relates to BP [33]. Moreover, the ARIC study also identified a product of microbial fermentation, 4-hydroxyhippurate, which can take part in BP regulation through oxidative stress [12]. These findings may be associated with metabolic abnormalities of gut flora and demonstrate the important role of gut microbes in the development of hypertension. Diet pattern is known to modulate the composition of the gut microbiota. Also, products of gut microbial metabolism act as signaling molecules and influence the host's metabolism [34]. Supplementing the diet that stimulates the expansion of specific microbes to improve metabolic regulation can be a therapy for metabolic diseases [34]. Recent meta-analysis of randomized, controlled trials on consumption of probiotics revealed that probiotics are a potential supplement and dietary constituent to improve blood pressure and prevent or control hypertension [35].

This study has several strong points. All individuals were free of hypertension at the time the blood samples were collected and this enabled us to investigate the association between baseline metabolites and the risk of incident hypertension. We used a matched case-control design with extreme phenotype to maximize the efficiency of the study. We also matched cases and controls for age and gender as well as baseline systolic BP and adjusted for risk factors at baseline to minimize potential confounding contributions. In addition, we applied a pathway approach to highlight the key associations between metabolic measures and hypertension.

Our study has some potential limitations. First, this is an exploratory research with small sample size and without replications study. Therefore the candidate metabolites of interest from this study should be replicated in studies using greater sample sizes. Second, to what degree the results presented here are ethnically specific is unknown. Previous studies showed there were great metabolic differences between populations [8]. Whether candidate metabolites identified from this study have a role in the development of hypertension in other populations requires further investigation. In addition, we did not include other factors that might influence the regulation of blood pressure.

In conclusion, our study identified multiple metabolites that associated with risk of new onset hypertension. These findings implied that low amino acid levels and gut microbiome might play an important role in the pathogenesis of hypertension. Further investigation is required to test whether measurements might help identify metabolic candidates for interventions to reduce hypertension risk and to elucidate the biological mechanisms of BP regulation.

Competing Interests

The authors stated that there is no conflict of interests regarding the publication of this paper. Research support played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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Clinical Study

Ramipril and Losartan Exert a Similar Long-Term Effect upon Markers of Heart Failure, Endogenous Fibrinolysis, and Platelet Aggregation in Survivors of ST-Elevation Myocardial Infarction: A Single Centre Randomized Trial

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Introduction. Blocking the renin-angiotensin-aldosterone system in ST-elevation myocardial infarction (STEMI) patients prevents heart failure and recurrent thrombosis. Our aim was to compare the effects of ramipril and losartan upon the markers of heart failure, endogenous fibrinolysis, and platelet aggregation in STEMI patients over the long term. *Methods*. After primary percutaneous coronary intervention (PPCI), 28 STEMI patients were randomly assigned ramipril and 27 losartan, receiving therapy for six months with dual antiplatelet therapy (DAPT). We measured N-terminal proBNP (NT-proBNP), ejection fraction (EF), plasminogen-activator-inhibitor type 1 (PAI-1), and platelet aggregation by closure times (CT) at the baseline and after six months. *Results*. Baseline NT-proBNP $\geq$ 200 pmol/mL was observed in 48.1% of the patients, EF $<$ 55% in 49.1%, and PAI-1 $\geq$ 3.5 U/mL in 32.7%. Six-month treatment with ramipril or losartan resulted in a similar effect upon PAI-1, NT-proBNP, EF, and CT levels in survivors of STEMI, but in comparison to control group, receiving DAPT alone, ramipril or losartan treatment with DAPT significantly increased mean CT (226.7 $\pm$ 80.3 sec versus 158.1 $\pm$ 80.3 sec, $p < 0.05$). *Conclusions*. Ramipril and losartan exert a similar effect upon markers of heart failure and endogenous fibrinolysis, and, with DAPT, a more efficient antiplatelet effect in long term than DAPT alone.

1. Introduction

Blocking the renin-angiotensin-aldosterone system in ST-elevation myocardial infarction (STEMI) patients prevents heart failure and recurrent thrombosis in particular by the use of angiotensin-converting-enzyme (ACE) inhibitors if there are no contraindications to their use [1, 2]. Early after STEMI they significantly improve outcomes, but according to guidelines their long-term use does not seem mandatory in asymptomatic STEMI patients without left ventricular systolic dysfunction or diabetes [1, 2]. An alternative to ACE inhibitors are angiotensin receptor blockers (ARBs) as demonstrated by the OPTIMAAL trial (Optimal Trial in Myocardial Infarction with the Angiotensin II Antagonist Losartan) [3].

Previous studies demonstrated similar short-term effects of losartan and ramipril in STEMI patients on markers of heart failure such as NT-proBNP and ejection fraction (EF), as well as on markers of endogenous fibrinolysis such as PAI-1 [4].

Regarding the effect on PAI-1 in the long term, studies indicated that ramipril seemed more efficient [3].

In hypertensive patients, ACE inhibitors prevent platelet aggregation, which is an important mechanism for recurrent coronary thrombosis [5–8]. Some ARBs, including losartan, exert an antiplatelet effect such as inhibition of platelet thromboxane A2-induced platelet aggregation as it was demonstrated in hypertensive patients [9]. In addition, losartan specifically prevents platelet adhesion by p-selectin blockade [9, 10].

In STEMI patients, in particular after primary percutaneous coronary intervention (PPCI), high residual platelet reactivity is associated with increased risk of recurrent

coronary thrombosis despite of dual antiplatelet therapy [11, 12]. Residual platelet reactivity can be monitored by several methods, including measuring closure times (CT) and being a simple, rapid assessment of high shear-dependent platelet function in whole blood, including platelet adhesion, activation, and aggregate formation [13–15].

Our goal was to evaluate whether six-month treatment by ramipril and losartan exerted any effect on PAI-1, ejection fraction (EF) of the left ventricle, and NT-proBNP and any antiplatelet effect, as measured by CT for the collagen/epinephrine (CEPI) in survivors of STEMI who were treated by PPCI and dual antiplatelet therapy (DAPT).

2. Methods

The study was approved by the National Ethical Committee of the Republic Slovenia (69/10/98). Written informed consent was obtained from all included patients. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki. The study was registered by Ema Europe (EudraCT number 2016-000243-14).

2.1. Patients Studied. We included patients with their first acute STEMI, admitted to the Department of Medical Intensive Care after PPCI was performed at the catheterization laboratory. Exclusion criteria were shock, severe pulmonary edema, hypotension, bronchospasm, severe infection with sepsis, acute renal and respiratory failure, prior treatment with ACE inhibitors or ARBs, and refusal [2].

After receiving written informed consent we randomized the included patients in a double-blind random fashion in to either losartan or ramipril groups—30 patients to ramipril, titrated to 10 mg daily, and 32 patients to losartan, titrated to 100 mg daily, according to blood pressure measurements. Seven patients discontinued therapy. Finally, we studied 28 patients who were randomly assigned ramipril and 27 who were assigned losartan, receiving therapy for six months.

In addition, the antiplatelet activity of the studied groups was compared to a small control group of 9 STEMI patients, treated only by DAPT without blocking the renin-angiotensin-aldosterone system. Dual antiplatelet therapy consisted of acetylsalicylic acid (ASA) and clopidogrel or ticagrelor or prasugrel.

2.2. Study Design. Our hypothesis was that no differences existed between the ramipril and losartan group of STEMI patients. To confirm the null hypothesis that no large effect size existed between the two studied groups, a sample size of more than 25 cases per group was needed (power = 0.8, alpha = 0.05).

In this prospective, randomized, double-blind monocenter study conducted at the Department of Medical Intensive Care of the University Clinical Centre Maribor in Slovenia, the studied STEMI patients were included within the first 24 hours of an in-hospital stay. Before randomization the patients were treated by PPCI and received all the treatments according to current ESC guidelines: ASA, an additional oral antiplatelet agent (either clopidogrel or ticagrelor or prasugrel), statin, and a beta blocker if indicated [1, 2, 12].

STEMI was additionally confirmed by the rise and fall of troponin I [1, 2, 16].

At the start of the study, pretreatment data were recorded, including age, gender, body mass index (BMI), prior arterial hypertension, diabetes, anterior location of acute STEMI, heart failure of Killip class ≥ II before randomization, treatment with PPCI, and treatments by oral antiplatelet agents (ASA with clopidogrel or prasugrel or ticagrelor).

At the start of the study, prior to randomization, and six months later, a physical examination and echocardiography were conducted, and blood samples were drawn.

Echocardiography was performed on an Phillips HDI 3000 ultrasound machine. We measured the ejection fraction (EF) by a modified biplane Simpson's method. The normal level for EF was 55% [17–19].

During the follow-ups over the next 6 months all the complications were recorded, in particular heart failure, which was defined as classes II–IV according to the Killip-Kimball classification [1, 2, 12]. Killip class II was characterized by protodiastolic gallop and/or tachycardia and pulmonary rales in the lungs were registered. In Killip class III signs of pulmonary edema were present, and in Killip class IV there were signs of cardiogenic shock [2, 19].

In case of pulmonary edema or cardiogenic shock the patients were excluded from the study and treated according to guidelines by the treating physician [2].

2.3. Blood Samples and Laboratory Methods. Blood samples to measure PAI-1 activity were drawn before and 6 months after randomization between 8:00 and 10:00 a.m. Blood samples were centrifuged and plasma was frozen and stored at −70°C. PAI-1 activity was measured by the chromogenic method (normal levels 0.3–3.5 U/mL, Berichrom PAI by Dade Behring, Marburg, Germany) [20, 21].

Blood samples to measure NT-proBNP were drawn just prior to and 6 months after randomization between 8:00 and 10:00 a.m. Plasma NT-proBNP levels were measured by the electrochemiluminescence immunoassay on an Elecsys 2010 analyzer (Roche Diagnostics, normal levels up to 20 pmol/L) [21, 22].

Blood samples to measure troponin I were drawn on hospital admission and once per day over the first few days after PPCI. Troponin I was measured by the immunochemical method (Siemens Healthcare Diagnostics Inc., Newark, USA, normal levels up to 0.045 μg/L) [2, 21]. Total serum cholesterol, HDL-cholesterol, and triglycerides were measured by the colorimetric method (Ektachem 250 Analyzer, Eastman Kodak Company, Rochester, USA). LDL-cholesterol level was measured by homogeneous assays [23]. The lipid profile was measured upon admission and after 6 months of treatment.

Platelet count measured by an automatic counter, the Sysmex XE-2100, Kobe, Japan; normal levels were 140–340 × 10^9/L upon admission, after 8 weeks, and after 6 months [24].

Residual platelet reactivity was measured by closure times (CT), using a platelet function analyzer device (PFA-100®). PFA-100 CT enabled the simple, rapid assessment of high

Table 1: Baseline clinical and laboratory data of all STEMI patients and comparisons between patients treated with ramipril and losartan.

Baseline clinical and laboratory data	All patients ($n = 55$)	Ramipril ($n = 28$)	Losartan ($n = 27$)	p
Mean age ± SD (years)	58.7 ± 9.9	59.1 ± 11.2	58.3 ± 8.4	0.774
Mean BMI ± SD	27.4 ± 4.3	26.9 ± 3.4	27.8 ± 5.0	0.447
Mean peak TnI ± SD (μg/L)	45.3 ± 38.5	42.2 ± 37.7	48.4 ± 39.9	0.553
Mean admission TnI ± SD (μg/L)	4.0 ± 8.1	4.6 ± 8.5	3.4 ± 7.7	0.588
Time to PPCI ± SD (hours)	4.7 ± 3.8	5.4 ± 4.2	4.0 ± 3.1	0.156

STEMI: ST-elevation myocardial infarction; TnI: Troponin I; SD: standard deviation; PPCI: primary percutaneous coronary intervention.

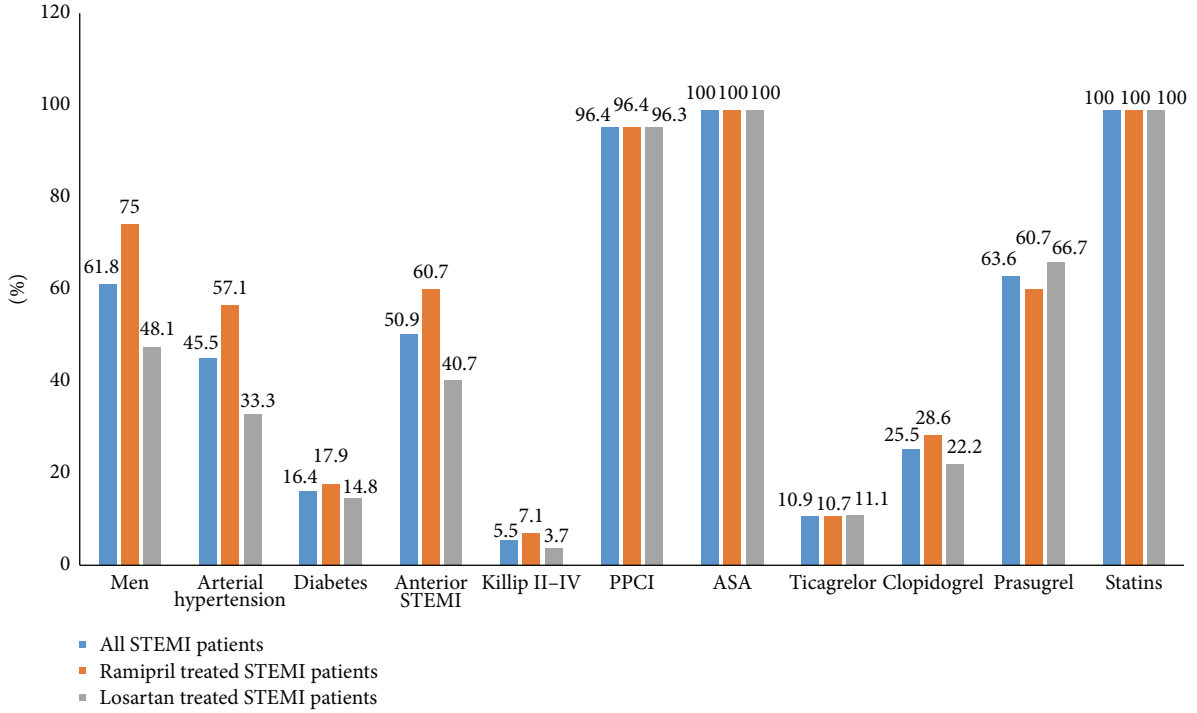

Figure 1: Baseline characteristics of all STEMI patients and comparison between ramipril and losartan treated STEMI patients. STEMI: ST-elevation myocardial infarction; PPCI: primary percutaneous coronary intervention; ASA: acetylsalicylic acid.

shear-dependent platelet function. Small amounts of citrated blood were needed (0.8 mL/cartridge; maximal CT results: 300 s) [13–15]. Blood samples were aspirated at high shear rates (5000–6000 s) through a capillary in the instrument cartridge to meet a membrane coated with collagen/epinephrine (CEPI) [13–15]. The membrane triggered platelet adhesion, activation, and aggregate formation, leading to occlusion of the membrane and cessation of blood flow [13–15]. Normal CT levels for CEPI were 82–150 seconds, but CT values > 300 seconds were nonclosure [13–15, 25].

