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# Roles and Clinical Applications of Biomarkers in Cardiovascular Disease 2017

Lead Guest Editor: Raffaele Serra

Guest Editors: Stefano de Franciscis and Laurent Metzinger





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# Contents

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**Roles and Clinical Applications of Biomarkers in Cardiovascular Disease 2017**

Raffaele Serra, Stefano de Franciscis, and Laurent Metzinger  
Volume 2017, Article ID 3093589, 2 pages

**Potential Biological Markers of Atrial Fibrillation: A Chance to Prevent Cryptogenic Stroke**

István Szegedi, László Szapáry, Péter Csécei, Zoltán Csanádi, and László Csiba  
Volume 2017, Article ID 8153024, 10 pages

**The Management of Cardiovascular Risk through Epigenetic Biomarkers**

Laurent Metzinger, Stefano de Franciscis, and Raffaele Serra  
Volume 2017, Article ID 9158572, 6 pages

**Hypofibrinolytic State in Subjects with Type 2 Diabetes Mellitus Aggravated by the Metabolic Syndrome before Clinical Manifestations of Atherothrombotic Disease**

Elsa Aburto-Mejía, David Santiago-Germán, Manuel Martínez-Marino, María Eugenia Galván-Plata, Eduardo Almeida-Gutiérrez, Mardía López-Alarcón, Jesús Hernández-Juárez, Antonio Alvarado-Moreno, Alfredo Leños-Miranda, Abraham Majluf-Cruz, and Irma Isordia-Salas  
Volume 2017, Article ID 6519704, 9 pages

**Clinic Predictive Factors for Insufficient Myocardial Reperfusion in ST-Segment Elevation Myocardial Infarction Patients Treated with Selective Aspiration Thrombectomy during Primary Percutaneous Coronary Intervention**

Jinfan Tian, Yue Liu, Xiantao Song, Min Zhang, Feng Xu, Fei Yuan, and Shuzheng Lyu  
Volume 2016, Article ID 3823809, 9 pages

**High Systolic and Diastolic Blood Pressure Variability Is Correlated with the Occurrence of Peripheral Arterial Disease in the First Decade following a Diagnosis of Type 2 Diabetes Mellitus: A New Biomarker from Old Measurement**

Chi-Hsiao Yeh, Hsiu-Chin Yu, Tzu-Yen Huang, Pin-Fu Huang, Yao-Chang Wang, Tzu-Ping Chen, and Shun-Ying Yin  
Volume 2016, Article ID 9872945, 8 pages

**Elevated Levels of Plasma Superoxide Dismutases 1 and 2 in Patients with Coronary Artery Disease**

Ji-Ren Peng, Ting-Ting Lu, Hao-Teng Chang, Xuan Ge, Bian Huang, and Wei-Min Li  
Volume 2016, Article ID 3708905, 9 pages

## Editorial

# Roles and Clinical Applications of Biomarkers in Cardiovascular Disease 2017

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Cardiovascular and Peripheral Vascular Disease (CPVD) is one of the major causes of mortality and morbidity worldwide.

Biomarkers can indicate several health or disease characteristics and they were found to be useful in order to improve diagnosis, prevention strategies, and treatment options in the field of CPVD. In fact, biomarkers may be used to better identify high-risk individuals, to diagnose disease conditions promptly and accurately, and to effectively prognosticate and treat patients with disease, targeting also specific biological sites.

As such, the present issue deals with both reviews and research articles focused on novel applications of biomarkers used to enhance the ability of the clinician to optimally manage the patient with CPVD. Furthermore, this special issue will discuss breakthrough biological and technological developments in biomarkers identification which are expected to revolutionize clinical research environments and healthcare in the area of CPVD.

The review by I. Szegedi et al. focused on biomarkers of atrial fibrillation and they based their study on four groups of biomarkers: markers of inflammation, markers of fibrosis, markers with hormonal activity, and other markers.

The review by L. Metzinger et al. considered the current evidences of epigenetic biomarkers dealing with the role of the main mechanisms of epigenetics in the area of cardiovascular risk. The main epigenetic processes showed in the review article are DNA methylation, posttranslational histone

modifications, and RNA-based mechanisms such as non-coding RNAs, which are mainly represented by microRNAs (miRNAs), and long noncoding RNAs (lncRNAs).

The study by E. Aburto-Mejía et al. supported the idea that characterization of emerging biomarkers of impaired fibrinolysis such as plasminogen activator inhibitor type-1 (PAI-1) should be measured for surveillance of transition from a healthy state through the development of the Metabolic Syndrome to atherothrombotic disease.

The article by F.-Y. Chu et al. showed that high systolic and diastolic blood pressure variability may be correlated with the occurrence of peripheral arterial disease in the first decade following a diagnosis of type 2 diabetes mellitus.

The study by J.-R. Peng et al. showed that plasma levels of superoxide dismutases 1 and 2 (SOD1 and SOD2) were elevated in patients with coronary artery disease (CAD) and they concluded that these enzyme levels may be useful in the future as biomarkers for diagnosis of CAD.

The paper by J. Tian et al. presented a study on clinic predictive factors for insufficient myocardial reperfusion in ST-segment elevation myocardial infarction patients treated with selective aspiration thrombectomy during primary percutaneous coronary intervention showing the importance of identifying specific risk factors in order to improve the effectiveness of selective thrombus aspiration.

We hope that this special issue will attract the interest of scientific community in order to stimulate and improve

further investigations leading to the discovery of novel biomarkers in the field of CPVD.

*Raffaele Serra  
Stefano de Franciscis  
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## Review Article

# Potential Biological Markers of Atrial Fibrillation: A Chance to Prevent Cryptogenic Stroke

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Stroke affects millions of people all over the world, causing death and disability. The most frequent type of this disease is ischemic stroke, which can be caused by different factors. In approximately 25 percent of cases, no obvious cause can be found. Recent observations have shown that paroxysmal atrial fibrillation could be responsible for a significant number of cryptogenic stroke events. Short- or long-lasting ECG monitoring could help with the diagnosis of transient arrhythmias. Unfortunately, these techniques either are expensive or require good patient compliance. An alternative option is the identification of biological markers that are specific for atrial fibrillation and can be used to predict arrhythmia. In this review, we give a summary of the recent advances in the research of arrhythmia markers. Based on their structure and function, we differentiated four groups of biomarkers: markers of inflammation, markers of fibrosis, markers with hormonal activity, and other markers. In spite of intensive researches, the optimal biological marker is still not available, but there are some promising markers, like NT-proBNP/BNP.

## 1. Introduction

Stroke is a common vascular disease manifesting in different subtypes, causing death and disability, and therefore it is an important challenge for healthcare systems. After an ischemic event, a thorough workup identifies the cause in about 75 percent of cases. In the remaining 20–25 percent, no causative factor can be found: these are called cryptogenic stroke events. In about 25–30 percent of these events, the underlying cause could be paroxysmal atrial fibrillation (PAF) [1–3]. Most studies defined PAF as an event that lasts longer than 30 seconds, referring to the AHA 2006 guidelines [4]. In atrial fibrillation (AF), the blood flow in the cavities of the heart is turbulent, which can precipitate thrombus formation and embolization [5]. Despite the fact that paroxysmal atrial fibrillation is frequently asymptomatic, the risk of stroke is the same as in permanent AF [6]. The diagnosis requires extended ECG monitoring (e.g., Holter ECG, outpatient monitoring [7, 8]) of the patient. If a cardiogenic mechanism is identified behind a stroke event, anticoagulation is warranted instead of antiplatelet therapy

[4]. Poststroke patients, however, are not optimal candidates for long-lasting ECG monitoring (insufficient compliance, frequent falls, aphasia, limb paresis, etc.). There is an increasing need for blood biomarkers that are capable of identifying patients at a significant risk of PAF. Currently, the optimal marker is still unknown. An ideal biological marker has the following characteristics: high sensitivity, high specificity, high predictivity, and rapid, simple, accurate, inexpensive, and reproducible detection in all relevant patients. In this review article, we aimed to summarize the most important recent observations.

## 2. Methods

PubMed, EMBASE, BioMed Research International, and Google Scholar were searched for published studies. We examined studies that have presented data on the association between atrial fibrillation and biological markers. We used the keywords “atrial fibrillation”, “biological marker”, and “stroke” and the names of the markers. Based on their structure and function, four groups of biomarkers can be

TABLE 1: Markers of inflammation.

Biomarker	Trials	Patients	Results	Potential DX efficiency in cryptogenic stroke
Pentraxin-3	1 prospective trial	382	Weak predictor of the recurrence of AF	+–
NLC	2 retrospective trials	126/32912	Useful in predicting stroke in patients with known AF	+

+–: questionable; +: potentially useful.

differentiated: markers of inflammation, markers of fibrosis, markers with hormonal activity, and other markers.

### 3. Markers of Inflammation

In the last few years, a number of trials have concluded that AF is triggered by inflammation, resulting in electrical and structural remodeling [9, 10]. Active inflammation can provoke AF, which in return causes an inflammatory response that further enhances atrial remodeling, resulting in arrhythmia, the so-called “AF begets AF” phenomenon. The progress is similar to a spiral: inflammation begets AF, and AF begets inflammation [11].

A new promising inflammatory marker is pentraxin-3 (PTX3), a member of the long pentraxin family. The C-terminal sequence of PTX3 is homologous with serum amyloid P component and the classic short pentraxin CRP. PTX3 is produced in large amounts by different cells (e.g., monocytes, macrophages, and endothelial cells) in local inflammatory lesions, whereas CRP is mostly produced by the liver [12].

A subgroup of 382 patients, who were selected from 36 centers with AF in their history but in sinus rhythm at randomization, was enrolled in the GISSI-AF biohumoral study that investigated the association between the plasma concentrations of pentraxin-3 (PTX3), high-sensitivity C-reactive protein (hsCRP), and interleukin-6 (IL-6), the echocardiographic findings, and the time of the first return of AF [13]. Recurrent AF developed in 204 patients during the one-year-long follow-up. Baseline plasma concentrations of IL-6, hsCRP, and PTX3 were measured and no significant differences between patients with or without recurrent AF were found. At 6 and 12 months of follow-up, IL-6 and PTX3 concentrations were significantly higher in patients with AF compared to those who were in sinus rhythm, and the hsCRP levels were higher in patients with the most recent episodes of AF. Baseline levels of PTX3, IL-6, and hsCRP were not significantly associated with a higher risk of AF recurrence. These markers can be elevated in AF patients, but they were found to be weak predictors of arrhythmia recurrence in this study.

The neutrophil-to-lymphocyte ratio (NLR) is a major marker of subclinical inflammation, and it is a widely investigated marker recently used in the prediction of cerebrovascular diseases [14, 15].

Ertas et al. carried out a retrospective study on 126 consecutive nonvalvular AF patients with or without thromboembolic stroke [16]. A group of 24 patients in sinus rhythm served as a reference point for the comparison with the

AF group. Based on NLR values at admission, the study population was divided into tertiles. A low NLR level ( $n = 84$ ) was defined as a value in the lower two tertiles and a high NLR level ( $n = 42$ ) was defined as a value in the third tertile. Stroke rates and CHADS2 scores were found to be significantly higher in the high NLR group compared to the low NLR group. Stroke patients had significantly higher mean white blood cell (WBC) counts and NLR values. Therefore, NLR can be used in patients with nonvalvular AF as an independent predictor of thromboembolic stroke.