2.4. Statistical Analysis. Statistical analyses were performed using the SPSS® statistical package, version 19 (SPSS Inc., Chicago, IL, USA) for Windows®. Data were expressed as mean ± standard deviations or percentages. Differences between the groups were tested by the two-sided Student's t-test for mean ± standard deviations and by the chi-square test for percentages. A p value < 0.05 was considered statistically significant.

3. Results

Baseline clinical and laboratory data of all included STEMI patients, as well as ramipril and losartan treated STEMI patients, are summarized in Table 1.

Between patients treated with ramipril and losartan there were nonsignificant differences in baseline clinical and laboratory data as illustrated in Table 1.

Comorbidities, the use of PPCI, and the use of antiplatelet agents are displayed in Figure 1. There were nonsignificant differences between the studied groups as shown in Figure 1.

Table 2 shows mean levels of NT-proBNP, EF, and PAI-1 at baseline and six months after randomization. Between ramipril and losartan there were only nonsignificant differences. Within ramipril and losartan group NT-proBNP

Table 2: Clinical data of all STEMI patients at baseline and after 6 months and comparisons between STEMI patients treated with ramipril and losartan after six months of therapy.

Clinical data (mean ± SD)	All ($n = 55$)	Ramipril ($n = 28$)	Losartan ($n = 27$)	p
NT-proBNP (pg/mL)				
Before treatment	222.4 ± 189.1	211.7 ± 181.1	233.9 ± 200.2	0.671
6 months after treatment	40.3 ± 56.1*	29.6 ± 21.4*	51.0 ± 75.6*	0.163
PAI-1 activity (U/mL)				
Before treatment	2.8 ± 2.1	2.6 ± 2.1	3.1 ± 2.1	0.408
6 months after treatment	2.8 ± 1.9	2.4 ± 1.8	3.1 ± 1.9	0.163
EF (%)				
Before treatment	53.5 ± 9.1	53.5 ± 9.4	53.6 ± 9.0	0.971
6 months after treatment	56.8 ± 8.3	57.0 ± 8.1	56.6 ± 8.5	0.858

Within group analysis: * $P < 0.05$.
NT-proBNP: N-terminal fragment of pro-brain-natriuretic peptide; PAI-1: plasminogen activator inhibitor type 1; EF: ejection fraction.

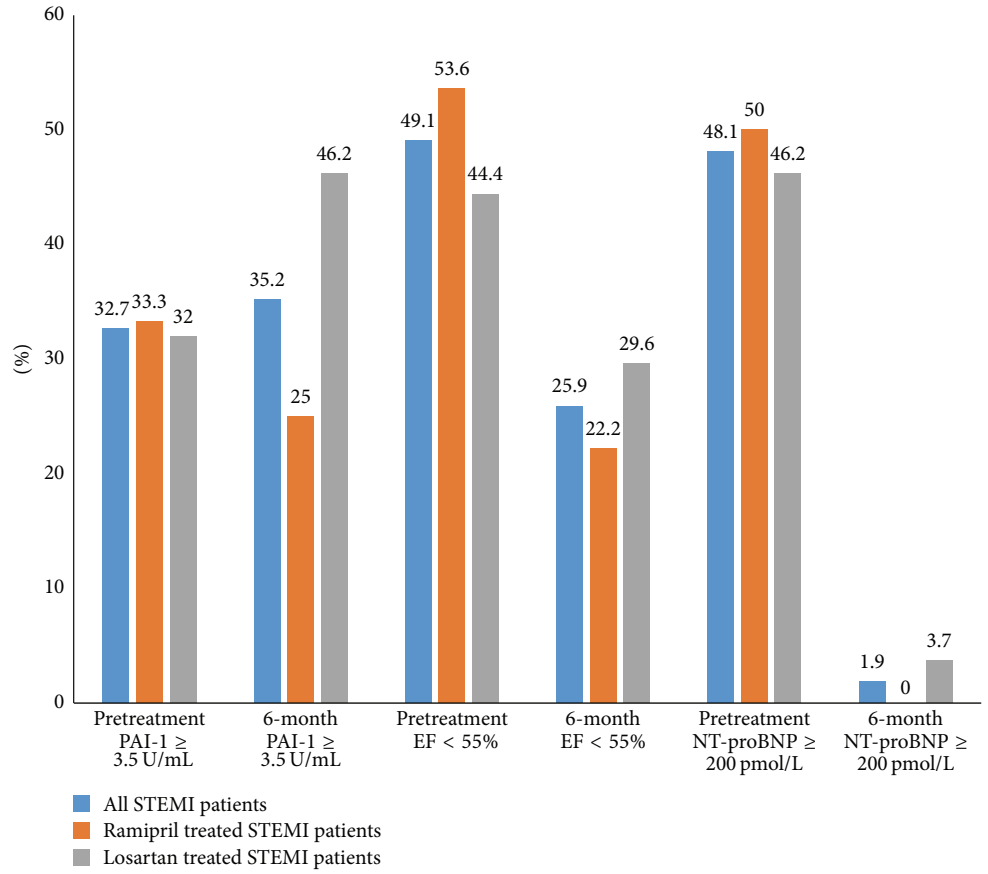

Figure 2: Proportions of all STEMI patients and ramipril and losartan treated STEMI patients with increased NT-proBNP and PAI-1 and decreased EF levels at baseline and six months later. NT-proBNP: N-terminal fragment of pro-brain-natriuretic peptide; PAI-1: plasminogen-activator-inhibitor type 1; EF: ejection fraction; SD: standard deviation, months in comparison to baseline, but PAI-1 and EF levels nonsignificantly.

decreased significantly within 6 months in comparison to the baseline, but mean PAI-1 and EF levels changed nonsignificantly as shown in Table 2.

Proportions of patients with NT-proBNP levels ≥ 200 pmol/L, EF < 55%, and PAI-1 ≥ 3.5 U/mL at baseline and after six months are displayed in Figure 2. Between STEMI patients treated with ramipril and losartan, there were nonsignificant differences regarding increased NT-proBNP, PAI-1 levels, and decreased EF levels as illustrated in Figure 2.

Table 3: Closure times for collagen/epinephrine and platelet counts in all STEMI patients and comparisons between ramipril and losartan treated STEMI patients after 8 weeks and six months of therapy.

Mean ± SD	All ($n = 55$)	Ramipril ($n = 28$)	Losartan ($n = 27$)	Control ($n = 9$)
After 8-week treatment				
CT (sec)	239.8 ± 73.5*	235.7 ± 84.6*	244.7 ± 59.4*	158.1 ± 80.3*
Platelet count (1×10^9/L)	202.5 ± 85.7*	190.7 ± 47.5*	216.7 ± 116.2	278.6 ± 158.1*
After 6-month treatment				
CT (sec)	226.7 ± 80.3*	226.9 ± 76.8*	226.3 ± 85.0*	158.1 ± 80.3*
Platelet count (1×10^9/L)	195 ± 70.9*	190.9 ± 44.3*	199.3 ± 91.6	278.6 ± 158.1*

CT: closure time for collagen/epinephrine; *there is a statistically significant p value (<0.05) between the control group and other groups.

Mean CT levels for CEPI are displayed in Table 3. In STEMI patients receiving either ramipril or losartan in addition to DAPT mean CT levels for CEPI after 8 weeks and 6 months were significantly increased in comparison to the control group, but between the ramipril and losartan group there were nonsignificant differences in mean CT levels after 8 weeks and 6 months of therapy as shown in Table 3.

4. Discussion

We demonstrated that in our asymptomatic patients after their first STEMI, treated by PPCI treatment by ramipril or losartan, exerted an equal effect upon NT-proBNP, PAI-1, and EF after six months as shown in Table 2 and Figure 2.

In addition we observed that both groups—treated with either ramipril or losartan—increased antiplatelet activity, measured by CT significantly when compared to controls as shown in Table 3. The control group consisted of STEMI patients, treated by DAPT only.

Several studies have demonstrated that the magnitude of NT-proBNP, released by an increased left ventricular wall stress induced by ischemia, strongly correlates with the size of acute ischemic necrosis in STEMI patients and its extension within the next few months [2, 26–28]. Even more, a decreased EF correlated with an increase in NT-proBNP over 100 pmol/L at baseline and after 6 months [27].

In our study the baseline NT-proBNP was estimated just before randomization—that is, approximately 20–24 hours after the start of chest pain. This is in accordance with the findings of several studies that the optimum timing to estimate prognostic levels of NT-proBNP should be 24–36 hours after the event [28].

When we stratified baseline NT-proBNP levels we observed that baseline NT-proBNP levels of ≥200 pg/mL were present in 48% of all STEMI patients, including 50% ramipril and 46.2% losartan treated patients. After 6-month treatment NT-proBNP levels were below 200 pg/L in >90% of STEMI patients—equally in the ramipril or losartan group. This effect upon NT-proBNP was already observed after 8 weeks of treatment with either ramipril or losartan in our previous study [4]. In our present study the effect of ramipril and losartan was even more pronounced after 6-month treatment.

In spite that fact that our STEMI patients, who were treated by PPCI 24 hours earlier, were asymptomatic at the start of random assignment to ramipril or losartan, baseline NT-proBNP levels ≥200 pg/mL were present in approximately 50% of patients. Luchner et al. demonstrated that NT-proBNP levels were higher in outpatients after myocardial infarction than in healthy controls, even in the absence of heart failure or significant systolic dysfunction. The reason might be most probably significant cardiac remodeling due to persistent renin-angiotensin-aldosterone system activation [27]. In addition, Weber et al. found that highest values of NT-proBNP were observed 24–36 hours after the start of chest pain, but admission levels were within normal. NT-proBNP levels strongly correlated with troponin T levels either on admission or later, confirming the release of NT-proBNP from ischemic cardiomyocytes [28]. This confirms the observations that NT-proBNP is released from myocardium as a response to ventricular wall stress, but also from ischemic cardiomyocytes [27, 28].

In our asymptomatic STEMI patients baseline mean troponin I level was of 3.9 μg/L and a mean peak level 45.3 μg/L, suggesting a moderate ischemic necrosis. In fact mean EF level was 53.5 ± 9.1% and baseline EF levels <55% in 49.1% of included STEMI patients—equally in ramipril and losartan groups (53.6% versus 44.4%). Mild systolic dysfunction improved gradually, but not earlier than six months later, when EF was <55% only in approximately 25% of STEMI patients—again equally in ramipril and losartan treated.

Brown et al. demonstrated in insulin-resistant hypertensives a greater decrease in PAI-1 antigen for ACE inhibition than for ARB after 6-week therapy, but the effect of both drugs (ramipril and losartan) was similar within the first 3-4 weeks, suggesting that ARBs may exert only a transient effect upon PAI-1 [29]. Regarding PAI-1 levels we demonstrated nonsignificant differences between STEMI patients treated with ramipril and losartan at baseline and after six months. Neither ramipril nor losartan affected PAI-1 levels significantly after 6 months.

In STEMI patients, in particular after PPCI with the use of stents, in particular DES, DAPT should be given for one year in order to prevent in-stent thrombosis and reinfarctions [1, 2, 12]. Novel antiplatelet agents such as ticagrelor and prasugrel are recommended as the first-choice ADP inhibitors in

addition to ASA, as they more successfully prevent recurrent thrombosis [1, 2, 12]. However, our results suggest that the blockade of renin-angiotensin-aldosterone system either by ramipril or losartan in addition to DAPT would improve antiplatelet activity further, as measured by CT after 8 weeks or 6 months in comparison to control groups. In contrast to our results, previous studies demonstrated that ARBs exert stronger antiplatelet effect than ACE inhibitors [7]. Schieffer et al. showed in a randomized trial in coronary patients after PCI that blockade of renin-angiotensin-aldosterone system with either ACE inhibitor or ARB reduced equally some of the inflammatory markers, but levels of IL-6, hsCRP, and platelet aggregation were reduced only by ARB, suggesting more pronounced antiplatelet effect by long-term use of an ARB than of an ACE inhibitor [7].

In our patients antiplatelet effect was estimated by CT and measured by PFA-100. This method is highly dependent on the von Willebrand factor (vWF) binding to the platelet membrane glycoprotein (GP) receptors Ib/IX/V and IIb/IIIa under high shear, but also on platelet count and hematocrit. Platelet count in all our STEMI patients and in the control group was normal [13, 15, 25]. Paniccia et al. compared several aggregometric methods in high-risk coronary patients, undergoing PCI, and discovered that PFA-100 CT, measured by CEPI cartridges, correlated significantly with other validated aggregometric methods [13].

Gianetti et al. in a randomized trial of standard versus tailored DAPT in STEMI patients measured platelet function also by PFA-100 with CEPI cartridges and concluded that this simple method could be a useful tool in acute coronary patients to identify high-on-treatment platelet reactivity [30].

PFA-100 is an example of a global platelet function assay that measures multiple platelet functions, including platelet adhesion and aggregation [14]. In spite of a need for better standardization, it can identify patients with high on-treatment platelet reactivity [15, 30]. Our STEMI patients were all treated by DAPT and then randomized to ramipril and losartan. It seems that adding ramipril or losartan significantly prolonged CT, which may reflect a significant decrease in platelet reactivity after blockade of renin-angiotensin-aldosterone system by ramipril or losartan.

Ono et al. demonstrated, in an experimental model, that losartan's antiplatelet effect is due to inhibition of platelet adhesion and aggregation via glycoprotein VI and was associated with losartan's molecular structure—phenyl group with the tetrazole ring [31]. In an animal study, Kalinowski et al. demonstrated that prevention of platelet adhesion and aggregation by losartan, its metabolite EXP3174, and valsartan are linked to NO release. At the same time, tested drugs could release NO directly, acting on either resting platelets or cultured endothelial cells [32].

On the other hand, Krämer et al. demonstrated, in hypertensive patients without coronary artery disease, that anti-inflammatory and antiplatelet properties of losartan were mainly mediated by another metabolite EXP3179 [9].

Therefore, in clinical settings, losartan may contribute to the prevention of coronary thrombosis and future coronary events by these two mechanisms, which are independent of its effect upon PAI-1. This effect would be of particular significance after STEMI in the long term, when DAPT is already discontinued.

Our results suggest that neurohormonal blockade by ramipril and losartan was equal after 6 months regarding decreased NT-proBNP levels. Systolic function—as measured by EF—in our asymptomatic patients was restored equally after six months by either ramipril or losartan. In addition antiplatelet activity was more significant when losartan or ramipril was added to DAPT in patients after STEMI, treated by PPCI, resulting in a similar and significant decrease in mean CT.

Our conclusions are that in asymptomatic STEMI patients after PPCI ramipril and losartan exert an important additional antiplatelet effect.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

The Diagnostic Role of Adiponectin in Pulmonary Embolism

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Background and Aims. Pulmonary thromboembolism (PTE) is a frequent disease with difficult diagnosis and high mortality. Misdiagnosis occurs in 2/3 patients and mortality rates reach up to 30%. The aim of our study was to investigate the role of adiponectin used in emergency service in diagnosis of PTE. *Materials and Methods*. 95 patients with suspected PTE included in the study. Plasma adiponectin and D-dimer levels were measured and chest X-ray and multidetector row computed tomography scan obtained. Diagnosis was supported by vascular filling defect on tomography. Control group consisted of patients with suspected PTE and normal chest computed tomography findings. *Results*. Mean D-dimer level was 4241.66 ± 1082.98 ng/mL in patients and 2211.21 ± 1765.53 ng/mL in the control group ($p \leq 0.05$). Mean adiponectin level was 5.46 ± 4.39 μg/mL in patients and 7.68 ± 4.67 μg/mL in the control group ($p \leq 0.05$). Wells and Geneva scores were higher in patients compared to the control group. *Conclusions*. As a result, we conclude that lower adiponectin levels have an important role in the diagnosis of PTE.

1. Introduction

Pulmonary thromboembolism (PTE) refers to the migration of a blood clot from systemic deep veins into the pulmonary vascular bed. It is a common occurrence with high mortality rates and presents itself as being difficult to diagnose [1]. No precise diagnosis has been provided in approximately 2/3 of the patients experiencing pulmonary thromboembolism and mortality rates of these patients have reached up to 30%. In cases where pulmonary thromboembolism is diagnosed accurately and proper treatment is provided, the mortality rate is likely to decrease by 3% [2]. Thus, physicians and protective health care planners should be aware of the rapid progress in epidemiology, pathophysiology, diagnosis, treatment, and protection strategies of PTE.

Today there is no biomarker for the diagnosis of pulmonary embolism specifically recommended in the guidelines. To introduce the latest guidelines D-dimer has been mentioned but from positive predictive value of elevated D-dimer levels is low and D-dimer testing is not useful for confirmation of pulmonary embolism. There is no standard for the diagnosis of pulmonary embolism. Some prediction rules have been developed, such as wells and Geneva scores. Both have been adequately validated and are used in daily practice. But today, despite all these rules many patients with pulmonary embolism cannot be diagnosed. Therefore we need new biomarkers that may help in the diagnosis.

Adiponectin, being an adipose tissue protein, accounts for 0.01% of human plasma proteins. Adiponectin is a glycoprotein containing 244 amino acids [3] and it has a significant role in regulating glucose and lipid metabolism over insulin-sensitive tissues in both humans and animals [3, 4]. The protective role of adiponectin in patients at high-risk for recurrent cardiovascular disease such as metabolic

Table 1: Baseline characteristics in the study groups.

	Control group ($n = 57$)	PTE group ($n = 38$)	p
Age (years)	59.48 ± 15.76	60.05 ± 18.38	NS
Sex (male/female)	23/34	14/24	NS
Heart rate (beat/min)	99.79 ± 21.69	105.97 ± 21.20	NS
Wells score	4.07 ± 1.90	5.04 ± 1.95	<0.05
Geneva score	6.03 ± 2.08	7.30 ± 2.39	<0.05
D-dimer (ng/mL)	2211.21 ± 1765.53	4241.66 ± 1082.98	<0.05
Adiponectin (μg/mL)	7.68 ± 4.67	5.46 ± 4.39	<0.05
WBC count ($10^3/\mu$L)	6.8 ± 1.9	7.1 ± 2.3	NS
Platelet count ($10^3/\mu$L)	228 ± 61	242 ± 73	NS
Hemoglobin (g/dL)	13.8 ± 1.3	13.5 ± 1.6	NS

PTE: pulmonary thromboembolism; $p \leq 0.05$ was accepted as statistically significant.
Wells score clinical probability: 0-1: low, 2–6: intermediate, >7: high.
Geneva score clinical probability: 0–3: low, 4–10: intermediate, >11: high.

syndrome has been proven. High adiponectin levels have an effect on decreasing mortality in all cardiovascular diseases [5]. Experimental studies have indicated that adiponectin has potential antiatherogenic and anti-inflammatory properties [6–8]. In *in vivo* studies conducted in human aortic endothelial cells, it has been shown that adiponectin in adhesion molecules regulating vascular inflammatory response leads to a dose-dependent reduction [9]. Adiponectin accumulates in the atherosclerotic vein wall in a dose-dependent manner and inhibits the cell migration induced by TNF-α [10]. It has been shown that hypoadiponectinemia in the carotid artery and arterial walls is associated with thickening of the intima and media layers [5].

The purpose of the present study was to investigate the relationship between serum adiponectin level and PTE.

2. Materials and Methods

After approval from the Ethics Committee, 95 patients hospitalized and monitored with PTE diagnosis were enrolled in the study conducted between January 2009 and May 2010 in the Emergency Medicine Clinic of Fırat University Hospital. Written informed consents of all patients agreeing to take part in the study were also taken. The diagnosis of pulmonary thromboembolism was made based on its compatibility with filling defect of PTE on multidetector computed tomography (MDCT) according to predefined standard protocol. The control group involved patients presenting to the emergency department with a suspicion of pulmonary embolism, yet this could not be detected on a Thorax CT scan.

2.1. Biochemical Analysis. D-dimer levels, which were drained into EDTA tubes, were measured in the emergency department via equipment called Biosite Triage Meter Plus® (San Diego, USA). The cutoff value was determined as 500 ng/mL. 5 mL of venous blood sample was drawn from all cases for adiponectin measurement. After allowing the blood to clot for 30 minutes, the blood was then separated into serum by centrifugation for 3 minutes at 5000 g. The separated serums were kept at −20°C until analysis time for adiponectin level. Adiponectin was analyzed via Assay Max Human adiponectin (Acrp30) ELISA kit® (ASSAYPRO®, 41 Triad South Drive, St. Charles, MO 63304, USA) through ELISA (enzyme-linked immunosorbent inc.) method. The results were recorded as μg/mL.

2.2. Statistical Analysis. In the study, data obtained were indicated as mean ± standard deviation. For statistics, the statistical package SPSS 11.00 (SPSS Inc.®, Software Chicago, IL, USA) was used. While Student's t-test was used for the analysis of continuous variables, chi-square test was utilized for the analysis of categorical data. Correlation analysis was made by Pearson correlation analysis method. A p value ≤0.05 was considered statistically significant. Statistical power of the study was calculated by 75%.

3. Results

A total of 95 patients, of whom 58 (61.1%) were females and 37 (38.9%) were males, were included in the study. Of 38 patients diagnosed with PTE, 24 were females and 14 were males. No statistical difference was detected between patients in terms of gender ($p > 0.05$).

Mean age of the patients was 59.83 ± 17.32 years. While average age of female patients was 60.05 ± 18.38, for the male patients it was 59.48 ± 15.76 years. In the overall evaluation, no statistical difference in terms of mean age was found ($p > 0.05$).

Clinically, the average heart rate in the patient group with PTE was measured 105.97 ± 21.20 beats/min.; in the non-patient group it was 99.79 ± 21.69 beats/min. ($p > 0.05$). Information regarding Wells and Geneva scores was shown in Table 1.

The Wells score used in the diagnosis of pulmonary thromboembolism was found as 5.04 ± 1.95 in the patient group with PTE, whereas it was 4.07 ± 1.90 ($p \leq 0.05$) in the control group. While the Geneva score was indicated as 7.30±2.39 in the patient group with PTE, in the control group it was measured as 6.03 ± 2.08 and the difference between these two groups was statistically significant ($p \leq 0.05$).

Table 2: The correlation analysis of the adiponectin.

Parameters	r	p
Adiponectin		
Systolic BP	−0.368	≤0.05
Geneva score	0.385	≤0.05
BNP	0.471	≤0.05

BNP: brain natriureticpeptide; BP: blood pressure; $p \leq 0.05$ was accepted as statistically significant.

Anatomically the most frequently occluded segment was the main pulmonary artery with 18.9%, followed by right pulmonary artery and its branches with 14.9% and left pulmonary artery and its branches with 6.4%.

The hospital stay of the patient group with PTE in the emergency department was measured as 185.79 ± 10.99 sec; however, in the control group, it was found 223.86 ± 13.53 sec and the difference was statistically significant ($p \leq 0.05$). Only 2 of the patients with PTE required intensive care treatment. Four patients (4.2%) died.

D-dimer level of all patient group included in the study was found as 3003.27 ± 1832.19 ng/mL and in the patient group with PTE diagnosis it was 4241.66 ± 1082.98 ng/mL; in the control group it was measured as being 2211.21 ± 1765.53 ng/mL and this finding was statistically significant ($p \leq 0.05$). D-dimer level in all patients with PTE diagnosis was found high. In none of the five patients with normal D-dimer level, PTE was detected. Negative predictive value (NPV) was indicated as 100%.

In all patient groups, mean adiponectin level was measured 6.70 ± 4.59 μg/mL, in the patient group with PTE diagnosis it was 5.46 ± 4.39 μg/mL and in the control group it was 7.68 ± 4.67 μg/mL; a statistically significant difference was detected between the two groups ($p \leq 0.05$). In the correlation analysis, adiponectin levels were significantly correlated with systolic blood pressure, Geneva score, and BNP level (Table 2).

4. Discussion

Pulmonary thromboembolism presents itself as a vital clinical problem which emerges in the form of a blockage in pulmonary artery branches due to an embolus that originates mostly from a venous system. The importance of PTE results from its high mortality and preventable nature, difficulty in diagnosis, and, lastly, difficulty in determining various treatment modalities for the patients already diagnosed. Strategies in diagnosis and treatment vary in terms of clinical manifestations and existence of hemodynamic disorder [11].

Laboratory findings of pulmonary thromboembolism are nonspecific. The most useful laboratory parameter for emergency departments is the D-dimer level. In a study by Perrier et al. [12], while the normal plasma level of D-dimer was eliminated by ELISA, PTE was excluded by nondiagnostic V/Q scintigraphy results. In our study, the D-dimer level of all patient groups with PTE was found high. The D-dimer level in the patient group was significantly higher than the control group ($p \leq 0.05$). In 5 out of 95 patients who were included in the study, D-dimer level was normal and on none of these patients' MDCT, PTE was detected. About this finding, NPV was indicated as 100%. Regardless of the test method, patients with high D-dimer level must undergo further examinations in clinically suspicious cases. A negative D-dimer result reliably excludes PTE diagnosis in patients with low and intermediate clinical probability of pulmonary embolism. Although the value of a positive D-dimer test is not specific, a negative D-dimer test is quite a reliable parameter in excluding PTE. A negative result in routinely used D-dimer test is treated very sensitively in excluding PTE diagnosis in cases with low clinical suspicion. However, negative D-dimer should not be predictive in excluding the diagnosis in cases with high clinical suspicion, and further examinations should be performed [13, 14]. We believe that the assessment of D-dimer level is a noninvasive subsidiary method in excluding PTE in patients with low and intermediate clinical suspicion and should particularly be used in emergency department settings.

Today, there are new biomarkers that can be used in the diagnosis of pulmonary embolism and are being investigated. In the new studies, serum copeptin and plasma miRNA-28-3p levels have been shown to be useful in the diagnosis of pulmonary embolism [15, 16]. In previous studies, MMP-9 and PAI-1 levels have been shown to be beneficial for the diagnosis of suspected pulmonary embolism [17]. In addition, the increased MMP levels have been shown to be associated with lung tissue damage and it was shown that MMP inhibition with doxycycline protects against acute pulmonary embolism-induced mortality and RV enlargement [18].

It has been determined that adipocytes do not only serve as essential tissues in storing fats but also play crucial key roles in controlling energy and homeostasis in metabolic and inflammatory signals. The effects of adiponectin in the body are as follows [19]: it increases insulin sensitivity; improves lipid level; acts as an anti-inflammatory agent; shows antiatherosclerotic effect; regulates angiogenic and endothelial function; and has antiapoptotic impact. Experimental studies have suggested that adiponectin has potential antiatherogenic and anti-inflammatory properties. In the studies regarding human aortic cells, it has been shown that adiponectin leads to a dose-dependent decrease in adhesion molecules, which regulate vascular inflammatory response [8, 9]. In some experimental animal models, it has been demonstrated that adiponectin may play a protective role against atherosclerotic agents due to its anti-inflammatory effect [20]. Matsuda et al. showed that when damage was done by a catheter in the vein wall of mice, which lack adiponectin, the veins of smooth muscle cells of these mice result in neointimal thickening by proliferation. They also reported that when the same mice were supplemented with adiponectin recombinant adenovirus, neointimal proliferation in the damaged arterial wall improved. They proved that increased serum adiponectin levels in APN-KO mice reduced significantly the progression of atherosclerotic lesions [21]. Hotta et al. demonstrated that serum adiponectin level was lower in diabetics with coronary artery disease than the diabetics without coronary artery diseases. It has also been reported in this study that serum adiponectin concentrations are negatively correlated with serum glucose, insulin, and

triglyceride levels but positively correlated with serum HDL cholesterol levels [22]. In the clinical study by Bang et al. (Bang, 2007 #3) including 231 patients, serum adiponectin level was found lowest in the group with intracranial atherosclerosis, highest in the cardioembolic group, and medium-low in the extracranial atherosclerosis and small artery blockage [23].