A cohort study conducted by Saliba et al. aimed to find the association between NLR, atrial fibrillation, and stroke [17]. 32,912 adult patients with AF, with no anticoagulation therapy at the baseline, and without previous stroke or TIA were analyzed retrospectively in a computerized database. The patients were followed up for the first occurrence of stroke or TIA from 1 January 2012 until 31 December 2012. 981 subjects developed stroke during a follow-up of 30,961 person-years (stroke rate: 3.17 per 100 person-years). The patients were grouped into quartiles based on NLR levels. The incidence rate of stroke increased in a dose-response manner across NLR quartiles, and therefore the study showed that there is a significant association between NLR level and the first episode of stroke in patients with atrial fibrillation.

Based on these studies, NLR is a promising marker in predicting paroxysmal AF, while the usefulness of PTX3 is questionable (Table 1).

### 4. Markers of Fibrosis

Galectin-3 (Gal-3) is a  $\beta$ -galactoside-binding lectin that seems to play a major role in the regulation of fibrosis and inflammation [18].

Ho et al. investigated the relation between plasma galectin-3 concentrations and the incidence of AF. Plasma levels of the peptide were measured in 3,306 members of the Framingham Offspring cohort (who participated in the sixth examination cycle between 1995 and 1998) [19]. They used Cox proportional hazard regression models to evaluate the association between baseline Gal-3 concentrations and the incidence of AF. They found that elevated Gal-3 plasma levels were associated with a higher risk of developing AF, but after adjusting for clinical risk factors to predict AF risk, the association was no longer significant [20].

An observational study managed by Gurses et al. also aimed to find the correlation between plasma galectin-3 levels and atrial fibrillation [21]. Seventy-six patients with paroxysmal or persistent AF and preserved left ventricular systolic function and 75 age- and gender-matched control

patients were enrolled in the study. All of them underwent transthoracic echocardiographic examination (TTE) to measure left atrium (LA) size and left ventricular (LV) function to exclude any structural disease of the heart. LA volume index (LAVI) was calculated on the basis of the patient's body surface area. Galectin-3 level was measured and it was significantly elevated in patients with AF in comparison with the control group. Serum galectin-3 levels were also significantly higher in patients with persistent AF than in those with paroxysmal AF. Multivariate regression analysis was performed demonstrating that serum galectin-3 levels and LAVI were independent predictors of AF. Gurses et al. found an independent correlation between LAVI and serum galectin-3 levels in patients with AF through linear regression analysis.

The observational study of Yalcin et al. was very similar, but this investigation was conducted using delayed enhancement magnetic resonance imaging (DE-MRI) to estimate the degree of atrial fibrosis and atrial electromechanical delay (AEMD), a noninvasive echocardiographic method to measure inter- and intra-atrial conduction delays [22]. Thirty-three patients with paroxysmal AF and unimpaired LV function were enrolled. The study demonstrated that serum galectin-3 levels had an independent correlation with the extension of LA fibrosis demonstrated by DE-MRI in paroxysmal AF patients. Serum galectin-3 levels also had a correlation with intra-left- and inter-AEMD, which is really important because they are accepted as noninvasive echocardiographic markers of atrial electrical remodeling.

TGF- $\beta$ 1 is expressed in endothelial cells, vascular smooth muscle cells, and myofibroblasts [23]. In the heart, TGF- $\beta$ 1 seems to be a factor that causes different diseases by inducing cardiac fibrosis, based on studies examining overexpression and knockout models [24]. In a mice study, the animals with increased expression of TGF- $\beta$ 1 were prone to develop atrial fibrillation as a result of a higher degree of atrial fibrosis [25].

TGF- $\beta$ 1 levels seem to be increased in humans with atrial fibrillation as well. In a study, Lin et al. examined the relation between TGF- $\beta$ 1 and atrial fibrillation in patients with essential hypertension (EH) [26]. 75 patients with AF secondary to EH were selected in the study and then divided into 2 subgroups: the paroxysmal AF group (pAF) consisting of 44 patients and the chronic AF group (cAF) consisting of 31 patients. 37 EH patients with sinus rhythm (SR) were selected in the EH + SR control group and 36 healthy subjects were assorted as normal controls (NC group). Clinical characteristics of the patients were collected and TTE examinations were also performed. Blood samples were taken in the morning from fasting and resting subjects for the assessment of TGF- $\beta$ 1 and CTGF (connective tissue growth factor) levels. TGF- $\beta$ 1 and CTGF serum levels were significantly higher in the EH groups than in the NC group. TGF- $\beta$ 1 and CTGF levels were the highest in the cAF group, followed by the pAF and SR groups. Lin et al. did not find significant differences in TGF- $\beta$ 1 and CTGF levels between the pAF group and the cAF group. In AF patients, there was an independent correlation between serum levels of TGF- $\beta$ 1 and left atrial diameter (LAD), the presence of AF, aldosterone, CTGF, and age. As a possible conclusion, serum TGF- $\beta$ 1 was found to indicate

the synthesis of CTGF causing enlargement and remodeling of the left atrium, which can lead to AF in EH patients.

Matrix metalloproteinase-9 (a member of the matrix metalloproteinase family) is an endopeptidase, synthesized and secreted in monomeric form as zymogen. It can degrade components of the extracellular matrix and it also takes part in various physiological and pathological processes including development, growth, and reproduction and, additionally, vascular, proliferative, and inflammatory diseases [27].

In their study, not only did Li et al. measure the plasma level of MMP-9, but also they investigated its significance in different stages of idiopathic AF [28]. The patients were categorized into 3 groups: paroxysmal AF, persistent AF, and permanent AF groups, each containing 25 patients. The control group consisted of 40 healthy individuals. Venous blood samples were taken. MMP-9 plasma levels in the AF groups showed significant elevation compared to the control group. From paroxysmal AF through persistent AF to permanent AF, the plasma levels of MMP-9 showed a significant gradual increase.

The growth/differentiation factor 15 (GDF-15) protein is a member of the transforming growth factor beta superfamily. It has several physiological functions including the regulation of proliferation and apoptosis in normal, injured, and transformed cells, but it also has pathological functions such as growth inhibition and overexpression in cancer cells [29]. Shao et al. aimed to find the correlation between the serum levels of GDF-15, NRG-1, and nonvalvular AF. Their study included 67 patients with nonvalvular AF and 67 healthy persons matched for age, sex, and atherosclerotic risk factors [30]. They collected baseline demographic and clinical characteristics and performed TTE. They measured the plasma levels of GDF-15, NRG-1 (a member of the epidermal growth factor (EGF) gene family playing a role in growth, cell survival, cardiovascular development, and metabolism [31]), and other basic laboratory parameters. Patients from the AF group had higher GDF-15 and NRG-1 levels and LAD values than non-AF patients. Patients with paroxysmal AF had a significantly higher serum level of GDF-15 compared to the control group. Likewise, NRG-1 levels were also higher in paroxysmal AF patients. According to multivariable analyses, GDF-15 was independently associated with paroxysmal AF.

Sonmez et al. examined a study population consisting of 52 patients diagnosed with nonvalvular AF and 33 age-matched subjects without AF in their history [32]. Their goal was to compare the serum levels of novel biomarkers between a group of AF patients and a group of healthy individuals. These markers were galectin-3, MMP-9, lipocalin-2 (NGAL, a novel adipokine associated with insulin resistance [33]), PIIINP (the amino-terminal peptide of type III procollagen, released into the blood during both synthesis and degradation of collagen type III [34]), hsCRP, and NLR. Correlation analyses found a significant correlation between NLR and LAVI (left atrial volume index), but not between hsCRP and LAVI. There was a strong correlation between galectin-3, MMP-9, PIIINP, and LAVI. MMP-9, galectin-3, and PIIINP levels were significantly higher in AF patients, but NGAL

TABLE 2: Markers of fibrosis.

Biomarker	Trials	Patients	Results	Potential DX efficiency in cryptogenic stroke
Galectin-3	1 prospective trial	3306	↑ Gal-3: ↑ risk of developing AF after adjusting for clinical risk factors: ⊙ significant	
	1 prospective trial	76	Galectin-3 ↑ in AF compared with the control galectin-3 ↑ in persistent AF compared with paroxysmal AF	+
	1 prospective trial	33	Galectin-3 level is an independent correlate of the extent of LA fibrosis in paroxysmal AF patients	
TGF-β1	1 prospective trial	75	TGF-β1 ↑ in cAF and pAF group compared with SR group; ⊙ difference in TGF-β1 levels between the cAF group and the pAF group	+–
MMP-9	1 prospective trial	75	MMP-9 levels ↑ gradually from paroxysmal AF through persistent AF, permanent AF	+
GDF-15	1 prospective trial	67	GDF-15 ↑ in paroxysmal AF independently associated with paroxysmal AF	+
Multimarker	1 prospective trial	52	Galectin-3, MMP-9, and PIIINP ↑ in AF	
	1 prospective trial	2935	PIIINP showed a nonlinear association with incident AF; ⊙ association between circulating TGF-β1 levels and incident AF	+

+–: questionable; +: potentially useful; ↑ increased; ⊙ no.

levels were not. NLR and hsCRP levels were also elevated in AF patients.

As a part of the Cardiovascular Health Study, Rosenberg et al. assessed the plasma levels of 2 fibrosis biomarkers, PIIINP and TGF-β1 [35]. PIIINP levels were measured in 2,935 participants, of whom 767 developed AF. PIIINP levels showed a nonlinear relationship with the risk of incident AF both before and after risk adjustment. A linear relationship was observed between the risk of AF and PIIINP levels approximately up to the median value, but unfortunately no significant association was identified beyond that point. TGF-β1 levels were assessed in 1,538 individuals with 408 cases of incident AF, but this marker's levels were not associated with AF risk. No association was found between TGF-β1 levels and the incidence of AF in unadjusted or adjusted models.

To summarize, galectin-3, MMP-9, GDF-15, and PIIINP are promising markers in the prediction of paroxysmal AF, while TGF-β1 shows a lower potential (Table 2).

## 5. Markers with Hormonal Activity

Natriuretic peptides (NPs) are produced in the heart and released into the circulation in response to pressure and

volume overload. NP levels provide information about the systolic and diastolic function as well as the right ventricular and valvular function [36]. In recent years, different NPs, such as brain natriuretic peptide (BNP), its N-terminal prohormone (NT-proBNP), and atrial natriuretic peptide (ANP), emerged as possible biological markers of atrial fibrillation. The connection between BNP and/or NT-proBNP has been investigated by numerous studies. Silvet et al. measured BNP levels in 72 outpatients with chronic atrial fibrillation (AF) and in 49 control subjects without AF. BNP levels were significantly higher in patients with AF [37]. Another study was performed at the Kagawa University School of Medicine Hospital that aimed to measure BNP levels in patients with acute ischemic stroke [38]. This cohort included 99 patients with acute cerebral infarction. 23 patients were excluded due to having myocardial infarction, heart failure, valve disease, or chronic renal failure. 36 of the remaining patients developed cardioembolic stroke with atrial fibrillation (23 with permanent and 13 with paroxysmal atrial fibrillation) and 40 had noncardioembolic stroke. BNP was evaluated on the first morning after admission, and TTE was also performed. In the cardioembolic stroke/atrial fibrillation group, plasma BNP levels, LAD, and the ratio of peak early

filling velocity to peak atrial systolic velocity (E/A) were significantly increased, while left atrial appendage flow was significantly reduced compared to noncardioembolic stroke patients. First-day BNP and LAA flow were useful in differentiating cardioembolic stroke with AF from noncardioembolic stroke.