Here, we endeavored to find answers for the questions that present the most important part of our study, "does adiponectin have a role in reducing mortality and morbidity in the early diagnosis and treatment of PTE?" "Can serum adiponectin level, therefore, be considered as a risk factor?" With regard to our findings, when we analyzed serum adiponectin levels of a total of 95 patients, we indicated a statistically significant difference between 38 patients diagnosed with PTE and 57 patients who were not diagnosed with PTE ($p \leq 0.05$). Adiponectin levels in the patient group with PTE were significantly low and these findings have an important role in the diagnosis of PTE.

5. Conclusion

As a result, we conclude that lower adiponectin levels have an important role in the diagnosis of PTE. Further studies are needed to support the findings so that patients with pulmonary embolism can receive accurate and immediate diagnosis in emergency departments.

Conflict of Interests

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

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Research Article

TBS Predict Coronary Artery Calcification in Adults

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Purpose. This study analyzes the association between the bony microarchitecture score (trabecular bone score, TBS) and coronary artery calcification (CAC) in adults undergoing health exams. *Materials and Methods*. We retrospectively collected subjects (N = 81) who underwent coronary computed tomography and bone mineral density studies simultaneously. CAC was categorized to three levels (Group 0, G0, no CAC, score = 0, N = 45; Group 1, G1, moderate CAC, score = 1–100, N = 17; Group 2, G2, high CAC, score ≧ 101, N = 19). Multinomial logistic regression was used to study the association between TBS and CAC levels. *Results*. CAC is present in 44.4% of the population. Mean TBS ± SD was 1.399 ± 0.090. Per 1 SD increase in TBS, the unadjusted odds ratio (2.393) of moderate CAC compared with no CAC was significantly increased (95% CI, 1.219–4.696, p = 0.011). However, there has been no association of TBS with high CAC (OR: 1.026, 95% CI: 0.586–1.797, p = 0.928). These relationships also existed when individually adjusted for age, sex, and multiple other covariates. *Conclusions*. Higher TBS was related to moderate CAC, but not high CAC; a possible explanation may be that bone microarchitecture remodeling becomes more active when early coronary artery calcification occurs. However, further researches are needed to clarify this pathophysiology.

1. Introduction

Atherosclerosis and osteoporosis share many risk factors, but their independent association is unclear [1]. The diagnosis of osteoporosis depends on area bone mineral density (BMD) measurements using dual energy X-ray absorptiometry (DXA) [2]. BMD has been inversely associated with subclinical and clinical cardiovascular disease (CVD), even after adjusting for potential confounding factors [3]. Previous studies have examined the relationship between trabecular volumetric BMD (vBMD) and aortic arterial calcification (AAC) or coronary artery calcification (CAC), with inconclusive results [3–6]. Cortical, but not trabecular, vBMD was associated with significantly decreased odds of AAC prevalence independent of other traditional risk factors [3]. With a decrease in vBMD, the adjusted odds of high AAC, compared with no AAC, were significantly increased; vBMD was related to high CAC in unadjusted, but not adjusted, models. No associations of vBMD with moderate AC or CAC were observed [5].

Practically, BMD evaluated by DXA studies was a presentation of both cortical and trabecular bone content. However, cancellous bone microarchitecture is the key determinant of bone strength, which is often measured by quantitative computed tomography (qCT). But this involves higher radiation exposure, is more expensive, and has a larger instrument requirement. TBS (trabecular bone score) is a texture parameter that can be computed from the two-dimensional lumbar spine DXA image [7]. TBS, a variogram, is related to bone microarchitecture (few large spans, i.e., low TBS, are mechanically weaker than a myriad of fine spans, i.e., high TBS) and is complementary to predict fracture risk, as well as lumbar spine BMD measurements. Therapeutic strategies for osteoporosis differ after inclusion of the influence from TBS [7].

The clinical application of TBS and the association of CAD have not been documented. The aim of this study is to

Table 1: Participants characteristics.

	No CAC (G0) (Score = 0) (N = 45)	Moderate CAC (G1) (Score = 1–100) (N = 17)	High CAC (G2) (Score ≧ 101) (N = 19)	p
Age (years)	53.9 ± 9.9[a]	53.6 ± 6.3[a]	62.3 ± 10.1[b]	0.004
Female (%)	21 (70)	3 (17.6)	6 (31.6)	0.092
Smoking (%)	4 (8.9)	1 (5.9)	1 (5.3)	0.848
HTN (%)	6 (13.3)	1 (5.9)	12 (63.2)	<0.001
DM (%)	1 (2.2)	5 (29.4)	3 (15.8)	0.007
HL (%)	2 (4.4)	1 (5.9)	2 (10.5)	0.652
Weight (kg)	66.5 ± 12.5	72.6 ± 13.7	67.1 ± 9.7	0.206
Height (cm)	163.3 ± 8.3	168.0 ± 9.0	162.1 ± 6.1	0.064
TCH (mg/dL)	191.8 ± 35.7	178.4 ± 26.1	192.3 ± 29.8	0.317
LDL (mg/dL)	125.0 ± 29.8	112.7 ± 19.4	121.4 ± 27.6	0.291
HDL (mg/dL)	51.1 ± 16.5	43.4 ± 7.9	47.4 ± 15.2	0.178
TG (mg/dL)	130.4 ± 70.6	175.1 ± 75.5	153.5 ± 147.6	0.237
Glucose (mg/dL)	100.8 ± 10.2[a]	124.3 ± 32.8[b]	107.8 ± 20.8[a]	<0.001
SBP (mmHg)	124.1 ± 24.8	124.4 ± 14.0	132.1 ± 24.8	0.427
DBP (mmHg)	77.4 ± 18.1	77.4 ± 9.8	83.0 ± 11.8	0.394
TBS	1.384 ± 0.083[a]	1.451 ± 0.081[b]	1.386 ± 0.101[ab]	0.024

Note: means with different superscripts indicate significant difference at $p < 0.05$ level, evaluated using Sidak post hoc adjustment.
HTN: hypertension; DM: diabetes mellitus; HL: hyperlipidemia; TCH: total cholesterol; LDL: low-density lipoprotein; HDL: high-density lipoprotein; TG: triglyceride; SBP: systolic blood pressure; DBP: diastolic blood pressure; TBS: trabecular bone score.

explore the relationship of TBS and CAC in adults undergoing a health exam.

2. Materials and Methods

2.1. Subjects. We retrospectively collected patients who had simultaneously undergone a coronary CT scan and BMD study from May 2014 to November 2015, after the introduction of the DXA equipment (HOLOGIC Discovery Wi) in the health examination center at Dalin Tzu Chi Hospital. The interval between the two tests varied from the same day to one month.

Health history (by interview or questionnaire), anthropomorphic characteristics, and laboratory data, including lipid profile (total cholesterol, LDL, HDL, and triglyceride), glucose levels, systolic and diastolic blood pressure, and smoking history, were recorded (Table 1).

2.2. Coronary Artery Calcification. CAC scoring was obtained on unenhanced axial images scanned before the coronary CT angiography. The scans were performed using a multidetector CT system (LightSpeed VCT, GE Medical Systems). CAC was quantified with the Agatston scoring method, which has been widely accepted [8]. Total calcium score was determined using the sum of individual scores from the four major coronary arteries (left main, left anterior descending, circumflex, and right coronary arteries).

2.3. TBS Measurement. TBS (trabecular bone score), a texture parameter, is computed from the DXA images. TBS can be quantified from local variations in pixels intensities and derived from the experimental variogram obtained from the gray levels of a DXA image. With TBS iNsight installed on the DXA device PC, it quantified the bone texture in 3 s by retrospectively automatic analysis from an existing DXA scan without additional examination or dosage for the patient.

2.4. Statistical Analysis. 45 subjects (54.9%) had a CAC score of zero and small sample size, so the CAC data analysis was treated as categorical 0 (CAC = 0), 1 (0 < CAC ≤ 100), and 2 (CAC > 100).

Differences in means or frequencies between characteristics statuses were tested by chi-squared test or ANOVA, as appropriate. Multinomial logistic regression was used to identify the significant predictors of coronary artery calcification after adjustment for other cofactors. Models of TBS predicting CAC were developed through addition of covariates to assess the strength and independence of the associations.

Covariates included age, sex, hypertension, diabetes, hyperlipidemia, systolic blood pressure, diastolic blood pressure, and measured laboratory data (total cholesterol, LDL, HDL, triglyceride, and glucose). Odds ratios were expressed as the effect of a 1 SD or unit increase in covariate or TBS in adjusted, unadjusted, or age-adjusted models.

Statistical analysis was performed using PASW Statistics 18 (SPSS Inc., Chicago, IL).

3. Results

3.1. Participant Characteristics. Total 81 subjects (51 males and 30 females) were collected from our database. Out of them, 36 (44.4%) had coronary artery calcification, described

Table 2: Odds of age-adjusted covariates at the multinomial logistic regression model for CAC.

Covariates	CAC group Age-adjusted OR (95% CI)		
	No CAC (G0)	Moderate CAC (G1)	High CAC (G2)
Age*	1.00	0.96 (0.53–1.74) [0.896]	2.87 (1.42–5.81) [0.003]
Male versus female	1.00	4.18 (1.04–16.83) [0.044]	2.31 (0.68–7.89) [0.181]
HTN	1.00	0.41 (0.05–3.71) [0.425]	8.05 (2.13–30.41) [0.002]
DM	1.00	21.02 (2.15–205.62) [0.009]	6.13 (0.56–67.25) [0.138]
Hyperlipidemia	1.00	1.39 (0.11–16.85) [0.797]	1.78 (0.21–14.89) [0.596]
SBP*	1.00	1.02 (0.57–1.81) [0.960]	1.31 (0.73–2.35) [0.375]
DBP*	1.00	1.00 (0.55–1.83) [0.988]	1.36 (0.76–2.44) [0.308]
TCH*	1.00	0.65 (0.36–1.18) [0.156]	1.02 (0.57–1.82) [0.958]
LDL*	1.00	0.64 (0.36–1.14) [0.129]	0.82 (0.45–1.52) [0.535]
HDL*	1.00	0.54 [0.27–1.08] [0.081]	0.70 (0.38–1.27) [0.081]
TG*	1.00	1.67 (0.90–3.10) [0.102]	1.72 (0.89–3.33) [0.108]
Glucose*	1.00	3.53 (1.59–7.85) [0.002]	1.90 (0.83–4.33) [0.129]

Values are odds ratios (95% CI) [p value].
*For 1 SD increase in age (9.92 y/o), SBP (22.93 mmHg), DBP (15.35 mmHg), TCH (32.68 mg/dL), LDL (27.53 mg/dL), HDL (14.96 mg/dL), and TG (95.47 mg/dL)
HTN: hypertension; DM: diabetes mellitus; SBP: systolic blood pressure; DBP: diastolic blood pressure; TCH: total cholesterol; LDL: low-density lipoprotein; HDL: high-density lipoprotein; TG: triglyceride.

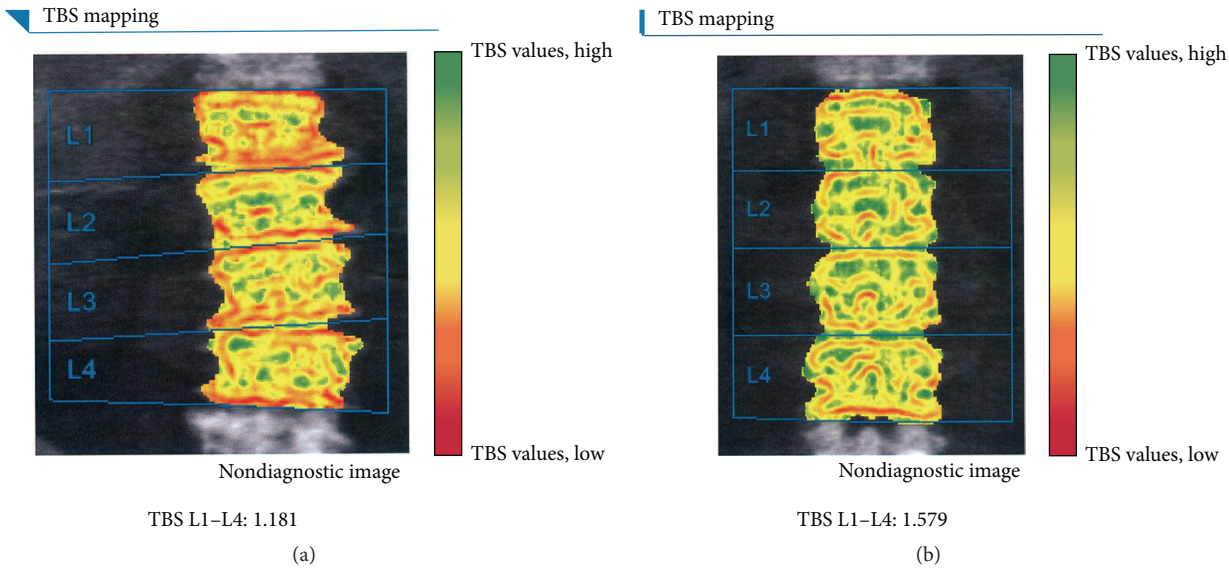

Figure 1: (a) A case in group 2 (high CAC), a 69-year-old female with hypertension and hyperlipidemia, height 151.0 cm, and weight 51.0 kg. CAC score is 1185; TBS value of L1–L4 showed 1.181. (b) A case in group 1 (moderate CAC), a 51-year-old male without any systemic disease, height 163.0 cm, and weight 68.0 kg. CAC score is 38; TBS value of L1–L4 showed 1.579.

as CAC > 0. The average TBS was 1.40 ± 0.09 (SD). Participants with high CAC were older and more likely to be hypertensive, compared to those with moderate or no CAC. A significant increase in glucose and TBS was observed with moderate CAC, as compared with the no CAC group (Table 1). In our cohort, no one had chronic kidney disease. One case had bilateral total hip replacement and was not included in the final analysis. An example of group 2 and another case from group 1 with their TBS figures were shown in Figure 1.

3.2. Predictors of CAC. A 9.92-year (1 SD) greater was associated with 2.87 times greater odds of high CAC, as compared with no CAC (Table 2). Male gender and diabetes after age-adjusted significantly increased the odds (4.18 and 21.02

Table 3: Odds of TBS with or without adjustment at the multinomial logistic regression model for CAC.

Covariates	No CAC (G0)	Moderate CAC (G1)	High CAC (G2)
TBS (unadjusted)*	1.00	2.39 (1.22–4.70) [0.011]	1.03 (0.59–1.80) [0.928]
TBS (adjusted for age)*	1.00	2.60 (1.28–5.29) [0.008]	1.63 (0.836–3.173) [0.152]
TBS (adjusted for sex)*	1.00	2.07 (1.02–4.20) [0.044]	0.89 (0.48–1.65) [0.707]
TBS (adjusted for age and sex)*	1.00	2.27 (1.07–4.78) [0.032]	1.45 (0.70–3.04) [0.320]
TBS (adjusted for HTN)*	1.00	2.43 (1.20–4.90) [0.014]	1.53 (0.78–3.00) [0.214]
TBS (adjusted for DM)*	1.00	2.63 (1.26–5.48) [0.010]	1.03 (0.58–1.82) [0.925]
TBS (adjusted for hyperlipidemia)*	1.00	2.43 (1.22–4.82) [0.011]	1.03 (0.59–1.79) [0.923]
TBS (adjusted for SBP)*	1.00	2.47 (1.24–4.89) [0.010]	1.14 (0.63–2.04) [0.670]
TBS (adjusted for DBP)*	1.00	2.42 (1.23–4.75) [0.011]	1.05 (0.60–1.84) [0.857]
TBS (adjusted for SBP and DBP)*	1.00	2.46 (1.24–4.88) [0.010]	1.12 (0.62–2.02) [0.700]
TBS (adjusted for smoking)*	1.00	2.41 (1.23–4.74) [0.011]	1.03 (0.59–1.80) [0.918]
TBS (adjusted for TCH, LDL, HDL, and TG)*	1.00	2.30 (1.12–4.74) [0.023]	0.90(0.48–1.68) [0.734]
TBS (adjusted for glucose)*	1.00	2.52 (1.16–5.45) [0.019]	1.02 (0.58–1.80) [0.949]
TBS (adjusted for age, LDL, SBP, and glucose)*	1.00	2.71 (1.20–6.12) [0.016]	1.73 (0.85–3.50) [0.128]

Values are odds ratios (95% CI) [p value].
*For 1 SD increase in TBS.
TBS: trabecular bone score; HTN: hypertension; DM: diabetes mellitus; SBP: systolic blood pressure; DBP: diastolic blood pressure; TCH: total cholesterol; LDL: low-density lipoprotein; HDL: high-density lipoprotein; TG: triglyceride.

times) of moderate CAC, relative to the no CAC group. Hypertensive subjects had 8.05 times greater age-adjusted odds in high CAC than in no CAC. Hyperlipidemia, SBP, DBP, total cholesterol, LDL, HDL, triglyceride, and glucose were not significantly associated with moderate CAC or high CAC after adjustment for age.

3.3. TBS and CAC. In unadjusted multinomial logistic regression analysis, per 1 SD increase in TBS, the odds of moderate CAC compared with no CAC were significantly increased 2.39-fold (95% CI, 1.22–4.70). The association remained significant after individually adjusting of age, sex, hypertension, diabetes, hyperlipidemia, systolic blood pressure (SBP), diastolic blood pressure (DBP), lipid profile, and glucose and after each combination adjustment for age and sex, SBP and DBP, even in an extensive adjusted model, which included age, LDL, SBP, and glucose. However, no significant relationship was observed between TBS and high CAC in unadjusted or adjusted analyses (Table 3).

4. Discussion

There is a link between osteoporosis and cardiovascular disease (CVD) [5]. Subjects who self-report a previous myocardial infarction had significantly higher odds of having low bone mineral density, when adjusting for CVD and osteoporosis risk factors, and this was not significantly associated in women but was significant in men [9]. Postmenopausal women with osteoporosis are at an increased risk for cardiovascular events, proportional to the severity of osteoporosis at the time of the diagnosis [10].

CAC had the role in developing CAD [11–13]; CAC and CT angiography in asymptomatic elderly patients can predict coronary artery disease [14]. Some studies showed a negative association between BMD (or vBMD) and score or presence of aortic calcification (AC)/coronary artery calcification (CAC) [5, 15, 16]. Their relationship may be age-related progression [3], shared risk factors (smoking), or common pathophysiological mechanisms (hormones or inflammatory cytokines) [5]. The association between cortical BMD (not trabecular vBMD) and AAC persisted even after adjustment for age, BMI, lifestyle factors, diabetes, and hypertension [3], while other studies showed an association of trabecular vBMD with AAC [5]. Their inconsistency with regard to results may be due to sex- and/or ethnicity-specific differences [3, 5]. Cortical and trabecular bone are known to have different turnover rates and age-related patterns [17]. The strongest predictors of AAC prevalence include increased age, male sex, smoking, higher BMI or waist circumference, hypertension, dyslipidemia, and diabetes [3, 18, 19].

In a study of volumetric BMD and vascular calcification measured by CT in middle-aged women, they divided the population of AC and CAC into three levels and found that lower trabecular BMD of the spine was significantly associated with high AC levels and also high CAC levels; the latter was not significant after adjusting for age [5]. In a recent Rotterdam Study, no association between CAC and BMD or fracture risk was found, except for BMD loss with higher follow-up CAC in women, which may be related to low estrogen levels [6].

Vascular calcifications (VCs) are of similar composition to bone minerals. Currently, intima-related VCs are commonly associated with atherosclerotic plaques (in the vicinity of lipid or cholesterol deposits) and lesions calcified lately, and lesions of media-related VCs calcified early (in the absence of lipid or cholesterol deposits) [20]. Even if medial and intimal calcification may share some common pathomechanisms and can occur together in patients, it is reasonable to maintain a distinction between the two [21]. VCs represent complex

biological process of calcium phosphate deposition and are related to regulation of osteogene expression, bone morphogenetic protein (BMP2), calcification inhibitors (osteoprotegerin, matrix-gla protein, fetuin-A), and inflammatory cytokines (TNF-α, CRP, and CD40–CD154) [20, 21]. Unfortunately, it is impossible, or at least extremely difficult, to distinguish between intimal and medial calcification in the coronary arteries [21].

The absence of CAC strongly excludes obstructive CAD, and CAC predicts the presence of coronary atherosclerotic plaque. However, the absence of any CAC does not exclude the presence of coronary atherosclerotic plaque, especially in patients aged <55 years. Plaque composition shifted from noncalcified to calcified plaque with increasing age, which may affect the vulnerability of these lesions over time [22]. CAC has also been associated with high serum concentration of some biomarkers, including undercarboxylated osteocalcin and fibroblast growth factor 23 [23, 24]. In patients on dialysis, high parathyroid hormone level and osteoporosis predict progression of CAC [25]; and bone volume/total volume (BV/TV) assessed by HR-pQCT were significantly lower in patients with CAC scores ≥100 [26].

The BMD T-score may not fully capture the fragility fracture risk, so the noninvasive analytic tool of TBS was developed. The TBS is a texture parameter that evaluates pixel gray-level variations in DXA images of the lumbar spine [2]. TBS decreases with age and appears to reflect qualitative aspects of skeletal structure complementary to BMD [7]. Quantitative computed tomography has the disadvantage of higher radiation exposure, increased expense, and larger instrument requirements. TBS measure the trabecular microarchitecture with simple DXA machine [27], which is cheaper, involves less radiation exposure, and only needs an immediately "1 click – 3 s" extra software analysis to the traditional lumbar spine BMD data, without additional exams or radiation doses. The TBS also can be retrospectively analyzed in the same machine from an existing DXA scan to quantify bone microarchitectural texture.

Our result suggested that TBS value (per 1 SD increase) positively predicted the group of moderate CAC (odds ratio = 2.39, $p = 0.011$) but had no association for the high CAC group (odds ratio = 1.03, $p = 0.928$). The relationship still existed even after adjusting for the covariates. This result is significantly different from previous studies with qCT and CAC [1, 26]. The difference might be possible due to the diverse methodologies. Since VCs have complex mechanisms, another possible explanation may be that early CAC is associated with a more complex variogram of bone microarchitecture during bone remodeling. However, at far-advanced CAC, the higher TBS had no significance in prediction value. It means that molecular cascades and procalcific microenvironment during "vascular calcification dynamics" change with the process of "bone microarchitecture formation." During early CAC, both are similar. In severe CAC, the direction of the kinetic equilibrium is stable and the progression of evolution makes the relationship between CAC and TBS not develop further. Exposure to high Ca concentrations may influence the development of low-turnover bone disease and coronary artery calcification (CAC) in patients on hemodialysis (HD) [28]. For cases under DXA measurement of lumbar spines, the BMD value may be overestimated for the cases with abdominal aorta calcification [29]. Although, in our CAC group 1, the mean TBS was higher than group 0, in our CAC group 2, the mean TBS was lower than CAC group 1. The projection interference, a potential confounder, may not be a factor that influences our results.

5. Conclusion

Atherosclerosis and vascular calcification are dynamic processes; both of them and bone microarchitecture reach a dynamic equilibrium in bone remodeling. Advanced age is significantly associated with high CAC (score > 100), while increased TBS is associated with moderate CAC ($0 <$ score ≤ 100), independent of age and other risk factors. These unusual findings are most likely due to the deferent biomechanism of diverse methodology or complex regulatory networks of VCs and need further research and a larger database for confirmation.

Conflict of Interests

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

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Research Article

Circulating Long Noncoding RNA UCA1 as a Novel Biomarker of Acute Myocardial Infarction

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Acute myocardial infarction (AMI) is the most serious cardiovascular disease with high morbidity and mortality. Recent studies have showed that long noncoding RNAs (lnc RNA) play important roles in pathophysiology of cardiovascular diseases, but the investigations are still in their infancy. An lnc RNA named urothelial carcinoma-associated 1 (UCA1) is found in tumors such as bladder cancers and lung cancer. And the UCA1 could be as a predictive biomarker for bladder cancer in urine samples or lung cancer in plasma, respectively. In normal states, UCA1 is specifically expressed in heart of adult, indicating that UCA1 might be as a biomarker for heart diseases such as AMI. To test the speculation, we detect the level of UCA1 in plasma of AMI patients and health control using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). In addition, we also test the level of miR-1 as it is reported to regulate the expression of UCA1. The results show that the level of plasma UCA1 is decreased at the early state of AMI patients and increased at day 3 after AMI. In addition, the UCA1 alteration is inversely associated with the expression of miR-1. These findings indicate that the circulating UCA1 could be used as a promising novel biomarker for the diagnosis and/or prognosis of AMI.

1. Introduction

Acute myocardial infarction (AMI) is the most serious cardiovascular disease with high morbidity and mortality [1]. Rapid and correct diagnosis of AMI has been a focus of research as it could prevent the progressive development of AMI and improve the cure and survival rate in patients [2]. Cardiac troponins are the most common biomarkers used for diagnosis of AMI in clinical practice. However, there are several conditions other than acute myocardial infarction which could result in elevated cardiac troponin levels. Therefore, there is still a clinical need for novel biomarker [3].

Increasing evidences suggest that noncoding RNAs (nc RNAs) including microRNAs (miRNAs) and long nc RNAs (lnc RNAs) play important roles in pathophysiology of cardiovascular diseases including AMI [4]. Of them, microRNAs (miR) with 21–25 nucleotides in length have been largely investigated in cardiovascular disease. For example, miR-1, miR-133, miR-499, and miR-208 are increased in serum of patients with AMI [5–8]. Recently, lnc RNAs with longer than 200 nucleotides in length also were found to regulate gene expression by diverse mechanisms [9]. In cardiovascular disease states, the profiles of lnc RNAs in plasma and serum have been found to be altered, suggesting broad opportunities for development of circulating lnc RNAs as blood-based markers for molecular diagnostics. For example, the circulating long noncoding RNA LIPCAR was explored as prognostic biomarkers for heart failure [10]. In addition, several lnc RNAs have been found to be regulated in the heart tissue during AMI [11]. However, studies about the circulating long noncoding RNA as biomarker of AMI are still in their infancy. To date, only two studies have analyzed altered circulating lnc RNAs in patients with myocardial infarction.

An ideal biomarker for AMI required high sensitivity and specificity as well as good accessibility and predictability [12]. An lnc RNA named urothelial carcinoma-associated 1 (UCA1) might meet the criteria. The UCA1 originally was identified in human bladder transitional cell carcinoma and highly expressed in lung cancer. In normal states, UCA1 is only expressed in heart and spleen after birth and higher

expression in heart [13, 14], indicating that UCA1 might be as a specific biomarker for heart. Besides the specificity, the UCA1 could be detected in freshly voided urine samples or plasma and be as a predictive biomarker for bladder cancer or lung cancer, respectively [13, 15]. In the early stages of AMI, pathological changes such as myocardial ischemia, hypoxia, edema, and necrosis occur rapidly, followed by the release of necrotic products, such as cardiac troponins (cTns), creatine kinase (CK), and brain natriuretic peptide (BNP), into the bloodstream [16]. The lnc RNA might be released from necrotic products and detected in the plasma, indicating UCA1 might be easily obtained. In addition, recent study shows that the UCA1 influences the cell proliferation, apoptosis, and cell cycle distribution of colorectal cancer [17]. Furthermore, UCA1 could protect cardiomyocyte against H_2O_2-induced cardiomyocyte apoptosis [18]. Whereas hypoxia or reoxygenation-induced cardiomyocyte apoptosis is one of the major causes of AMI, indicating that UCA1 might be as the predictive biomarker for the diagnosis and/or prognosis of AMI.

The objectives of the study were to test whether UCA1 could be as the biomarker of AMI. We tested the level of UCA1 in AMI and non-AMI subjects using qRT-PCR and compare its diagnostic value with that of cTnI and CK-MB.

2. Method and Material

2.1. Participants. Between 2014 November and 2015 January, we studied 49 AMI patients at ages of 30–75 and 15 non-AMI subjects at ages of 37–63 from the Second Hospital of Jilin University (Changchun, China). AMI was diagnosed based on combination of several parameters: ischemic symptom plus increased cardiac troponin I (cTnI) and creatine kinase-MB (CK-MB), pathological Q wave. ST-segment elevation or depression was defined by the European Society of Cardiology/American College of Cardiology. Baseline ECG was recorded in all patients. Written consents were obtained from all subjects studied and the study protocol was approved by the ethics committee of the Jilin University.