In the Cardiovascular Health Study (CHS), Patton et al. found a connection between AF and NT-proBNP [39]. NT-proBNP levels were measured in 5,447 patients (2 of them were excluded due to missing baseline ECG results). NT-proBNP levels showed a strong association with prevalent AF. After a median follow-up of 10 years (maximum of 16 years), 1,126 cases of incident AF were registered (a rate of 2.2 per 100 person-years). NT-proBNP levels proved to be greatly predictive of incident AF.

Within the settings of the Multi-Ethnic Study of Atherosclerosis (MESA), 5,518 patients were enrolled to investigate a possible association between serum NT-proBNP levels and AF [40]. NT-proBNP levels were measured from frozen serum samples drawn at enrollment. The associations between NT-proBNP and gender, age, and ethnicity/race were also investigated. Patton et al. followed up the patients for a median of 7.6 years. During this time, 267 of them developed AF. The average NT-proBNP level was higher in subjects with AF. NT-proBNP was statistically significantly associated with incident AF.

Kara et al. investigated the association of BNP with incident AF in a large population-based cohort study [41]. The patients did not have a history of prior stroke, coronary heart disease, heart-device therapy, open heart surgery, or prevalent AF at baseline. 3,067 subjects were involved in the logistic regression analysis. They examined the association of BNP as a continuous and binary variable (with predefined gender-specific BNP thresholds) with new onset of AF as well as its value for risk prediction beyond traditional AF risk factors. Higher BNP levels showed association with excessive incidence of AF (e.g., resulting in a fourfold risk in a five-year period for subjects with a BNP level over 31 pg/mL for men and 45 pg/mL for women in the crude model). After adjustment for traditional AF risk factors and CAC (coronary artery calcium), the association stayed statistically significant. The associations were more explicit in younger patients. To summarize these results, elevated levels of BNP were found to be associated with significant excess of incident AF independently of traditional risk factors of AF in the general population. In the future, the gender-specific BNP thresholds may help in the detection of subjects with unknown or future AF that can lead to stroke events.

Rodríguez-Yáñez et al. performed an interesting prospective study enrolling patients with cryptogenic stroke from February 2008 to July 2011 according to the TOAST criteria [42]. Of the 1,050 evaluated patients, 264 were qualified for the study. Blood samples were taken within the first 24 hours from the onset of the stroke for the measurement of proBNP levels. Then, the patients were followed up at 3 and 6 months by a neurologist and later by a primary care physician for 2 years in order to register the development of AF. Fifteen patients (5.6%) developed AF during the follow-up period. Patients who developed AF were older and more frequently

had a history of hypertension. Forty-eight patients (18.2%) died during the follow-up period. Higher NT-proBNP levels were detected in patients who developed AF, compared with those who did not. Based on these findings, high NT-proBNP levels measured during the acute phase of stroke in cryptogenic stroke patients are associated with a fivefold increase in the risk of developing AF in the following 2 years.

The TARGET AF was a prospective cohort study of stroke survivors performed in the stroke center of Nice University Hospital, which aimed to identify a relevant marker of delayed AF in the selected patients in whom AF was detected by early and prolonged monitoring [43]. 373 patients were included in the study and 53 of them were excluded due to diagnosis of AF at baseline. 20 patients with sinus rhythm at baseline but with AF in their history were also excluded. Plasma BNP was measured in blood samples taken at admission. Holter ECG monitoring was started immediately at admission and was stopped at discharge in all patients. Newly diagnosed AF was documented in 52 patients (AF prevalence of 17.33%) suggesting the association of early and prolonged monitoring with an increased AF detection rate. Plasma BNP values were significantly higher in patients with AF. The use of all examined parameters together did not provide significant additional diagnostic value over BNP (diagnostic properties of BNP level: sensitivity: 98.08%; specificity: 71.37%; negative predictive value: 99.4%). The most important result of the study is that BNP level has a really strong negative predictive value in stroke patients that can be related to delayed AF.

Fibroblast growth factor-23 (FGF-23) is a bone-derived hormone that plays an important role in the homeostasis of phosphate. FGF-23 reduces gastrointestinal phosphate absorption, inhibits the production of 1,25-dihydroxyvitamin D, and promotes urinary phosphate excretion [44, 45].

Mathew et al. examined participants from the Multi-Ethnic Study of Atherosclerosis (MESA) and the Cardiovascular Health Study (CHS) to investigate the relation between FGF-23 and AF. 6,398 patients were from the MESA study, and 1,350 participants were from the CHS [46]. Incident AF was identified using inpatient and outpatient physician claims data, systematic reviews of hospital discharge diagnoses, and study ECGs over 7.7 and 8.0 years of median follow-up. Cox proportional hazard models were used to test associations between FGF-23 and the risk of developing AF. A series of multivariable models were also constructed. In MESA participants and CHS participants, 291 and 229 incident AF events were observed, respectively. In both MESA and CHS participants, higher FGF-23 concentrations were associated with higher unadjusted incidence rates of AF. Later adjustments for demographics and potential confounding characteristics were carried out. In these analyses, each twofold higher FGF-23 concentration was associated with a 41% higher risk of AF in MESA patients and a 29% higher risk of AF in CHS patients, proving that higher circulating FGF-23 concentrations increase the risk of incident AF. Other biomarkers of mineral metabolism, eGFR, urine ACR, and heart failure events were also accounted for, and the associations remained significant. Adjusting for FGF-23 attenuated the association of low eGFR with incident AF in MESA patients, suggesting

TABLE 3: Markers with hormonal activity.

Biomarker	Trials	Patients	Results	Potential DX efficiency in cryptogenic stroke
NT-pro BNP/BNP	1 prospective trial	72	↑ BNP levels in patients with AF compared to those without AF	++
	1 prospective trial	76	First-day BNP and LAA flow are helpful in differentiating cardioembolic stroke with AF from noncardioembolic stroke	
	1 prospective trial	5445	NT-proBNP was an important predictor of incident AF, also after adjustment for covariates	
	1 prospective trial	5518	NT-proBNP was significantly associated with incident AF and is a strong predictor of it	
	1 prospective trial	3067	↑ BNP levels were associated with significant excess of incident AF and independent of traditional AF risk factors	
	1 prospective trial	264	↑ ProBNP levels determined during the acute phase of stroke ↑ 5-fold the risk of developing AF in cryptogenic stroke patients in the following 2 years	
FGF-23	1 prospective trial	300	BNP level has a really strong negative predictive value in patients with stroke that can be related to AF	+
	1 prospective trial	7748	FGF-23 concentrations were associated with higher unadjusted incidence rates of AF ↑ FGF-23 concentration ↑ the risk of incident AF	

+: potentially useful; ++: very promising; ↑: increased.

that FGF-23 may mediate, in part, the known association of CKD with AF.

NT-proBNP (and BNP) was found to be elevated in AF by several studies. Studies that evaluated the connection of the natriuretic peptide with AF in cryptogenic stroke patients were also performed, determining well-defined cut-off values that are able to predict paroxysmal events in cryptogenic patients. FGF-23 is also a promising marker, but currently we do not have enough information about it (Table 3).

## 6. Markers with Other Functions

Circulating procoagulant microparticles (MPs) are small membrane vesicles that are derived from different cells (e.g., platelets, endothelial cells, leukocytes, lymphocytes, and erythrocytes) in response to activation, injury, and/or apoptosis [47]. Platelet microparticles (PMPs) are procoagulant membranous vesicles produced by activated platelets. PMP levels are elevated in stroke, coronary artery disease (CAD), hypertension, and diabetes.

Choudhury et al. aimed to find the correlation between serum platelet microparticle levels and nonvalvular atrial fibrillation [48]. The study had 3 hypotheses: (1) PMP levels

are higher in patients with AF compared to levels in both disease control subjects (i.e., patients with diabetes or stroke, CAD, or hypertension who are in sinus rhythm) and healthy control subjects (i.e., patients without cardiovascular diseases who are in sinus rhythm); (2) PMP levels correlate with levels of soluble P-selectin (sP-selectin) which is a platelet activation marker; (3) in patients with AF, PMP levels are related to the underlying factors that contribute to the comprehensive risk of stroke secondary to AF. The study team performed a case-control study of 70 AF patients, 46 disease controls, and 33 healthy control patients. The levels of PMPs and sP-selectin were significantly higher in both AF patients and disease control subjects compared to healthy control subjects, but no difference was found between AF patients and disease control subjects. There was not any difference in PMP levels between patients with paroxysmal and permanent AF and between those who received antiplatelet or anticoagulant therapy (aspirin and warfarin, resp.). A significant correlation was not observed between PMP and sP-selectin levels and the clinical characteristics that contribute to the elevated risk of stroke in patients with AF. Through multiple regression analysis in the combined cohort of AF patients and the disease control subjects, the presence/absence of AF did not

prove to be an independent determinant of PMP and sP-selectin levels.

Ederhy et al. also suggested that procoagulant MP levels in the circulation would be increased in AF, so they elaborated a hospital-based case-control study design, involving 45 patients with AF and 90 control subjects: 45 with cardiovascular risk factors and 45 without [49]. The levels of 3 different MPs were screened: platelet-derived MPs, Annexin V-positive MPs, and endothelial-derived MPs. Annexin V-positive MP levels were elevated in patients with AF compared with control subjects with and without cardiovascular risk factors. The levels of platelet-derived MPs and endothelial-derived MPs were similar in patients with AF and control subjects with cardiovascular risk factors but were significantly higher than in control subjects without cardiovascular risk factors. Finally, the presence of AF strongly predicted Annexin V-positive MP levels. Based on these data, circulating procoagulant MPs can be increased in persistent and/or permanent AF and might indicate a hypercoagulable state that could lead to atrial thrombosis and thus thromboembolism.

Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide synthase (NOS) capable of causing NO deficiency and an increased risk of thrombosis [50]. Cengel et al. investigated patients in three groups: 17 patients whose AF was detected for the first time within the first 24 hours of presentation (group I), 25 patients with permanent chronic AF lasting at least 1 year or more (group II), and 18 healthy people as the control group (group III) [51]. Plasma ADMA, SDMA, and L-arginine concentrations were compared. In patients with acute onset of AF, ADMA levels were significantly higher when compared to patients with chronic AF and the healthy control group. ADMA levels were higher in all patients with AF than in the control group of healthy people. This information indicates that endothelial dysfunction and a prothrombotic state develop in a very early phase of AF.

MicroRNAs (miRNAs) are a class of 19–25-nucleotide noncoding RNAs with a broad spectrum of functions including the regulation of cellular differentiation, proliferation, development, and death [52]. Different miRNAs with different functions are known, and they are widely investigated, but their value as blood biomarkers is still not clear.

In a study conducted by Liu et al., 105 patients were enrolled for miRNA investigation [53]. 15 participants were selected for in-depth sequencing of plasma miRNAs: 5 people with paroxysmal AF, 5 people with persistent AF, and 5 healthy individuals. The other 90 participants were randomly classified as test subjects using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Blood samples were taken from all enrolled patients to carry out the in-depth analysis. Massively parallel signature sequencing (MPSS) was also performed. 22 specific miRNAs showed dysregulation in each group. Four candidate microRNAs (miRNA-375, miRNA-146a, miRNA-19a, and miRNA-150) were suitable for further investigation in an independent cohort of 90 plasma samples using TaqMan miRNA quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). The expression levels of these miRNAs were

significantly downregulated in patients with AF, but only miRNA-150 demonstrated significant characteristic changes. Its expression levels were reduced by a factor of approximately 17 times in PAF patients relative to controls and a factor of approximately 20 times in PersAF relative to controls. Based on the median expression results of miRNA-150, no significant differences were found between the levels of miRNA-150 among the healthy controls, PAF patients, and PersAF patients. Moreover, no correlation was found between miRNA-150 levels and the presence or absence of antiarrhythmic drug therapy in AF patients. miRNA-150 levels were independently associated with age and LAD. Parallel plasma CRP measurements showed that the levels of CRP were negatively correlated with the plasma levels of miRNA-150.

McManus et al. analyzed the association between circulating miRNAs and AF [54]. 2,445 individuals from the Framingham Heart Study (FHS) were enrolled. The expression of 385 miRNAs isolated from whole blood was measured using TaqMan chemistry-based assays. After the measurement and statistical analysis, the expression of several miRNAs (miR-150-5p, miR-328, miR-331-3p, and miR-28-5p) was found to be negatively associated with prevalent AF. After adjustment for age, sex, isolation batch, RNA quality, concentration, and 260/280 ratio, the association with AF remained significant only with miR-328, which was found in a relatively high number in patients. Unfortunately, after further adjustments for clinical AF risk factors linked to atrial size and/or pathological atrial remodeling, including weight, height, systolic and diastolic blood pressure, antihypertensive medication (including beta-blocker) use, current smoking, prevalent heart failure, myocardial infarction, and diabetes mellitus, this association was attenuated. The association between higher miR-328 and AF is really interesting, because this miRNA promotes atrial electrical remodeling, thus AF by reducing L-type Ca<sup>2+</sup> channel density [55].

The potential in using PMPs and ADMA as predictors of AF is promising. MicroRNAs are quite intriguing, because they have a lot of different types, thus a lot of potential, but more investigations are needed in the future (Table 4).

## 7. Conclusion

The investigated markers have different functions; some of them are connected to inflammation (NLR, pentraxin-3, CRP, and IL-6), while others contribute to the fibrosis of the atrium (MMP-9/TIMP, TGF- $\beta$ 1, PIIINP, galectin, and CTGF). Inflammation and fibrosis go hand in hand, so the separation of these markers can be difficult sometimes. Some markers have a characteristic hormonal effect (NT-proBNP, FGF-23), and we can find markers that play a role in protein catabolism (ADMA) or posttranscriptional changes (microRNA), but there are some markers with complex functions and structure as well (circulating procoagulant microparticles). Most of the analyzed markers have promising data, but at present none of them fulfills the criteria of an optimal biomarker. NT-proBNP/BNP are the most promising candidates.

TABLE 4: Markers with other functions.

Biomarker	Trials	Patients	Results	Potential DX efficiency in cryptogenic stroke
Circulating procoagulant microparticles	1 prospective trial	70	↑ PMPs in both AF patients and disease control subjects compared to healthy control subjects; ∅ difference between AF patients and disease control subjects; ∅ difference in PMP levels between patients with paroxysmal and permanent AF and between those receiving anticoagulant therapy	+
	1 prospective trial	45	Circulating procoagulant MPs can be ↑ in persistent and/or permanent AF and might reflect a hypercoagulable state that could lead to atrial thrombosis and thromboembolism	
ADMA	1 prospective trial	42	ADMA levels in patients with acute AF ↑ compared to patients with chronic AF and healthy controls	+
MicroRNA	1 prospective trial	10	The expression levels of these 4 miRNAs ↓ in patients with AF; the miRNA-150 levels ↓ by a factor of approximately 17 times in paroxysmal AF patients relative to controls and a factor of approximately 20 times in persistent AF relative to controls	+
	1 prospective trial	2445	Circulating levels of miR-328 that were associated with prevalent AF adjustment for risk factors that promote atrial remodeling attenuated the association	

+: potentially useful; ↑: increased; ↓: decreased; ∅: no.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

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## Review Article

# The Management of Cardiovascular Risk through Epigenetic Biomarkers

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Epigenetic sciences study heritable changes in gene expression not related to changes in the genomic DNA sequence. The most important epigenetic mechanisms are DNA methylation, posttranslational histone modification, and gene regulation by noncoding RNAs, such as microRNAs (miRNAs) and long noncoding RNAs (lncRNAs). Cardiovascular diseases (CVD) are responsible for at least one-third of premature deaths worldwide and represent a heavy burden of healthcare expenditure. We will discuss in this review the most recent findings dealing with epigenetic alterations linked to cardiovascular pathophysiology in patients. A particular focus will be put on the way these changes can be translated in the clinic, to develop innovative and groundbreaking biomarkers in CVD field.

## 1. Introduction

Epigenetics is the study of all the heritable changes in gene expression that do not involve changes to genomic DNA sequences in themselves. Epigenetic mechanisms represent a stable cellular memory that allows the propagation of gene activities from one generation of cells to another. The three main epigenetic processes are represented by DNA methylation, posttranslational histone modifications, and RNA-based mechanisms such as noncoding RNAs, represented mainly by microRNAs (miRNAs), and long noncoding RNAs (lncRNAs) [1, 2].

Cardiovascular diseases (CVD) are responsible for one-third of all deaths worldwide and accounting for an important burden of healthcare expenditure. Previously, epigenetic modifications were reported to play a pivotal role in processes underlying CVD, including atherosclerosis, inflammation, and hypertension [3]. To date, most of the limitations for the full understanding of the genetic influence on cardiovascular diseases (CVD) are probably due to the static simple evaluation of the DNA code. In this context, epigenetics,

through the study of several dynamic pathways, modify also the genome's functionality under exogenous influence, which could identify novel mechanisms and targets in the control of gene regulation, with significant acquisitions in CVD knowledge of its genetic risk and pathophysiology [1–3]. Indeed, epigenetic modifications such as histones modifications, DNA methylation, and small noncoding RNAs occur in response to environmental changes. Pollution and diet will profoundly change these epigenetic modifications and trigger susceptibility to CVD. For example, as will also be discussed in this review, DNA methylation has been associated with atherosclerosis [4], abdominal aortic aneurysm [5], and coronary heart disease [6].

This review will thus focus on the role of the main mechanisms of epigenetics in the area of cardiovascular risk.

## 2. Literature Search

In the context of CVD, we decided to search for relevant articles in the three main areas of interest of epigenetics: DNA methylation, posttranslational histone modifications,

and RNA-based mechanisms. PubMed, Scopus, and Science Direct databases were used for the search strategy. A PubMed search using the terms cardiovascular + epigenetic + biomarker found 261 results in May 2017.

### 3. DNA Methylation

DNA methylation, partly controlled by DNA methyltransferases, is usually associated with transcriptional repression, while demethylation is associated with transcriptional activation, influencing gene expression by altering DNA promoter accessibility to RNA polymerase, and consequently gene transcription [7]. A PubMed search using the terms cardiovascular + methylation + biomarker + biomarker found 185 results in May 2017.

In this context, the concept of global methylation refers to the overall level of methylcytosines in the genome, expressed as percentage of total cytosines. In fact, a large proportion of methylation sites within the genome are found in repeat sequences and transposable elements, such as Alu and long-interspersed nuclear element (LINE-1) and this correlates with the total genomic methylation content, and, for this reason, LINE-1 is used as a surrogate for the overall methylation of the genome [3]. Interestingly, LINE-1 methylation is increased in males [21], while it is not affected by either age or natural cycle of hormones.

Several research teams have studied the possibility that DNA methylation could be correlated with the risk of CVD. Several evidences showed that global DNA methylation assessed at LINE-1 sequences was inversely and independently related to CVD risk; thus more proportions of global DNA methylation are indicative of a higher CVD risk [3, 22, 23]. Very recently, a Swedish team performed an epigenome-wide association study to identify disease-specific alterations in DNA methylation in blood samples of a Swedish population of 729 patients, afflicted with hypertension, myocardial infarction, stroke, thrombosis, and cardiac arrhythmia [24].

For this, they used an Illumina Infinium BeadChip. Differential DNA methylation was detected in more than 200 CpG-sites in patients with a history of myocardial infarction. Among these sites, 42 genes were related to cardiac function. The authors concluded that individuals with a history of MI have an altered pattern of DNA methylation at numerous genomic loci linked to CVD. These sites are thus potential biomarkers for CVD [24]. DNA methylation in specific genes can also be helpful to predict response to a specific treatment. Two elegant studies by Gallego-Fabrega et al. have shown that changes in DNA methylation patterns of PPM1A and TRAF3 are, respectively, associated with vascular recurrence in aspirin-treated patients and clopidogrel response and recurrence of ischemic events in patients with stroke [25, 26].

### 4. Posttranslational Histone Modifications

Eukaryotic DNA is wrapped around an octamer of the core histones that build the fundamental unit of chromatin, the nucleosome. These chromatin elements are unstable and they change rapidly in response to any external stimuli, and any permanent changes to DNA can lead to the

development of defective organs or the development of a disease [1]. Very sparse studies tend to show that histone modifications are linked with CVD. In normal mice fed with propylthiouracil (PTU, an inhibitor of T3 production), Pandya et al. have shown that the distribution of histone 3 lysine 4 trimethylation at a myosin-heavy chain related locus was reversibly altered in ventricles, suggesting that histone 3 lysine 4 trimethylation modification is an epigenetic marker associated with changes in myosin-heavy chain gene regulation [27]. Histone acetylation is a dynamic process regulated by histone acetyltransferases and histone deacetylases (HDACs). The balance between these two enzyme families is crucial to regulating gene expression and could be incriminated in CVD development [28]. In rats, inhibiting a specific type of HDAC in the heart promotes cardiac stem cell-promoted cardiac regeneration which in turn induces a partial restoration of cardiac function [29]. These animal studies give hope that histone modification is a promising way to develop innovative biomarkers and therapies in the CVD field.

To date, there are however very few significant reports in human related to posttranslational histone modifications in the area of CVD risk to be discussed. Ek et al., using a genomewide DNA methylation study, have shown that growth-differentiation factor 15 is associated with myocardial infarction and that, interestingly, growth-differentiation factor 15 mRNA was regulated by a specific small RNA, miR-21, leading us to the next chapter in our review [30].

### 5. RNA-Based Mechanisms

*5.1. Noncoding RNAs in the Management of Cardiovascular Risk.* Noncoding RNAs including microRNAs (miRNAs) and the recently discovered long noncoding RNAs (lncRNAs) are defined as a novel class of endogenous RNAs that regulate the human genome and are not translated into proteins. They have been shown to be implicated in cardiovascular physiopathology. A large number of long noncoding RNAs and microRNAs were in recent years implicated in cellular and animal models of cardiovascular diseases (CVD) and were shown to be deregulated in patients with CVD. We will discuss here their potential role as biomarkers in the CVD context. Also, as the miRNAs and lncRNAs we discuss may represent novel targets to treat or prevent CVD, we will see how they could be used for the delivery of new treatments in CVD using groundbreaking techniques.

*5.2. miRNAs as Biomarkers in CVD.* It has been now 17 years since the existence of microRNAs (miRNAs) was shown in human. These short endogenous interfering RNAs are coded by the human genome, represent a new class of thousands of RNAs of approximately 20 to 25 nucleotides (see specialized Internet databases, such as miRbase [http://www.mirbase.org/cgi-bin/mirna\\_summary.pl?org=hsa](http://www.mirbase.org/cgi-bin/mirna_summary.pl?org=hsa)), and have emerged as regulators of numerous physiological and pathological processes [31], including CVDs [32, 33]. Their number is now estimated to be around 2,000 but a recent study using thorough RNA purification from human cells and next generation

sequencing claims that there exist up to 5,000 miRNAs, with more than a half being human specific [34].