2.2. Isolation of Human Plasma. For lnc RNA and miRNA detection, whole blood (WB) samples (5 mL per patient) were collected from subjects via a direct venous puncture into tubes containing sodium citrate, centrifuged at 1000 g for 5 min, and then the supernatant (plasma) was carefully transferred into an RNase-free tube for extraction of RNA.

2.3. RNA Isolation and Real-Time Quantitative RT-PCR (qRT-PCR). For measuring microRNAs, miRNA was extracted from the plasma samples using the miRcute miRNA Isolation kit (TIAN GEN, Applied Biosystem). The samples performed both poly-(A) tailing and reverse transcription with the miScript reverse transcription kit (TIAN GEN). miR-1 was quantitated by using TaqMan miRNA quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) assay according to the protocol of the manufacturer. U6 RNA was performed as a miRNA internal control.

For measuring lnc RNAs, 1 μg total RNA was reverse transcribed using MulV reverse transcriptase (Transgen, Cat. AT101-02) and random hexamer primers in a 20 μL reaction. The SYBR Green PCR Master Mix Kit (Transgen, Cat. AQ131-01) was used in real-time PCR for relative quantification of miRNAs and of UCA1; in this study U6 and 18S were used as an internal control, respectively. qRT-PCR was performed on 7500 FAST Real-Time PCR System (Applied Biosystems). The RT primers used were (1) miR-1 forward: GGGGTGGAATGTAAAGAA and miR-1 reversed: TGCGTGTCGTGGAGTC; (2) U6 forward: GCTTCGGCAGCACATATACTAAAAT and U6 reversed: CGCTTCACGAATTTGCGTGTCAT; (3) UCA1 forward: ACGCTAACTGGCACCTTGTT and UCA1 reversed: TGGGGATTACTGGGGTAGGG; (4) 18S forward: CAGCCACCCGAGATTGAGCA and 18S reversed: TAGTAGCGACGGGCGGTGTG.

Analysis of relative gene expression levels was performed using the formula $2^{-\Delta CT}$ with $\Delta CT = CT_{(\text{target gene})} - CT_{(\text{control})}$.

2.4. Statistical Analysis. Data were described as means ± SD and median for general characteristics of subjects. All statistical analyses were performed using the SPSS16.0 software. Differences between different groups were assessed using One-Way ANOVA comparison method. Value of $P < 0.05$ was considered to indicate statistical significance. Receiver operating characteristic (ROC) curves were established to evaluate the predictive power of circulating UCA1 between the AMI and non-AMI subjects. The area under the ROC curve (AUC) was used to assess the predictive power. The sensitivity and specificity were calculated according to the standard formulas.

3. Results

3.1. The Baseline Clinical Characteristics of the Study Subjects. A total of 49 AMI patients and 15 non-AMI subjects were studied to determine the association of circulating UCA1 levels with AMI. The baseline clinical characteristics of the study subjects are shown in Table 1. There were significant differences in cTnI and CK-MB between AMI and non-AMI subjects ($P < 0.01$, $P < 0.01$, resp.). There were no statistical differences in age, HDL, LDL, EF%, hypertension, and history of diabetes and smoking status between the AMI and non-AMI subjects.

3.2. The Level of Circulating UCA1 in the Patients with AMI. To investigate whether UCA1 could be as the novel biomarker for the diagnosis of AMI, its expression levels in plasma of AMI patients and health subjects were analyzed. In the non-AMI subjects, the level of circulating UCA1 was very low and the mean CT value was 28.43 by 45 cycles of Q-PCR. The results (Figure 1) showed that the expression of UCA1 was significantly decreased in plasma of patients with AMI, compared with non-AMI subjects ($P < 0.05$). However, we also found the levels of UCA1 were increased in some samples. We speculated that the level of circulating UCA1 in patients with AMI might be correlated with the time after onset of AMI.

Table 1: Clinical characteristics of patients.

Characteristics	AMI ($n = 49$)	Non-AMI ($n = 15$)	P
Age (years)	61.02 ± 11.83	58.13 ± 10.18	0.42
Male/female (n/n)	30/19	7/8	0.75
HDL C (mmol/L)	1.05 ± 0.35	1.28 ± 0.23	0.52
Current smoking, n (%)	25 (51%)	5 (33%)	0.161
EF%	55.02 ± 8.72	69.54 ± 6.78	0.72
LDL C (mmol/L)	2.76 ± 0.76	2.13 ± 0.84	0.68
Diabetes, n (%)	12 (24.5%)	4 (26.7%)	0.938
CK-MB (U/L)	74.35 ± 52.04	3.05 ± 1.47	<0.01
cTnI (ng/mL)	5.34 ± 3.57	0.032 ± 0.022	<0.01
Hypertension, n (%)	31 (63.2%)	6 (53.3%)	0.324

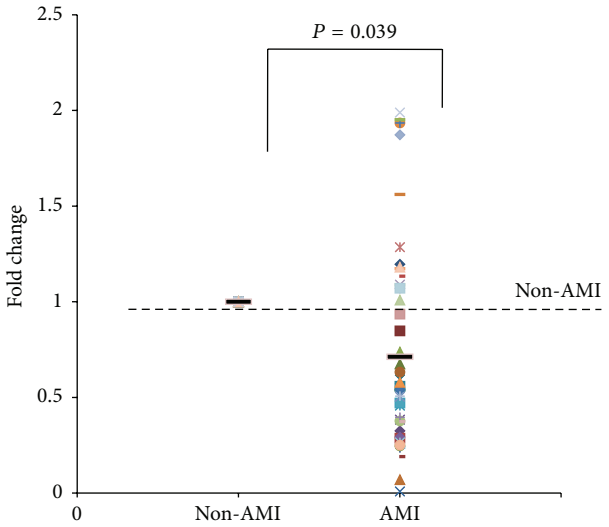

Figure 1: The level of UCA1 in plasma of AMI patients. The expression levels of UCA1 in plasma of hospitalized AMI patients ($n = 49$) and non-AMI subjects ($n = 15$) were analyzed by qRT-PCR. The results are shown as the means, P, versus non-AMI subjects.

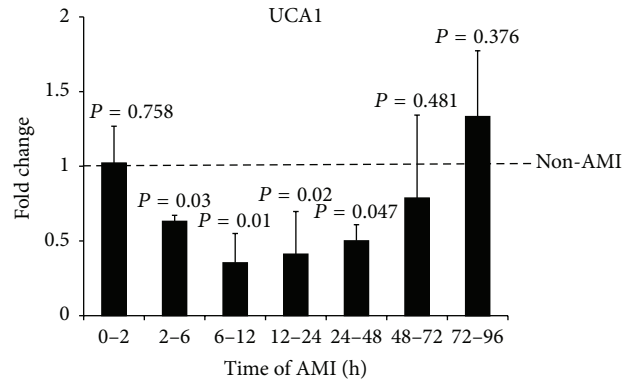

Figure 2: The levels of circulating UCA1 in different time of onset of AMI. The expression level UCA1 in plasma of AMI patients ($n = 49$) were analyzed by qRT-PCR according to the time after onset of AMI. The results are shown as the means, P, versus non-AMI subjects.

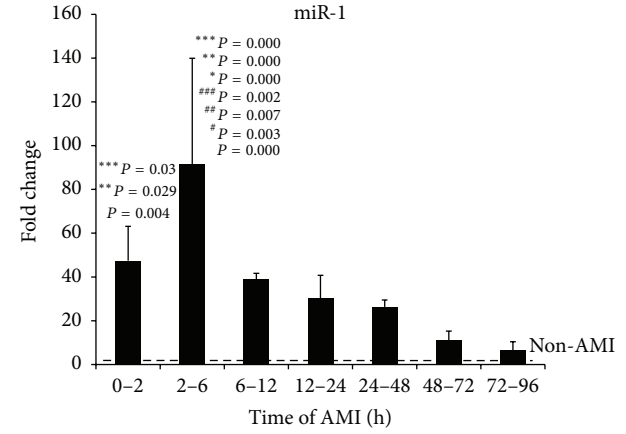

Figure 3: The levels of circulating miR-1 in different time of onset of AMI. The expression level of miR-1 in plasma of AMI patients ($n = 49$) were analyzed by qRT-PCR according to the time after onset of AMI. The results were shown as the means, P, versus non-AMI subjects; $^{\#}P$, versus 0–2 h; $^{\#\#}P$, versus 6–12 h; $^{\#\#\#}P$, versus 12–24 h; $^{*}P$, versus 24–48 h; $^{**}P$, versus 48–72 h; $^{***}P$, versus 72–96 h.

To test the speculation, the patients with AMI were divided into 7 groups according to the time after AMI: 0–2 hours (2 cases), 2–6 hours (3 cases), 6–12 hours (6 cases), 12–24 hours (12 cases), 24–48 hours (13 cases), 48–72 hours (9 cases), and 72–96 hours (4 cases). As shown in Figure 2, the levels of UCA1 in AMI patients were decreased in 2–6 h, 6–12 h, 12–24 h, and 24–48 h after AMI ($P < 0.05$). The level of UCA1 was decreased from 2 h after AMI and was lowest in 6–12 h. Then, the level of circulating UCA1 began to recover and back to the control value in 48–72 h after onset of AMI. The level of UCA1 in 72–96 h after onset of AMI was higher than non-AMI subjects.

3.3. The Level of Circulating miR-1 in the Patients with AMI. It was reported that miR-1 inhibited the expression of UCA1 [19]. Therefore, we investigate whether the expression levels of UCA1 are associated with miR-1. The level of plasma miR-1 was also tested according to the time after AMI. The results (Figure 3) showed that the level of miR-1 was increased from 0 to 2 h, compared with health control ($P < 0.05$), and was highest in 2–6 h. Then, it began to decrease and returned to the normal level at 72–96 h. There was statistical significance between the patients at 2–6 h after onset of AMI and health subjects or other patients ($P < 0.05$). The results showed that the expression of UCA1 was negatively correlated with the expression of miR-1.

3.4. The Effect of Hypertension and Diabetes on Circulating UCA1. Many AMI patients have the history of hypertension and diabetes. We further investigated the influence of hypertension and diabetes on the level of UCA1. As the level of UCA1 was lowest at 6–48 h after AMI, those patients were divided into AMI alone group, AMI + hypertension

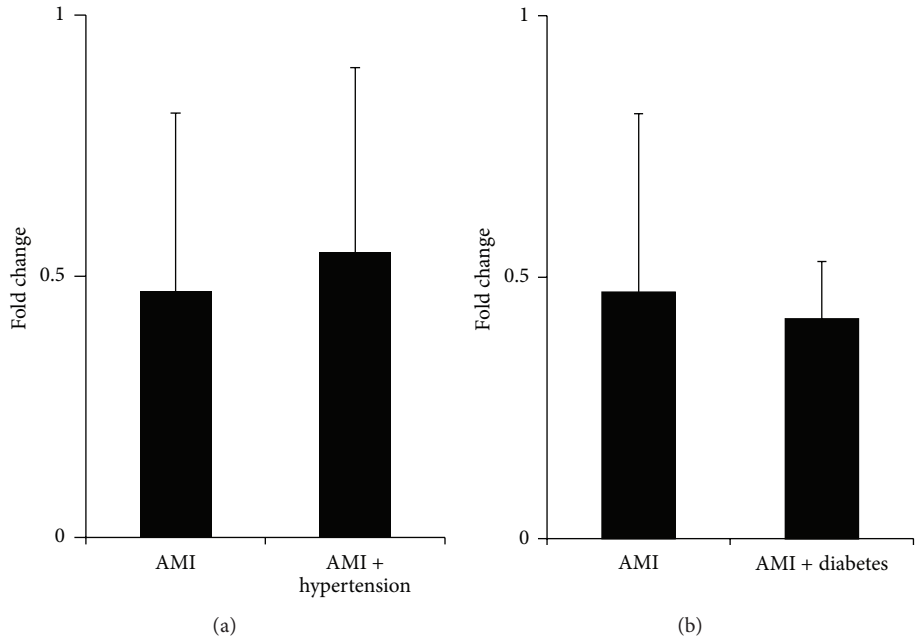

Figure 4: The influence of hypertension and diabetes on level of UCA1 in plasma of AMI patients. The expression level UCA1 in plasma of AMI patients ($n = 34$) at 2–48 h was analyzed by qRT-PCR according to the patients with or without hypertension or diabetes. (a) AMI and AMI combined hypertension subjects. (b) AMI and AMI combined diabetes subjects. The results are shown as the means, P, versus non-AMI subjects.

group, and AMI + diabetes group and the level of UCA1 in different groups was analyzed. The results (Figure 4(a)) showed that there were no significant differences in the UCA1 expression between AMI sand AMI combined with hypertension subjects. Similarly, there were no significant differences in the UCA1 expression between AMI and AMI combined with diabetes (Figure 4(b)). Those results indicated that the UCA1 was not correlated with hypertension or diabetes.

3.5. The Predictive Power of UCA1 for AMI. To evaluate the predictive power of circulating UCA1 for AMI, we performed ROC analysis for 49 patients with AMI. As shown in Figure 5(a), the area under the ROC curve (AUC) for UCA1 was 0.757 (95% confidence interval) and the AUC measured for cTnI was 0.957, while the AUC measured for UCA1-cTnI was 0.981. As shown in Figure 5(b), the area under the ROC curve (AUC) for UCA1 was 0.757 (95% confidence interval) and the AUC measured for CK-MB was 0.9592, while the AUC measured for UCA1-CK-MB was 0.983. This result demonstrated that UCA1 had marked sensitivity and specificity for AMI, but it was not superior to cTnI and CK-MB for the diagnosis of AMI.

4. Discussion

In this study, we investigated whether plasma lnc RNA UCA1 could be as biomarker of AMI. Interestingly, the levels of plasma UCA1 were decreased at the early state of AMI patients and increased at day 3 after AMI, which was not affected in AMI patient with hypertension or diabetes. Moreover, the level of UCA1 was negatively correlated with the expression of miR-1.