**5.3. miRNA Biogenesis.** In the canonical pathway, a large RNA precursor, pri-miRNA, is transcribed and matured in the nucleus by the RNA polymerase II. It is subsequently cleaved near the hairpin stem base, by RNase III Drosha and protein partner, Di George syndrome critical region 8. Thus the pre-miRNA precursor, a hairpin of approx. 60 to 70 nucleotides, is obtained which is then released in the cytoplasm. It will be cleaved in its stems by the RNase III Dicer. This results in the double stranded miRNA/miRNA\* duplex (21–25 base-pairs) which is unwound by RNA-induced-silencing complex (RISC). RISC will then carry the mature miRNA to target messenger RNAs resulting in gene silencing. Most of the time the binding will take place in the 3' untranslated region and will decrease target mRNA levels and translation [35]. Noncanonical pathways such as the mirtron pathway (in this instance miRNAs arise from introns) have also been described [36].

**5.4. MicroRNAs as Predictors of CVD Outcome.** As is abundantly explained in this special issue, CVD are one of most common causes of premature death in the general population, with an increasing number of individuals at risk. New biomarkers are needed to better stratify the risk of progression or the risk of specific complications associated with CVD, and noncoding RNAs are prime candidates. A PubMed search using the terms cardiovascular + mirna + biomarker brings 969 results in May 2017, further highlighting the interest in the field.

It was reasonable to hypothesize that miRNAs would be differentially expressed between the general population and patients at risk of developing CVD or suffering from CVD. For instance, we have shown that several miRNAs are differentially expressed in the carotid plaque between symptomatic patients (having developed a stroke of ischemic transient episode) and asymptomatic patients (having carotid plaques removed by surgery but without pathological episode) [37]. Often however the clinician does not have access to the pathological tissue. In a groundbreaking work, Mitchell et al. [38] have demonstrated that circulating miRNAs are present in the human blood, where they are carried in complex with the chaperon argonate 2 and/or lipoproteins or by microvesicles, conferring stability and protection against RNases [39]. Correlations exist between blood miRNA levels and pathologies, indicating that miRNAs are potential non-invasive biomarkers [40]. It is thus reasonable to hypothesize that miRNA seric or plasma expression is a most promising avenue to evaluate early risk and patient outcome, but also individual patient response to various treatments or surgical procedures. At first, miRNAs were considered tissue and cell specific, and it was thus expected that their serum expression would reflect pathophysiology of concerned organs. This is now controversial and most miRNAs are rather enriched for a peculiar tissue. miRNAs are usually quantified using reverse transcription-quantitative PCR, so the appropriate internal control gene is very important for obtaining accurate results of miRNA expression. However, there still has been

controversy about the choice of gene used as an internal control [41]. Currently, the most widely used and accepted internal controls for miRNA qPCR are spiked-in exogenous nonhuman miRNA (e.g., synthetic *Caenorhabditis elegans* miR-39), to avoid further experimental bias [41]. Please note that urinary miRNA also represents a potential novel source to discover noninvasive biomarkers for CVD [42].

We now know that cardiospecific miRNAs are highly stable in the blood [37]. The pioneering work of Dimmeler et al. highlighted a change in miRNA levels in the serum of patients with coronary artery disease (CAD) compared to controls [43], and miRNAs are now potential biomarkers for CVD [32]. Since this pioneering study, others have shown that miRNAs are biomarkers able to predict CVD outcome. For example, Seronde et al. have shown, in a cohort of 340 patients, that decreased seric levels of miR-423-5p were associated with a poor long-term outcome in acute heart failure patients [8], indicating the value of this miRNA as a prognostic biomarker of acute heart failure. The largest cohort published to date (1114 patients) indicates that seric levels of miR-132, miR-140-3p, and miR-210 were able to precisely predict cardiovascular death (the main outcome) in patients suffering from acute coronary syndrome diagnosis [9].

miRNAs are interesting biomarkers to predict CVD outcome in populations that are particularly at risk. This is, for instance, true in the chronic kidney disease (CKD) population that is especially at risk for CVD [10, 11]. Our experimental data [12, 13] suggests that miR-126, miR-143, miR-145, miR-155, and miR-223 are potential circulating biomarkers for the diagnosis and prognosis of patients with CKD to assess the levels of miRNA expression at various stages of CKD, such as vascular calcifications [13], justifying further studies to correlate alterations of miRNA levels with CVD outcome in CKD patients. Also the levels of several of these miRNAs are sensitive to circulatory stresses and could provide valuable data about the way patients regulate their blood pressure [14].

Gupta et al. have shown that miR-22 regulates cardiac autophagy, especially in the myocardium of senior patients, and that the circulating levels of miR-22 give prognostic information on eventual progression towards heart failure, giving interesting clues about miR-22's role as biomarker candidate for CVD in the elderly population [15]. miRNAs have also potential to predict the cardiovascular toxicity of drugs and could, for example, help to detect anthracycline-induced cardiac damage. Piegari et al. have shown that miR-34 can predict the cardiotoxic effects of the anticancer drug doxorubicin, at least in a murine model [16].

As miRNAs are potential biomarkers in type 2 diabetes mellitus, obesity, and cardiovascular diseases, they have also been proposed to monitor the physiological effects of exercise in the general population [44]. For example, miR-146a and miR-221 levels were decreased and miR-149\* was increased after acute exercise [17], whereas endurance exercise altered the expression of miR-1, miR-133, and miR-206 [19]. The same was also true for patients at risk, such as a population of chronic kidney disease patients where acute exercise increased miR-150 levels and decreased miR-146a levels [18].

TABLE 1: miRNAs implicated in CVD.

	Pathophysiology	Ref
miR-423	Acute heart failure	[8]
miR-132, -140, -210	Cardiovascular death	[9]
miR-126, miR-143, miR-145, miR-155, miR-223	Vascular calcifications linked to chronic kidney disorders	[10–14]
miR-22	Cardiac autophagy	[15]
miR-34	Cardiotoxic effect of doxorubicin	[16]
miR-146a, -150, miR-221, miR-149*	Acute exercise	[17, 18]
miR-1, -133, -206	Endurance exercise	[19]
miR-1	Strength training	[20]

miR-1 was also described as a potential biomarker in an elderly population submitted to eccentric and conventional strength training [20].

Table 1 resumes the main miRNAs implicated in CVD.

**5.5. Long Noncoding RNAs (lncRNAs).** We now know that miRNAs are regulated at least in part by lncRNAs, long transcripts (>200 nt) that do not code for proteins. The biogenesis and molecular mechanism of action of miRNAs and lncRNAs have been described in greater detail in other reviews [26, 33, 44, 45]. According to recent databases 56018 and 46475 lncRNA genes have been, respectively, found for human and mouse. lncRNAs differ from miRNAs, as they regulate gene expression not only at the transcriptional and but also at the posttranscriptional and chromatin remodeling levels. They also have high tissular specificity and are not evolutionarily conserved. lncRNA can act as decoy for microRNA to allow microRNAs' target mRNA to escape from degradation (reviewed by [44]). The use of lncRNAs as innovative biomarkers in the field is still in its infancy compared to the miRNA field. Some really promising candidates, such as LIPCAR [46] or UCA1 [47], have been published in the CVD field, and more are yet to come.

**5.6. Gene Therapy via MicroRNAs: Can It Work in the CVD Field?** As miRNAs are promising biomarkers, they are also logical candidates to design innovative gene therapies, especially as several genes are involved in most cardiovascular disorders [48]. And miRNAs are master gene regulators able to regulate up to 100 mRNA targets, often implicated in convergent pathways [33]. We will however have to keep in mind several potential problems, such as loss of the therapeutic RNAs due to nucleases and/or uptake by macrophages and inefficient endocytosis by target cells.

Several successes have been accomplished in human patients, using antisense based miRNA inhibitors such as miravirsin, with minimal side effects [49]. On the other side, we can also use overexpression of miRNAs depending on the tissue and clinical context. Chad Mirkin's team has developed nanotechnologies based on gold nanoparticles that can be covalently linked with mature miRNA duplexes or antisense sequences. Interestingly, in rodents, they can cross

the blood-brain/blood-tumor barriers when administered intravenously [50, 51]. miRNA-based therapies are at present mostly developed in the cancer field, but the CVD field will follow without doubt, with exciting times ahead of us.

## 6. Conclusion

Epigenetics is a relatively new science that has a tremendous potential to introduce new biomarkers in the CVD field and also new avenues for innovative therapies. There are several potential benefits of the use of innovative epigenetic biomarkers such as DNA methylation of specific genes (i.e., TRAF3 or PPM1A methylation patterns as explained in this review) or miRNAs (i.e., miR-132, miR-140-3p, and miR-210 as predictors of cardiovascular death). Compared to classic biochemical biomarkers, they can provide valuable data about gene functions and phenotypes that would be helpful concerning CVD diagnosis, outcome, prognosis, treatment monitoring, and stratification. However one has to keep in mind that, as with other scientific fields in their infancy, a significant number of clinical studies with large cohorts will now have to be implemented to confirm that interest. Also, biologists will have to harmonize the methods of detection in order to avoid biases linked to technical issues.

## Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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## Research Article

# Hypofibrinolytic State in Subjects with Type 2 Diabetes Mellitus Aggravated by the Metabolic Syndrome before Clinical Manifestations of Atherothrombotic Disease

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**Background.** Metabolic and genetic factors induce plasminogen activator inhibitor type-1 (PAI-1) overexpression; higher PAI-1 levels decrease fibrinolysis and promote atherothrombosis. **Aim.** To assess PAI-1 antigen levels among subjects with type 2 diabetes mellitus (T2DM) plus Metabolic Syndrome (MetS) before clinical manifestations of atherothrombosis and the contribution of metabolic factors and 4G/5G polymorphism of PAI-1 gene on the variability of PAI-1. **Methods.** We conducted an observational, cross-sectional assay in a hospital in Mexico City from May 2010 to September 2011. MetS was defined by the International Diabetes Federation criteria. PAI-1 levels and 4G/5G polymorphism were determined by ELISA and PCR-RFLP analysis. **Results.** We enrolled 215 subjects with T2DM plus MetS and 307 controls. Subjects with T2DM plus MetS had higher PAI-1 levels than the reference group ( $58.4 \pm 21$  versus  $49.9 \pm 16$  ng/mL,  $p = 0.026$ ). A model with components of MetS explained only 12% of variability on PAI-1 levels ( $R^2 = 0.12$ ;  $p = 0.001$ ), with  $\beta = 0.18$  ( $p = 0.03$ ) for hypertension,  $\beta = -0.16$  ( $p = 0.05$ ) for NL HDL-c, and  $\beta = 0.15$  ( $p = 0.05$ ) for NL triglycerides. **Conclusion.** Subjects with T2DM plus MetS have elevated PAI-1 levels before clinical manifestations of atherothrombotic disease. Metabolic factors have a more important contribution than 4G/5G polymorphism on PAI-1 plasma variability.

## 1. Introduction

Plasminogen activator inhibitor type-1 (PAI-1) is the principal inhibitor of fibrinolysis [1]. PAI-1 concentrations are

determined by age, gender, ethnicity, circadian rhythm, adipose tissue distribution, sympathetic nerve activity, smoking status, and chronic inflammation [1]. Higher levels of PAI-1 are associated with an increased risk of atherothrombosis

by at least two mechanisms: by decreased fibrinolysis and by inhibition of vascular smooth muscle cell migration and proliferation, predisposing to formation of atheroma plaques prone to rupture, by a thin fibrous cap of collagen separating the lipid core from the arterial lumen [2, 3]. Insulin resistance, obesity, dyslipidemia, and endothelial dysfunction also induce PAI-1 overexpression and are associated with an augmented cardiovascular risk [4]. The cluster of those traits in a same individual is known as the Metabolic Syndrome [5, 6]. The increase in the number of metabolic risk factors in the same person increases the risk for ischemic heart disease and stroke [5, 6]. In Mexico, more than 17 million adults have the Metabolic Syndrome, and 3.5 million are already diagnosed with diabetes [7]. Previously, we reported a frequency of 68% of the Metabolic Syndrome in an urban Mexican sample [8]. Furthermore, high prevalence of patients with coronary artery disease in our country has Metabolic Syndrome (43.4%) [9].

Paradoxically, the reduction of blood glucose levels does not decrease cardiovascular event incidence in patients with type 2 diabetes mellitus (T2DM), suggesting that impaired synthetic and secretory capacity of endothelial cells, increased platelet reactivity, cellular stress, and increased circulating procoagulant and proinflammatory molecules contribute to the enhanced cardiovascular risk in diabetic subjects [10]. In addition, ~50% of individuals with atherothrombotic disease are lacked of traditional cardiovascular risk factors, suggesting a genetic contribution [11]. Previously, we identified the allele 4G of the PAI-1 gene as an independent risk factor for ST elevation myocardial infarction in young Mexican individuals and higher PAI-1 plasma concentrations in homozygous for the allele 4G [12]. In contrast, the allele 4G was not associated with an increased risk of ischemic stroke in a young Mexican sample [13]. PAI-1 plasma concentrations vary across populations, at least in part by differences in ethnicity and genetic background [14].

Therefore, the purpose of the present research was to assess the PAI-1 plasma concentrations among subjects with T2DM aggravated by the Metabolic Syndrome before clinical manifestations of atherothrombotic disease in addition to evaluating the contribution of metabolic factors and the 4G/5G polymorphism of the PAI-1 gene on the plasma variability of PAI-1 among a sample of Mexican subjects with T2DM plus the Metabolic Syndrome.

## 2. Materials and Methods

We conducted an observational, cross-sectional assay in a secondary care level hospital at Mexico City from May 2010 to September 2011. We screened consecutive apparently healthy members of the medical staff, relatives of outpatients whom came to medical consultation, and those who were in routine follow-up for diabetes mellitus. The recruitment was made by invitation through printed announcements and personal appeal to people to participate in the survey if they were interested to know their glucose tolerance status and cardiovascular risk factors. We included all individuals  $\geq 20$  years old who accepted to participate. Informed written

consent was obtained from all subjects before enrollment. The study protocol was reviewed and approved by the Human Ethical Committee and Medical Research Council of the Mexican Institute of Social Security (IMSS) and conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

The exclusion criteria were subjects with previous or current diagnosis of atherothrombotic disease (i.e., myocardial infarction, angina, stroke, transient ischemic attack, and peripheral artery disease), cancer, autoimmune disorders, acute and chronic infectious diseases, and hepatic or renal failure, those under immunosuppressive therapy, and transplant receivers.

Demographic and clinical data were collected using a questionnaire in a private interview performed by a physician; the information contained in the survey included age, gender, smoking status, previous diseases, and familial history of diabetes and cardiovascular disease. Anthropometric parameters were taken from all the participants interviewed; the same physician measured all the subjects. Waist circumference (WC) was measured at the midpoint between the last rib and the iliac crest with subjects standing and wearing only undergarments. Body weight was measured by precision scale, while subjects were minimally clothed without shoes. Height was measured in a standing position without shoes using tape meter, while the shoulders were in a normal state. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Blood pressure was measured after a 5-minute rest in a seated position. Two readings were taken in 5-minute interval between these two separated measurements, and thereafter the mean of the two measurements was considered to be the participant's blood pressure. Blood samples were obtained from all the participants by puncture of the antecubital vein at morning (between 8:00 and 10:00 am) after an overnight fast of at least 8 hours. Biochemical measurements included fasting plasma glucose (FPG), glycosylated hemoglobin (HbA1c), high-density lipoprotein cholesterol (HDL-c), and triglycerides. Buffy coat and plasma were collected and frozen at  $-70^{\circ}\text{C}$  for subsequent biochemical and genetic analysis. PAI-1 serum levels and 4G/5G polymorphism of PAI-1 gene were determined in all subjects with T2DM plus the Metabolic Syndrome in accord with the International Diabetes Federation (IDF) criteria and the reference group.

T2DM was defined as a FPG  $\geq 126$  mg/dL or HbA1c  $\geq 6.5\%$  or previous diagnosis [15]. The Metabolic Syndrome was defined as central obesity (a waist circumference  $\geq 90$  cm for men and  $\geq 80$  cm for women according to IDF criteria for Hispanic population) plus any one of the following factors: (1) raised triglycerides  $\geq 150$  mg/dL or under specific drug treatment; (2) reduced HDL-c  $< 40$  mg/dL in males and  $< 50$  mg/dL in females or under specific drug treatment; and (3) a systolic blood pressure  $\geq 130$  mm Hg or diastolic blood pressure  $\geq 85$  mm Hg or use of antihypertensive treatment [16]. The subjects were considered smokers if they were currently smoking (regularly or occasionally, including also former smokers defined as people who stopped smoking at least one year before the examination). A familial history of cardiovascular disease was defined as acute myocardial infarction, stroke, or sudden death in a first-degree male

relative below 55 years of age or a female relative below 65 years of age. Subjects without T2DM and the Metabolic Syndrome were considered as the reference group.

**2.1. Determination of PAI-1 Antigen Plasma Levels.** PAI-1 plasma concentrations were determined from blood samples collected between 8:00 and 10:00 am to avoid variations due to circadian rhythm and stored in tubes containing citrate as anticoagulant. Samples were centrifuged at 3000g for 25 min at 4°C to avoid the contamination of plasma with platelets. Then, they were stored in aliquots of 0.5 mL at -70°C until use. The plasma concentration of PAI-1 was determined immunoenzymatically by enzyme-linked immunosorbent assay (ELISA) (Coaliza PAI-1, Chromogenix, Milan, Italy).

**2.2. Deoxyribonucleic Acid Extraction and Genotyping.** Genomic deoxyribonucleic acid (DNA) was obtained from leukocyte concentrate of peripheral blood using the commercial QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Genotyping of the 4G/5G polymorphism in the PAI-1 promoter region was performed by polymerase chain reaction (PCR) using the following oligonucleotides: 5'-CACAGAGAGAGTCTGGCCACGT-3' (sense) and 5'-CCAACAGAGGACTCTTGGTCT-3' (antisense) [17]. The reaction conditions were as follows: initial denaturation at 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 30 s, alignment at 60°C for 30 s, and an extension step at 72°C for 30 s, followed by a final linear extension step at 72°C for 1 min. Amplification products of 99 bp (5G) and 98 bp (4G) were obtained. The PCR products were subjected to digestion with the specific restriction enzyme *Bs*II (New England Biolabs, Beverly, Massachusetts, USA) at 55°C. The DNA fragments were separated by electrophoresis in 3% agarose gel (Bio-Rad Laboratories, Hercules, California, USA) and visualized using SYBR Safe DNA Gel Stain (Invitrogen). All samples were processed in duplicate. Some samples were subject to sequencing.

**2.3. Statistical Analysis.** Continuous variables with normal distribution were expressed as mean  $\pm$  standard deviation; those with nonparametric distribution were expressed as median and interquartile range. Categorical data were expressed as total number and percentage. Continuous data were submitted to normality tests; variables not normally distributed were transformed to natural logarithm (NL) before any statistical analysis. Continuous variables were compared between subjects with T2DM plus the Metabolic Syndrome and the reference group by Student's *t*-test. Categorical variables were compared between both groups by  $X^2$  test. The allele frequency of the 4G/5G polymorphism conforming to Hardy-Weinberg equilibrium proportions was tested using  $X^2$  test. PAI-1 antigen plasma levels were compared between homozygous 4G/4G, 5G/5G, and heterozygous 4G/5G carriers in diabetic subjects plus the Metabolic Syndrome and the reference group using one-way analysis of variance (ANOVA). A Pearson correlation analysis was performed to evaluate the association between continuous

explanatory variables (such as age, WC, HDL-c, triglycerides, and FPG) and PAI-1 plasma levels. A Spearman correlation analysis was performed to evaluate the relationship between categorical explanatory variables (such as gender, elevated blood pressure, smoking status, and genotype) and PAI-1 plasma levels. A multivariable linear regression analysis was performed to develop a model that includes only the variables with linear correlation in the bivariate analysis (as the explanatory variables) with PAI-1 plasma levels (as the response variable) to estimate the independent contribution of each feature to variation in PAI-1 plasma concentrations. A *p* value  $\leq$  0.05 (two-tailed) was considered statistically significant. All statistical analyses were performed using SPSS (Statistical Package for the Social Sciences) statistical software package (version 15: SPSS Inc., Chicago, IL, USA).

### 3. Results

A total sample of 215 subjects with T2DM plus the Metabolic Syndrome was recruited among an urban population from Mexico City between May 2010 and September 2011. We enrolled 307 subjects without T2DM or the Metabolic Syndrome as the reference group. Comparison of clinical and biochemical characteristics between the two groups is shown in Table 1. Individuals with T2DM plus the Metabolic Syndrome were older (with a mean age of  $56 \pm 10$  versus  $48.5 \pm 13.7$  years,  $p < 0.0001$ ), with higher levels of FPG (136 (112–181) versus 90 (85–95) mg/dL,  $p < 0.0001$ ), HbA1c (5.4 (4.4–7.5) versus 3.9 (3.6–4.2) %,  $p < 0.0001$ ), BMI ( $30.8 \pm 5$  versus  $26.5 \pm 4.2$  kg/m<sup>2</sup>,  $p < 0.0001$ ), WC ( $100 \pm 9.9$  versus  $87.4 \pm 11.9$  cm,  $p < 0.0001$ ), and triglycerides (205 (134–270) versus 118 (88–150) mg/dL,  $p < 0.0001$ ) and lower levels of HDL-c (35 (30–41) versus 46.4 (37.2–55.4) mg/dL,  $p < 0.0001$ ), when compared with the reference group as was expected. The group of subjects with T2DM plus the Metabolic Syndrome had central obesity plus at least one of the following conditions: 87.9% ( $n = 189$ ) had lowered HDL-c, 70% ( $n = 152$ ) had elevated triglycerides, and 60% ( $n = 129$ ) had elevated blood pressure. There were no differences regardless of gender, smoking status, and familial history of atherothrombotic disease between both groups. There was a statistical significant difference in PAI-1 plasma levels between subjects with T2DM plus the Metabolic Syndrome and the reference group ( $58.4 \pm 21$  versus  $49.9 \pm 16$  ng/mL,  $p = 0.026$ ) Figure 1.

The genotype distribution of 4G/5G polymorphism of PAI-1 gene in the group with T2DM plus the Metabolic Syndrome was 4G/4G, 11.6% ( $n = 25$ ), 4G/5G, 48.4% ( $n = 104$ ), and 5G/5G, 40% ( $n = 86$ ), with an allelic frequency of 35.8% ( $n = 154$ ) for the allele 4G (Table 2). There were no statistical differences in genotype distribution and allelic frequency when compared with the reference group. Subjects with T2DM plus the Metabolic Syndrome homozygous for the allele 4G/4G had the highest PAI-1 plasma levels (56.7 (48.5–73.7) ng/mL), followed by the heterozygous allele 4G/5G (52.7 (43.9–70.2) ng/mL), with the lowest concentration for the homozygous allele 5G/5G (50.1 (39.1–63.3) ng/mL), but without significant statistical difference between the three

TABLE 1: Comparison of clinical and biochemical characteristics between diabetic subjects with the Metabolic Syndrome and the reference group.