The UCA1 played important role on embryonic development and only was expressed in heart and spleen of normal adult after birth [14]. The UCA1 might play important role in protecting heart. UCA1 promoted glucose consumption and lactate production in bladder cancer cells [20], indicating that the UCA1 might be important in providing the energy needed for heart. In addition to involvement in glucose metabolic processes, UCA1 also could promote cell proliferation and inhibit cell apoptosis in bladder cancer [21]. It was also supported that UCA1 could inhibit the expression of p27 [22], while upregulation of p27 significantly enhanced cell apoptosis [23]. Consistently, it was found that UCA1 inhibited the expression of miR-1 and protected the cardiomyocyte against H_2O_2-induced apoptosis [24]. Our study showed that the circulating UCA1 was decreased in the plasma of patients with AMI, indicating that the cardiomyocyte apoptosis in AMI might be associated with downregulation of UCA1.

Interestingly, the UCA1 was decreased early but upregulated afterwards in the plasma of patients after myocardial infarction. Similarly, global transcriptomic analyses uncovered that LIPCAR (the mitochondrial long noncoding RNA uc022bqs.1) was downregulated early but upregulated afterwards in the plasma of patients after myocardial infarction [10]. The level of UCA1 was regulated by miR-1. It was found that miR-1 decreased the expression of UCA1 in bladder

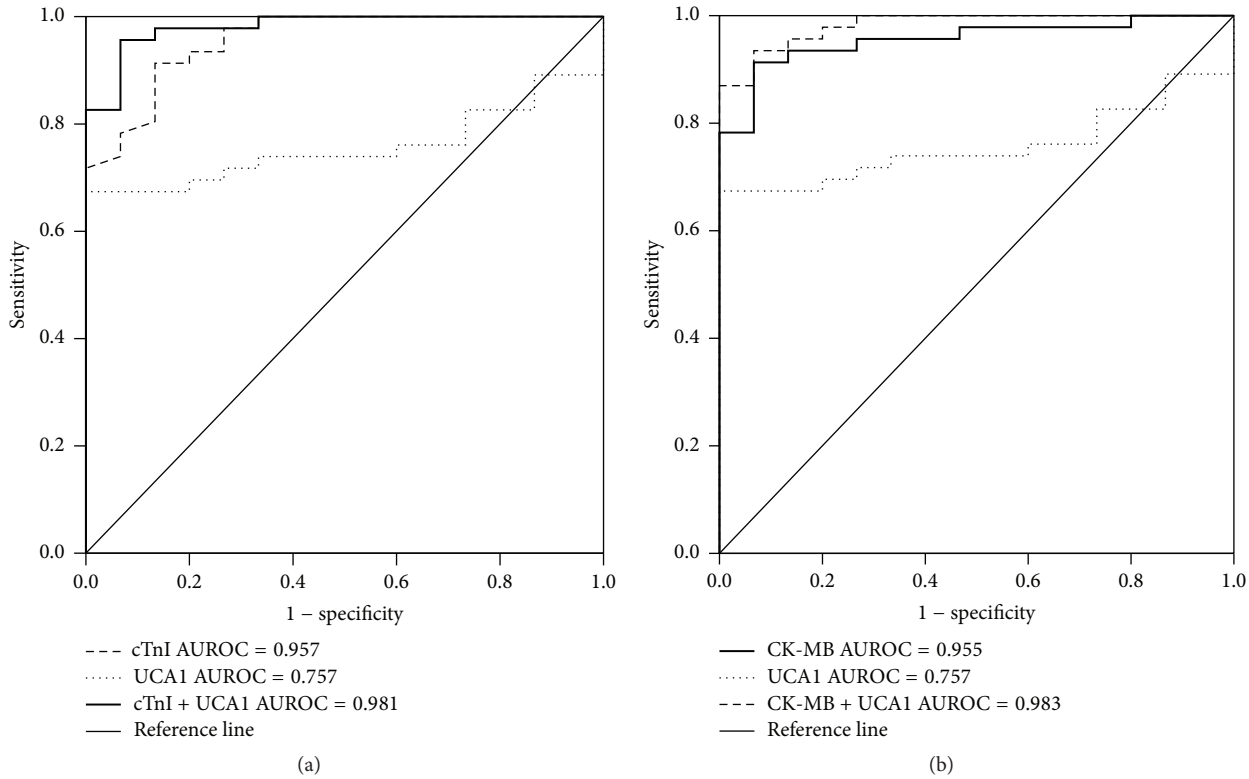

Figure 5: Comparisons of the sensitivity and specificity of the diagnosis by plasma UCA1 and cTnT in the AMI patients. ROC curves were constructed to evaluate the diagnostic values of UCA1 for the AMI patients in comparison with cTnI and CK-MB. (a) Comparison of UCA1 and nTnI. (b) Comparison of UCA1 and CK-MB. AUC: area under the ROC curve.

cancer cells in an Ago2-slicer-dependent manner [19]. In addition, the binding site of miR-1 and UCA1 was also found, indicating that miR-1 could directly inhibit the expression of UCA1 [19]. The miR-1 was markedly increased in the plasma of the rats or patients with AMI [25]. Our study also showed that the UCA1 was negatively correlated with the expression of miR-1. The UCA1 was decreased at the early state of AMI and increased at day 3 after AMI, while the miR-1 was increased in the early state of AMI and returned to the normal level on day 3 after the onset of AMI. The miR-1 was found to exacerbate cardiac injury by affecting the expression of a host of protective proteins such as BCL2 and HSP60 [26] and also by inhibition of UCA1. In addition, some microRNAs are selectively deplenished from plasma during their passage through the myocardial circulation. For example, circulating levels of miR-126 decreased during transcoronary passage in patients with evidence of myocardial injury, suggesting consumption during transcoronary passage. Those microRNAs might be consummated by some circulating lnc RNAs [27].

Limitations. It should be noted that the consideration of circulating lnc RNA (UCA1) as a biomarker for AMI is at present based on our results from a relatively small sample size and larger clinical studies are definitely required to establish the case. UCA1 had marked sensitivity and specificity for AMI, but it was not superior to cTnI and CK-MB for the diagnosis of AMI. However, UCA1 as an additional factor might enhance the clinical use of cTnI and CK-MB. While the fact that circulating was restored back to normal in AMI patients on discharge may give an insinuation for the potential of a prognostic marker as well. In addition, it should be concerned that the UCA1 was highly expressed in many cancers such as bladder cancer and lung cancer and could be as predictor biomarker for those cancers. Furthermore, UCA1 is also expressed in the spleen in normal states, indicating that the level might be changed with immune dysfunction. Therefore, those conditions must be excluded when UCA1 was used as biomarker for AMI. Application of detection technology for lnc RNA is challenged. Although real-time PCR is the gold standard for gene expression quantification and accepted as being a powerful technique in comparative expression analysis in life sciences and medicine [19], it was very expensive and time-consuming for a large quantity of samples. Last, the emerging application of circulating lnc RNAs for disease diagnosis is restrained by the limited knowledge that we have of their biology. For instance, it remains unclear whether lnc RNAs contribute to the disease or if they become altered as a consequence of the disease itself.

Taken together, our data demonstrates that UCA1 is decreased in the early state of AMI and negatively correlated with miR-1, suggesting that UCA1 participates in the pathophysiology of AMI interaction with miR-1. Although UCA1

as independent biomarker displays sensitivity and specificity compared to cTnI and CK-MB, it could be as additional biomarker to enhance sensitivity and specificity than cTnI and CK-MB. It is unquestionable that the more we learn about lnc RNA expression patterns in AMI disease, the higher the chances for an improved diagnosis and better prognosis will be [28].

Ethical Approval

The authors declare that their studies have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Consent

All persons gave their informed consent prior to their inclusion in the study.

Disclosure

They also confirm that all the listed authors have participated actively in the study and have seen and approved the submitted paper.

Conflict of Interests

The authors have no conflicting financial interests.

Acknowledgments

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Research Article

Gender-Specific Association of *ATP2B1* Variants with Susceptibility to Essential Hypertension in the Han Chinese Population

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Previous genome-wide association studies (GWASs) found that several *ATP2B1* variants are associated with essential hypertension (EHT). But the "genome-wide significant" *ATP2B1* SNPs (rs2681472, rs2681492, rs17249754, and rs1105378) are in strong linkage disequilibrium (LD) and are located in the same LD block in Chinese populations. We asked whether there are other SNPs within the *ATP2B1* gene associated with susceptibility to EHT in the Han Chinese population. Therefore, we performed a case-control study to investigate the association of seven tagSNPs within the *ATP2B1* gene and EHT in the Han Chinese population, and we then analyzed the interaction among different SNPs and nongenetic risk factors for EHT. A total of 902 essential hypertensive cases and 902 normotensive controls were involved in the study. All 7 tagSNPs within the *ATP2B1* gene were retrieved from HapMap, and genotyping was performed using the Tm-shift genotyping method. Chi-squared test, logistic regression, and propensity score analysis showed that rs17249754 was associated with EHT, particularly in females. The MDR analysis demonstrated that the interaction of rs2070759, rs17249754, TC, TG, and BMI increased the susceptibility to hypertension. Crossover analysis and stratified analysis indicated that BMI has a major effect on the development of hypertension, while *ATP2B1* variants have a minor effect.

1. Introduction

Because of its high prevalence and substantial impact on several cardiovascular diseases, hypertension is considered a major contributor to the global health burden [1]. Approximately 95% of hypertensive patients are diagnosed with essential hypertension (EHT), which is defined as high blood pressure (BP) with no identifiable cause [2]. EHT is one of the most common complex genetic disorders, with heritability ranging from 31% to 68% [3]. However, attempts to identify the genetic basis of EHT have been frequently unsuccessful and of relatively low yield [4]. The inability to identify the genetic basis of EHT may be due to the cumulative impact of multiple genes interacting with a variety of environmental factors in the pathogenesis of hypertension [5, 6].

In 2009, based on a genome-wide association study (GWAS) conducted by the Cohorts for Heart and Aging Research in Genome Epidemiology (CHARGE) Consortium, genetic polymorphisms of *ATP2B1* were found to be significantly related to systolic blood pressure (SBP), diastolic blood pressure (DBP), and hypertension [7]. These SNPs were also replicated in the European populations by the Global Blood Pressure Genetics (Global BPgen) Consortium [8]. Moreover, combined analysis of these two datasets further confirmed that only *ATP2B1* variants reached genome-wide significance threshold ($P < 5 \times 10^{-8}$) with SBP (rs2681492), DBP (rs2681472), and hypertension (rs2681472) [9]. Similarly, in a study of the Korean Association Resource (KARE), rs17249754, which is located near the *ATP2B1* gene, was found to be strongly associated with SBP [10]. Moreover, in a

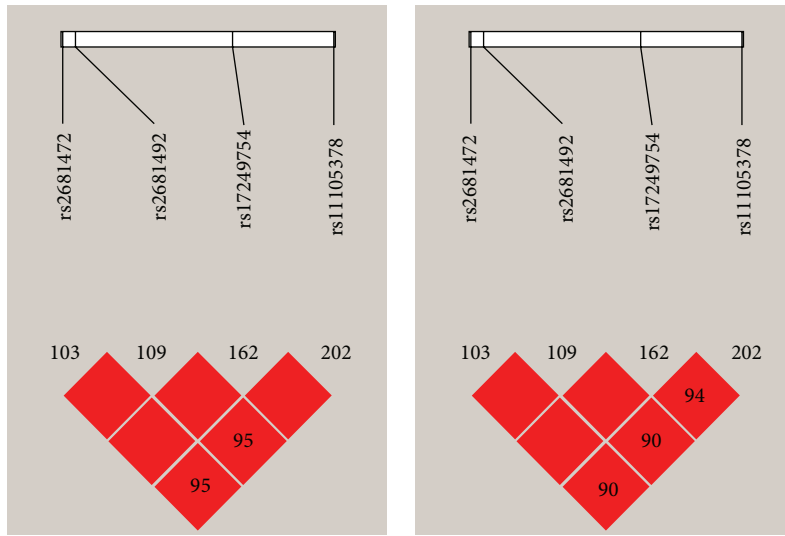

FIGURE 1: The patterns of linkage disequilibrium for 4 SNPs with D' (a) and r^2 (b).

study by the Japanese Millennium Genome Project, another *ATP2B1* variant, rs11105378, was found to have the most significant association with hypertension ($P = 4.1 \times 10^{-11}$), and the association was cross-validated by replication analysis with the Global BPgen dataset ($P = 5.9 \times 10^{-4}$) [1]. Meta-analysis of GWASs in East Asians indicated that rs17249754 was associated with SBP ($P = 7.7 \times 10^{-20}$) and DBP ($P = 1.9 \times 10^{-13}$) [11].

Although *ATP2B1* was confirmed to be associated with blood pressure or hypertension in various populations, the "significant" SNPs (rs2681472, rs2681492, rs17249754, and rs1105378) found in the GWASs are in strong linkage disequilibrium (LD) and are located in the same LD block (HapMap CHB $D' > 0.95$, $r^2 > 0.9$) in the Chinese population (Figure 1). We wondered whether there are other SNPs within the *ATP2B1* gene associated with the susceptibility to EHT in the Han Chinese population. In the current study, we conducted a replication analysis to test the association of seven tagSNPs within the *ATP2B1* gene and EHT in the Han Chinese population. Subsequently, we analyzed the interaction among different SNPs and nongenetic risk factors for EHT, which provided additional information on the role of *ATP2B1* variants.

2. Materials and Methods

2.1. Ethics Statement. The protocol of this study was approved by the medical ethics committee of Ningbo University. The health records and blood samples of the participants were collected with informed written consent.

2.2. Study Participants. The details of the study participants have been described previously [12]. Briefly, we collected more than 10,000 health records from our established database of Ningbo Chronic Diseases Cohort. The participants in this database are 30 to 75 years old, Han Chinese, living in Ningbo City (East coast of China) for at least three generations without migration history. Patients with secondary hypertension, severe cardiovascular diseases, diabetes, kidney diseases, or other major chronic illnesses according to their health records were excluded before case-control paring. Hypertension in this study was defined as sitting systolic blood pressure (SBP) ≥140 mmHg and/or diastolic blood pressure (DBP) ≥90 mmHg or self-reported use of antihypertensive medication. Participants with SBP ≤120 mmHg and DBP ≤80 mmHg were recruited as controls. Subsequently, 902 hypertensive cases and 902 normotensive controls, matched for age and sex, were selected with informed consent.