Characteristic	MetS <i>n</i> = 215	Reference group <i>n</i> = 307	<i>p</i> value
Age (years)	56 ± 10	48.5 ± 13.7	<0.0001
Women, <i>n</i> (%)	138 (64.2)	206 (67.1)	0.55
FPG (mg/dL)	136 (112–181)	90 (85–95)	<0.0001
HbA1c (%)	5.4 (4.4–7.5)	3.9 (3.6–4.2)	<0.0001
BMI (kg/m <sup>2</sup> )	30.8 ± 5	26.5 ± 4.2	<0.0001
WC (cm)	100 ± 9.9	87.4 ± 11.9	<0.0001
Men ≥ 90 cm, <i>n</i> (%)	77 (35.8)	54 (17.5)	<0.0001
Women ≥ 80 cm, <i>n</i> (%)	138 (64.2)	138 (44.9)	<0.0001
HDL-c (mg/dL)	35 (30–41)	46.4 (37.2–55.4)	<0.0001
Men < 40 mg/dL, <i>n</i> (%)	63 (29.3)	49 (15.9)	0.001
Women < 50 mg/dL, <i>n</i> (%)	126 (58.6)	111 (36.1)	<0.0001
Hypertension, <i>n</i> (%)	129 (60)	40 (13)	<0.0001
Triglycerides (mg/dL)	205 (134–270)	118 (88–150)	<0.0001
≥150 mg/dL, <i>n</i> (%)	152 (70)	79 (25.7)	
PAI-1 (ng/mL)	58.4 ± 21	49.9 ± 16	0.026
Current smoking, <i>n</i> (%)	50 (23)	62 (20.2)	0.46
FH of AT, <i>n</i> (%)	41 (19)	43 (14)	0.16

Continuous variables with normal distribution are expressed as mean ± standard deviation. Continuous variables with nonnormal distribution are expressed as median (interquartile range). Categorical variables are expressed as total number and percentages. MetS: the Metabolic Syndrome; FPG: fasting plasma glucose; HbA1c: glycosylated hemoglobin; BMI: body mass index; WC: waist circumference; HDL-c: high-density lipoprotein cholesterol; PAI-1: plasminogen activator inhibitor type-1; FH of AT: familial history of atherothrombotic disease.

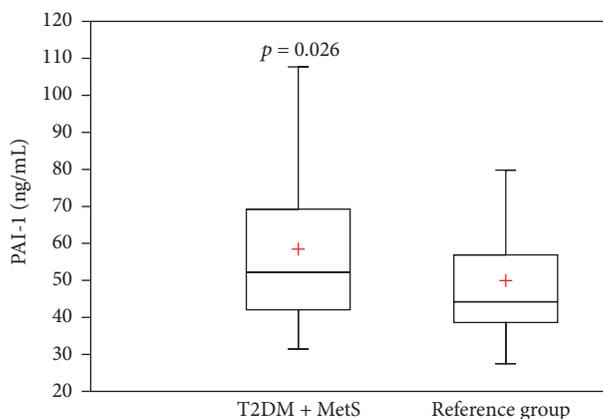


FIGURE 1: Box plots showing PAI-1 antigen plasma levels between diabetic subjects plus the Metabolic Syndrome and the reference group. + = Mean; *p* = *p* value; PAI-1 = plasminogen activator inhibitor type-1; T2DM = type 2 diabetes mellitus; MetS = the Metabolic Syndrome.

genotypes by ANOVA test (4G/4G versus 5G/5G, *p* = 0.58; 4G/5G versus 5G/5G, *p* = 0.39; 4G/4G versus 4G/5G, *p* = 0.98) (Figure 2(a)). The reference group showed similar PAI-1 plasma levels for the three genotypes; the homozygous 4G/4G had a median of 44.2 (43–45.4) ng/mL, the heterozygous 4G/5G had a median of 44.4 (40.7–58.1) ng/mL, and the 5G/5G had a median of 43.2 (36.5–57.7) ng/mL, without statistical significance (4G/4G versus 5G/5G, *p* = 0.60;

TABLE 2: Genotype distribution and allele frequencies of -675 4G/5G polymorphism of the PAI-1 gene in diabetic subjects with the Metabolic Syndrome and the reference group.

	MetS <i>n</i> = 215	Reference group <i>n</i> = 307	<i>p</i> value*
Genotype, <i>n</i> (%)			
4G/4G + 4G/5G	129 (60)	185 (60.3)	1.0
5G/5G	86 (40)	122 (39.7)	
4G/4G	25 (11.6)	26 (8.5)	0.30
4G/5G	104 (48.4)	159 (51.8)	
5G/5G	86 (40)	122 (39.7)	
Allelic frequency, <i>n</i> (%)			
4G	154 (35.8)	211 (34.4)	0.67
5G	276 (64.2)	403 (65.6)	

Categorical variables are expressed as total number and percentages. PAI-1: plasminogen activator inhibitor type-1; MetS: the Metabolic Syndrome; \*:  $\chi^2$  test.

4G/5G versus 5G/5G, *p* = 0.88; 4G/4G versus 4G/5G, *p* = 0.64) (Figure 2(b)).

Correlation coefficients between PAI-1 plasma levels and metabolic factors in subjects with T2DM plus the Metabolic Syndrome are shown in Table 3. PAI-1 plasma concentrations were positively associated with elevated natural logarithm of triglycerides (*r* = 0.24; *p* = 0.004) and hypertension (*r<sub>s</sub>* = 0.22; *p* = 0.01). In contrast, there was a negative relationship

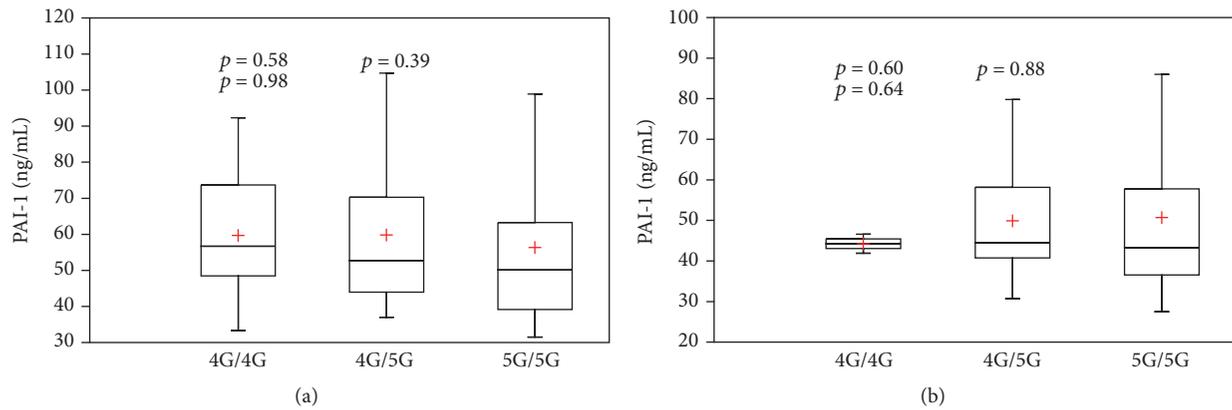


FIGURE 2: Box plots showing PAI-1 antigen plasma levels between diabetic subjects plus the Metabolic Syndrome (a) and the reference group (b), according to genotype distribution of -675 4G/5G polymorphism of the PAI-1 gene. (a) = 4G/4G versus 5G/5G,  $p = 0.58$ ; 4G/4G versus 4G/5G,  $p = 0.98$ ; 4G/5G versus 5G/5G,  $p = 0.39$ , (b) = 4G/4G versus 5G/5G,  $p = 0.60$ ; 4G/4G versus 4G/5G,  $p = 0.64$ ; 4G/5G versus 5G/5G,  $p = 0.88$ ; + = mean;  $p = p$  value; PAI-1 = plasminogen activator inhibitor type-1.

TABLE 3: Correlation of PAI-1 antigen plasma concentrations with metabolic factors of diabetic subjects plus the Metabolic Syndrome.

Total = 215 subjects					
	WC	NL HDL-c	NL Tg	FPG	Hypertension
PAI-1	0.15	<b>-0.21</b>	<b>0.24</b>	0.01	<b>0.22</b>

Cells show correlation coefficients (significant in bold,  $p \leq 0.05$ ). PAI-1: plasminogen activator inhibitor type-1; WC: waist circumference; NL HDL-c: natural logarithm of high-density lipoprotein cholesterol; NL Tg: natural logarithm of triglycerides; FPG: fasting plasma glucose.

TABLE 4: Variability in PAI-1 antigen plasma levels explained by the components of the Metabolic Syndrome.

Explanatory variables	$\beta$ (95% CI)	$p$ value
Hypertension	0.18 (0.35 to 0.012)	0.03
NL triglycerides	0.15 (0.01 to 0.33)	0.05
NL HDL-c	-0.16 (-0.33 to -0.01)	0.05
$R^2 = 0.12$ , $F$ -statistic = 0.001		

Explanatory variables are listed vertically;  $\beta$ : standardized correlation coefficient (95% confidence interval);  $R^2$ : determination coefficient represents percentage of variance explained by the explanatory variables of the model; NL HDL-c: natural logarithm of high-density lipoprotein cholesterol; NL triglycerides: natural logarithm of triglycerides.

between PAI-1 plasma levels and natural logarithm of HDL-c ( $r = -0.21$ ;  $p = 0.01$ ). The rest of the variables did not have linear correlation: abdominal obesity measured by WC ( $r = 0.15$ ;  $p = 0.08$ ), FPG ( $r = 0.01$ ;  $p = 0.88$ ), age ( $r = -0.05$ ;  $p = 0.53$ ), gender ( $r = 0.27$ ;  $p = 0.53$ ), smoking status ( $r = 0.07$ ;  $p = 0.42$ ), and 4G/5G polymorphism ( $r = 0.06$ ;  $p = 0.45$ ).

The variability on PAI-1 plasma levels explained by the components of the Metabolic Syndrome is shown in Table 4. The model included NL HDL-c, NL triglycerides, and hypertension; this model only explained 12% of variance in PAI-1 plasma levels ( $R^2 = 0.12$ ;  $p = 0.001$ ). Components of the Metabolic Syndrome were much more determinant in

PAI-1 variability than the 4G/5G polymorphism, with a standardized correlation coefficient ( $\beta$ ) statistically meaningful for hypertension:  $\beta = 0.18$  (0.35 to 0.012),  $p = 0.03$ ;  $\beta = -0.16$  (-0.33 to -0.01 for NL HDL-c),  $p = 0.05$ ; and  $\beta = 0.15$  (0.01 to 0.33),  $p = 0.05$ , for NL triglycerides.

#### 4. Discussion

We analyzed 215 subjects with T2DM plus the Metabolic Syndrome according to IDF criteria without clinical manifestation of atherothrombotic disease from a secondary care level hospital at Mexico City and compared them with the reference group ( $n = 307$ ). The overall sample of subjects with T2DM plus the Metabolic Syndrome had elevated waist circumference according to parameters for Hispanic population plus any one of the following factors: lowered HDL-c (87.9%), hypertriglyceridemia (70%), and hypertension (60%). Similar results were reported from a nationally representative subsample randomly selected in the Mexican National Health and Nutrition Survey 2006 (ENSANUT 2006), with prevalence of 49.8% (95% CI: 47.5 to 52.1) cases with the Metabolic Syndrome using the IDF definition regardless of geographical region and socioeconomic status, predominantly women (52.7%) [7]. The frequency of metabolic factors reported by the ENSANUT 2006 study in a subsample of subjects with T2DM plus the Metabolic Syndrome was as follows: in first place, 83% (95% CI: 76.5 to 88) for reduced HDL-c, followed by 65% (95% CI: 56.5 to

72.6) for hypertension, and 46.7% (95% CI: 38.8 to 54.7) for elevated triglycerides [7].