2.3. Measurement of Clinical Parameters. With informed written consent, two milliliters of venous blood was collected with ethylene diamine tetraacetic acid as an anticoagulant. Subsequently, the serum levels of total cholesterol (TC), high-density lipoprotein (HDL), and triglyceride (TG) were measured enzymatically using a Hitachi automatic biochemistry analyzer 7100. Clinical information, including body mass index (BMI), and weekly alcohol and cigarette consumption were also obtained. In this study, people who consumed ≥70 g of alcohol per week for more than 1 year were defined as individuals with alcohol abuse. Moreover, people who smoked ≥70 cigarettes per week for more than 1 year were defined as individuals with a smoking habit.

2.4. SNP Genotyping. All 7 tagSNPs were retrieved from HapMap using the tagger pairwise method in CHB as follows: R^2 cutoff = 0.8 and minor allele frequency (MAF) cutoff = 0.1. Genomic DNA was extracted from whole blood through the standard phenol-chloroform method. Genotyping was performed using the Tm-shift genotyping method [13]. To

Table 1: Baseline characteristics of the investigated participants.

Variables	Case	Control	*P* value
Number	902	902	N/A
Male/female	390/512	390/512	N/A
Age (y)	56.92 ± 7.36	56.59 ± 7.43	$P = 0.45$
TG (mM)	2.02 ± 1.68	1.64 ± 1.12	$P < 0.01$
HDL (mM)	1.41 ± 0.35	1.41 ± 0.33	$P = 0.71$
TC (mM)	5.33 ± 1.01	5.18 ± 0.93	$P < 0.01$
BMI (Kg/m^2)	24.65 ± 3.25	23.22 ± 2.88	$P < 0.01$
Smoking habit	171	147	$P = 0.14$
Alcohol abuse	152	148	$P = 0.80$

TG: triglyceride; HDL: high-density lipoprotein; TC: total cholesterol; BMI: body mass index.

confirm the genotyping results, 100 samples were randomly selected and sequenced with bidirectional coverage by BGI Tech Solutions Company.

2.5. Statistical Analysis. Continuous variables are presented as the mean ± SD and analyzed by *t*-test between two groups. Statistical analyses of the allele frequencies between the hypertensive and normotensive subjects and between males and females were performed using the chi-squared test. Logistic regression was used to control the confounding variables. *P* values, odds ratios (ORs), and 95% confidence intervals (CIs) were calculated using SPSS 18.0 (SPSS Inc., Chicago, IL, USA). The propensity score analysis was performed using STATA 13.0 according to the method described by Rosenbaum and Rubin [14]. The Hardy-Weinberg equilibrium (HWE) test for genotype distribution was performed for the controls using PEDSTATS [15]. Multifactor dimensionality reduction (MDR), stratified analysis, and crossover analysis were used to identify and characterize interactions among SNPs and nongenetic factors [16]. *P* values were adjusted for the total number of tested SNPs using the Bonferroni correction method ($\alpha = 0.05/7 \approx 0.0071$).

3. Results

Table 1 shows the baseline characteristics of the participants. Each group consists of 390 males and 512 females, and the mean ages of the hypertensive participants and controls were similar, demonstrating that the case and control groups were well matched. Serum levels of TC and TG and BMI were significantly higher in the hypertensive groups than those in the control group ($P < 0.01$). However, the serum level of HDL and the percentage of participants with a smoking habit or alcohol abuse were not different between two groups.

Table 2 shows the genotypes of each SNP. The success rate of genotyping was 99%, and all SNPs did not deviate from HWE ($P > 0.05$). Based on the prevalence, OR, and MAF in this study, the genetic power calculator indicated that the sample size is large enough to perform a case-control analysis with 80% power [17]. According to the *P* values and ORs, only G allele of rs17249754 is associated with EHT ($P = 0.005$, OR (95% CI) = 1.21 (1.06–1.39)) after correction for multiple testing. However, rs2070759, rs3741895, rs2854371, rs11105357, rs957525, and rs11105358 were not associated with EHT. Inputting all covariates including age, gender, HDL, TC, TG, BMI, smoking habit, and alcohol abuse, the propensity score analysis indicated that still only G allele of rs17249754 is associated with EHT ($P = 0.007$, OR = 1.21). After control of confounding variables including TC, TG, and BMI, logistic regression also confirmed rs17249754 is associated with EHT ($P = 0.007$, OR = 1.21).

Considering gender difference in EHT [18], the genotyping results were further stratified by gender. Interestingly, both the A allele of rs2070759 and the G allele of rs17249754 were significantly associated with EHT only in women (for rs2070759, $P = 0.008$, OR (95% CI) = 1.27 (1.06–1.51); for rs17249754, $P = 0.017$, OR (95% CI) = 1.25 (1.04–1.49)).

MDR was used to analyze the interaction among SNPs and nongenetic risk factors for EHT, and the software output the best model for "BMI" and "rs2070759, rs17249754, TG, TC, and BMI" with 10/10 cross-validation consistency (Table 3). To determine the manner in which BMI and *ATP2B1* variants interact to cause hypertension, we performed a stratified analysis. The result showed that when BMI ≥25, neither SNP is associated with hypertension ($P > 0.05$). However, when BMI <25, the A allele of rs2070759 or the G allele of rs17249754 showed a significant association with hypertension (Table 4), indicating that BMI has a major effect and that the *ATP2B1* variants have minor effects. Additional crossover analysis also confirmed that BMI had the primary effect (Table 5).

4. Discussion

Although dozens of GWASs have been conducted to identify genetic markers for BP traits or hypertension over the past two decades, *ATP2B1* may be the first gene that has been cross-validated in different GWASs. The present study confirmed *ATP2B1* variant rs17249754 as strong susceptibility for EHT in the Han Chinese population. The SNP rs17249754 is associated with BP variation and EHT based on several GWASs in different ethnic populations [1, 10, 11, 19], which is also in strong linkage disequilibrium with other genome-wide significant SNPs, such as rs2681472, rs2681492, and rs1105378, within the *ATP2B1* gene. Similar findings in different ethnic groups further strengthen the hypothesis that the *ATP2B1* gene is a susceptibility locus of likely global significance for BP variation and the development of hypertension.

The *ATP2B1* gene encodes the plasma membrane calcium ATPase isoform 1, which plays a critical role in intracellular calcium homeostasis due to its capacity for removing bivalent calcium ions from eukaryotic cells against very large concentration gradients [20]. Although the pathophysiological implications of *ATP2B1* gene on the development of hypertension are still unclear, results from *ATP2B1* knockout mouse studies suggested that ATP2B1 may play an important role in the regulation of BP through alterations of calcium handling and vasoconstriction in vascular smooth muscle cells [21]. *ATP2B1* mRNA expression levels in umbilical artery smooth

Table 2: Association statistics for the *ATP2B1* variants and hypertension.

SNP	Genotype	Group	Genotype			MAF	*P* value	OR	95% CI
rs3741895	AA/AG/GG	Case	778	122	0	0.07	0.954	0.99	0.77–1.29
		Control	778	121	0	0.07			
		Male case	331	58	0	0.07	0.433	0.86	0.58–1.27
		Male control	338	50	0	0.06			
		Female case	447	64	0	0.06	0.533	1.12	0.79–1.59
		Female control	440	71	0	0.07			
rs2854371	CC/CT/TT	Case	519	339	44	0.24	0.879	1.01	0.87–1.18
		Control	510	347	41	0.24			
		Male case	226	143	21	0.24	0.648	0.95	0.75–1.20
		Male control	226	146	15	0.23			
		Female case	293	196	23	0.24	0.553	1.06	0.87–1.30
		Female control	284	201	26	0.25			
rs2070759	AA/AC/CC	Case	266	453	183	0.45	0.036*	1.15	1.01–1.31
		Control	223	476	203	0.49			
		Male case	107	201	82	0.47	0.879	1.02	0.83–1.24
		Male control	109	194	87	0.47			
		Female case	159	252	101	0.44	0.008*	1.27	1.06–1.51
		Female control	114	282	116	0.50			
rs11105357	CC/CT/TT	Case	722	172	8	0.10	0.472	0.92	0.74–1.15
		Control	733	163	6	0.10			
		Male case	320	64	6	0.10	0.407	1.15	0.83–1.59
		Male control	309	76	5	0.11			
		Female case	402	108	2	0.11	0.088	0.78	0.58–1.04
		Female control	424	87	1	0.09			
rs957525	AA/AG/GG	Case	546	314	40	0.22	0.705	1.03	0.88–1.21
		Control	541	313	45	0.22			
		Male case	245	132	12	0.20	0.239	1.16	0.91–1.48
		Male control	231	141	17	0.22			
		Female case	301	182	28	0.23	0.615	0.95	0.77–1.17
		Female control	310	172	28	0.22			
rs11105358	CC/CG/GG	Case	43	311	547	0.22	0.160	1.12	0.96–1.32
		Control	35	293	574	0.20			
		Male case	24	124	242	0.22	0.903	1.02	0.80–1.29
		Male control	18	134	238	0.22			
		Female case	19	187	305	0.22	0.076	1.22	0.98–1.51
		Female control	17	159	336	0.19			
rs17249754	AA/AG/GG	Case	102	417	383	0.34	0.005*	0.82	0.72–0.94
		Control	143	416	343	0.39			
		Male case	46	182	162	0.35	0.128	0.85	0.69–1.05
		Male control	59	185	146	0.39			
		Female case	56	235	221	0.34	0.017*	0.80	0.67–0.96
		Female control	84	231	197	0.39			

P values were obtained from the comparison of two allele frequencies. OR: odds ratio; CI: confidence interval. * *P* value was less than 0.05.

Table 3: MDR analysis of gene-environment interaction.

Best model	Testing odds ratio	Testing X^2	Cross-validation consistency
BMI	2.25 (95% CI: 1.19–4.24)	6.36 ($P = 0.012$)	10/10
BMI, TG	2.00 (95% CI: 1.10–3.61)	5.27 ($P = 0.021$)	9/10
rs2070759, rs17249754, TG, TC, and BMI	1.83 (95% CI: 1.01–3.30)	4.05 ($P = 0.044$)	10/10

Table 4: Stratified analysis of interaction between BMI and *ATP2B1* variants.

SNP	Genotype	BMI	Group	Number			*P* value	OR	95% CI
rs2070759	AA/AC/CC	<25	Case	147	268	104	0.022*	1.21	1.03–1.42
			Control	156	359	164			
		≥25	Case	115	185	83	0.349	0.89	0.71–1.13
			Control	70	114	39			
rs17249754	AA/AG/GG	<25	Case	59	241	219	0.011*	0.80	0.68–0.95
			Control	104	331	244			
		≥25	Case	43	176	164	0.61	0.94	0.74–1.20
			Control	37	85	101			

P values were obtained from the comparison of two allele frequencies. OR: odds ratio; CI: confidence interval.
* *P* value was less than 0.05.

Table 5: Crossover analysis of interaction between BMI and *ATP2B1* variants.

SNP	BMI	Allele	Case	Control	*P* value	OR	95% CI
rs2070759	<25	C	476	687	1	1	NA
	<25	A	562	671	0.022*	1.21	1.03–1.42
	≥25	C	351	192	$P < 0.001$*	0.38	0.31–0.47
	≥25	A	415	254	$P < 0.001$*	0.42	0.35–0.52
rs17249754	<25	A	359	539	1	1	NA
	<25	G	679	819	0.011*	0.80	0.68–0.95
	≥25	A	262	159	$P < 0.001$*	0.40	0.32–0.51
	≥25	G	504	287	$P < 0.001$*	0.38	0.31–0.46

P values were obtained from the comparison of two allele frequencies. OR: odds ratio; CI: confidence interval.
* *P* value was less than 0.05.

muscle cells were found to be significantly different among rs11105378 genotypes, which may be a potential mechanism by which changes in the *ATP2B1* gene product levels are involved in BP regulation [1]. According to HapMap CHB, rs17249754 and rs1105378 are in strong linkage disequilibrium ($D' = 1$, $r^2 = 0.95$) in Chinese populations; therefore, rs17249754 was genotyped instead of rs1105378 in the present study. In our replication study, we also found that rs1105378 is significantly associated with hypertension ($P < 0.01$). Therefore, the SNPs rs2681472, rs2681492, and rs17249754 are in strong linkage disequilibrium with rs1105378 and may be a genetic marker for the development of hypertension, whereas rs1105378 may have a biological function.

Another finding of the present study is that *ATP2B1* variants are associated with EHT only in women. According to the World Health Organization's (WHO) "Global Status Report on Noncommunicable Diseases 2014" (http://www.who.int/nmh/publications/ncd-status-report-2014/en/), hypertension occurs at a lower rate and at a later age in females than males in all WHO regions. The impact of gender on the prevalence, presentation, and long-term outcome of hypertension has long been a topic of active research. Recent data from several large epidemiological studies showed that awareness, treatment, and control rates of hypertension are higher among women than men, which may cause the gender difference in hypertension [22, 23]. The pathophysiological mechanisms underlying the disparity in blood pressure levels between the two genders are poorly defined, although many hypotheses have been proposed, with hormonal hypotheses prevailing [24]. Similar to our study, several previous studies also found a gender-specific association between gene polymorphisms and EHT [25–27]. Therefore, further basic research is of paramount importance to uncover the genetic and biological mechanisms mediating potential gender differences in hypertension.

EHT is a typical complex disease [28], with dozens of risk factors, such as obesity, physical inactivity, high-fat diet, cigarette smoking, alcohol abuse, excessive salt intake, and mental stress [29–31]. Growing evidence indicates that interactions among multiple genes and environmental factors may increase the susceptibility to EHT [32]. Our previous study has shown that interaction analysis may provide somewhat more information than a single genetic association study [12, 33]. In the present study, MDR analysis demonstrated that BMI itself and the interaction between *ATP2B1* variants and BMI increase the susceptibility to hypertension. Because BMI represents the internal metabolic status and physiological environment [34], it is not surprising that BMI has a major effect in the development of hypertension, while the *ATP2B1* variants have a minor effect. With the development of statistical methods for the evaluation of gene-gene and gene-environment interactions, more missing inheritability will be identified and more specific mechanisms will be discovered [35, 36].

In conclusion, we confirmed the association of *ATP2B1* variants with the susceptibility to EHT in the Han Chinese population, especially in the females. Moreover, the interaction of BMI and *ATP2B1* variants increased the susceptibility to hypertension, with BMI having a major effect and *ATP2B1* variants having a minor effect.

Conflict of Interests

The authors declare no conflict of interests.

Authors' Contribution

Jin Xu, Hai-xia Qian, and Su-pei Hu contributed equally to this work.

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