In the present study, the group of subjects with T2DM plus the Metabolic Syndrome showed higher mean PAI-1 antigen levels of  $58.4 \pm 21$  ng/mL compared with the reference group ( $49.9 \pm 16$  ng/mL) with statistical significance ( $p = 0.026$ ). Patients with T2DM plus the Metabolic Syndrome had PAI-1 antigen levels above the cutoff considered as normal (reference value: 2 to 47 ng/mL) even when some of them were under pharmacologic treatment and showed FPG and HbA1c levels within ranges considered as adequate. Metabolic factors frequencies and PAI-1 plasma concentrations vary among populations. PAI-1 was dramatically higher in Italian Caucasian subjects with obesity and the Metabolic Syndrome by the National Cholesterol Education Program Third Adult Treatment Panel (NCEP ATP-III) definition when compared with healthy subjects without obesity ( $p < 0.0001$ ) [18]. A previous report from European based-sample population showed a positively and independently association between PAI-1 antigen levels and the presence of the Metabolic Syndrome according to the NCEP ATP-III criteria, reporting a median of 126 (81.4–194.2) ng/mL for those subjects with the Metabolic Syndrome versus 57.3 (35.5–99.7) ng/mL without it ( $p < 0.001$ ) [19]. In contrast, data from multicentric cross-sectional Spanish population-based survey reported higher levels of PAI-1 in the presence of the Metabolic Syndrome or diabetes mellitus in the bivariate analysis but without statistical significance in the multivariate analysis [20]. In a sample of Malaysian subjects, there was no difference in PAI-1 antigen levels when diabetic subjects with the Metabolic Syndrome were compared against normal individuals, with a median of 28.4 (26.5–30.5) ng/mL versus 30.2 (27.1–33.7) ng/mL, respectively [21]. The variability of PAI-1 antigen levels between populations of subjects with the Metabolic Syndrome may be related to differences in (1) selection criteria for the Metabolic Syndrome, (2) the prevalence of components of the Metabolic Syndrome in the population, (3) the sample size, and (4) the lack of analysis for the effect of pharmacologic therapy in patients with dyslipidemia, hypertension, and T2DM. The pleiotropic effects of statins in reduction of cardiovascular events beyond blood cholesterol reduction include an antithrombotic property of most statins except pravastatin which downregulate the expression of PAI-1 via inhibition of Rho family proteins [22]. Also, clinical trials suggest that Angiotensin-Converting Enzyme (ACE) inhibitors may favorably modify markers of hemostasis such as PAI-1, although the data reported by different authors are still not clear [23]. There is evidence that some molecules as Angiotensin II can act as a potent fibrogenic molecule independent of its effects on blood pressure by stimulating extracellular matrix synthesis through induction of transforming growth factor- $\beta$  (TGF- $\beta$ ) expression and increasing PAI-1 gene transcription [24]. In contrast, there are some drugs such as pioglitazone, which not only improve insulin sensitivity but also can retard preclinical atherogenesis in patients with T2DM, at least in part by a reduction in PAI-1 expression [25]. Although in our sample some individuals were under treatment with antihypertensive drugs, hypoglycemic medication, or statins,

they exhibited abnormal levels of PAI-1 when compared with the reference group, which may contribute to an increased risk for atherothrombotic disease. More studies about the pharmacologic effects in PAI-1 levels are needed.

After the inclusion of all the explanatory variables correlated with PAI-1 antigen levels in a multivariable linear regression model, we found that metabolic factors with the strongest contribution in the variability of PAI-1 antigen levels in patients with T2DM plus the Metabolic Syndrome were hypertension ( $\beta = 0.18$ ;  $p = 0.03$ ), NL HDL-c ( $\beta = -0.16$ ;  $p = 0.05$ ), and NL triglycerides ( $\beta = 0.15$ ;  $p = 0.05$ ). Elevated plasma prorenin levels are commonly found in diabetic patients; and, also, it has been demonstrated that prorenin at high concentration binds and activates prorenin/renin receptor [(p)RR] on vascular smooth muscle cells in vitro, leading to increased expression of PAI-1 via Angiotensin II-independent and dependent mechanisms, suggesting that elevated prorenin levels in diabetes may contribute to progression of atherothrombotic disease [26]. Hypertension might have a predominant role in the formation of atheroma plaque rather than rupture. In a previous report by our group, hypertension represented the second cardiovascular risk factor in patients with acute myocardial infarction [12]. In addition, treating hypertension only reduces coronary heart disease (CHD) risk by about 25%; treating hypercholesterolemia in hypertensive patients reduces CHD risk more than 35%, suggesting a relationship and a synergic effect between dyslipidemia and hypertension [27]. Both metabolic factors, hypertension and dyslipidemia, represent an important trait for the development of atherothrombosis and might contribute to an increased cardiovascular risk by mechanisms that include a hypofibrinolytic state. PAI-1 could be a novel marker for evaluation of cardiovascular risk in patients with hypertension. PAI-1 antigen levels should be monitored in hypertensive patients, and treatment should be encouraged to prevent a hypofibrinolytic state.

In subjects with T2DM and the Metabolic Syndrome, the genotype distribution was 11.6%, 48.4%, and 40% for the alleles 4G/4G, 4G/5G, and 5G/5G, respectively, with an allelic frequency of 35.8% for the risk allele 4G, with any statistical difference with the reference group. Those results are consistent with a previous report in healthy subjects from the west of Mexico (with an allelic frequency of 34.1% for the allele 4G), among a control group of young individuals ( $\leq 45$  years old) in a previous publication by our group (with an allelic frequency of 28.4% for the allele 4G), and in Mexican children with obesity and without it (4G allelic frequency of 32.9% versus 26.4%) [28, 29]. In contrast, variations in prevalence of allele 4G have been reported between populations. The Insulin Resistance Atherosclerosis Study (IRAS) showed a different genotype distribution of the 4G/5G polymorphism of PAI-1 gene among African Americans (28%), Hispanics (38%), and non-Hispanic whites (52%) for the allele of risk [14]. In a sample of three different South African ethnic groups, the frequency of allele 4G was lower in the African (0.13) than Indian (0.54) or White (0.58) individuals [28]. In our sample, homozygous subjects with T2DM plus the Metabolic Syndrome with the allele 4G had the highest PAI-1 antigen levels when compared with the

homozygous 5G without statistical significance (56.7 (48.5–73.7) versus 50.1 (39.1–63.3) ng/mL,  $p = 0.58$ ). In several epidemiological, clinical, and basic studies, the allele 4G has been associated with increased PAI-1 plasma levels regardless of the effect of the Metabolic Syndrome related factors [14, 30, 31]. However, the contribution of 4G/5G polymorphism in PAI-1 variability seems to be lower. In a sample of 1328 white unrelated participants from the Framingham Heart Study, the 4G/5G polymorphism explained only 2.5% of the residual variance in circulating PAI-1 levels, with the 4G allele being associated with a higher PAI-1 concentration [32]. In a cohort of 1032 white subjects without clinical evidence of atherosclerosis from southern Italy, the contribution of 4G/5G polymorphism was small ( $\approx 1\%$ ) compared with BMI and triglycerides (20%) on PAI-1 variability [33]. A sample of 510 male survivors of myocardial infarction and 543 controls from the HIFMECH Study reported a percentage of variance explained by the 4G/5G polymorphism of 1.12% ( $p = 0.004$ ) [34]. In our sample, the 4G/4G genotype was not correlated with PAI-1 plasma levels, and therefore it was not included in the model. Differences in the genetic background and prevalence of metabolic traits between populations are determinants in the variability of PAI-1 expression and the development of cardiovascular disease, limiting the results to a specific ethnic group.

The present study exhibits a hypofibrinolytic state in a selective group of individuals with a particular environmental and genetic background, with higher PAI-1 antigen levels before clinical manifestations of atherothrombotic disease. In a previous publication, we identified higher levels of C reactive protein and fibrinogen in T2DM individuals when compared with subjects with normal glucose tolerance. Proinflammatory conditions and prothrombotic and hypofibrinolytic state might increase the risk to develop cardiovascular disease [35].

Strengths of our research include the similarities between our sample and the population-based sample from the ENSANUT 2006 study, as well as the enrolment of subjects with similar severity of the Metabolic Syndrome. Some limitations include the lack of analysis of the effect of treatment with statins, ACE inhibitors, Angiotensin receptor blockers, and insulin sensitizing drugs in PAI-1 plasma concentration. Future analysis must include the possible effect of medication on PAI-1 variability and the relationship of PAI-1 levels in patients with hypertension.

## 5. Conclusions

Subjects with T2DM aggravated by the Metabolic Syndrome have elevated PAI-1 antigen plasma levels before clinical manifestations of atherothrombotic disease. In our sample, metabolic factors (hypertension, low HDL-c, and hypertriglyceridemia) have a more important contribution than the 4G/5G polymorphism on PAI-1 plasma levels of Mexican subjects with T2DM plus the Metabolic Syndrome. However, metabolic factors only explained 12% of PAI-1 variability.

As we have shown in previous studies, PAI-1 plasma concentrations were higher in young patients with acute

myocardial infarction, and hypertension was the second more frequent cardiovascular risk factor, followed by dyslipidemia [12]. Previous and recent findings support the idea that characterization of emerging biomarkers of impaired fibrinolysis such as PAI-1 should be measured for surveillance of transition from a healthy state through the development of the Metabolic Syndrome to atherothrombotic disease and for prevention purposes in individuals with high risk of atherothrombotic disease in order to help us identify vulnerable groups for the correct targeting treatments and avoid future atherothrombotic complications such as myocardial infarction and stroke.

## Competing Interests

The authors declare that they have no competing interests.

## Authors' Contributions

Elsa Aburto-Mejía and David Santiago-Germán equally contributed to the work.

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## Research Article

# Clinic Predictive Factors for Insufficient Myocardial Reperfusion in ST-Segment Elevation Myocardial Infarction Patients Treated with Selective Aspiration Thrombectomy during Primary Percutaneous Coronary Intervention

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**Background.** Insufficient data are available on the potential benefit of selective aspiration and clinical predictors for no-reflow in STEMI patients undergoing primary percutaneous coronary intervention (PPCI) adjunct with aspiration thrombectomy. **Objective.** The aim of our study was to investigate clinical predictors for insufficient reperfusion in patients with high thrombus burden treated with PPCI and manual aspiration thrombectomy. **Methods.** From January 2011 till December 2015, 277 STEMI patients undergoing manual aspiration thrombectomy and PPCI were selected and 202 patients with a Thrombolysis in Myocardial Infarction (TIMI) thrombus grade 4~5 were eventually involved in our study. According to a cTFC value, patients were divided into Group I (cTFC > 40), namely, insufficient reperfusion group; Group II (cTFC ≤ 40), namely, sufficient reperfusion group. **Results.** Univariate analysis showed that hypertension, multivessel disease, time from symptom to PCI (≥4.8 hours), and postaspiration cTFC > 40 were negative predictors for insufficient reperfusion. After multivariate adjustment, age ≥ 60 years, hypertension, time from symptom to PCI (≥4.8 hours), and postaspiration cTFC > 40 were independently associated with insufficient reperfusion in STEMI patients treated with manual aspiration thrombectomy. Upfront intracoronary GP IIb/IIIa inhibitor (Tirofiban) was positively associated with improved myocardial reperfusion. **Conclusion.** Fully identifying risk factors will help to improve the effectiveness of selective thrombus aspiration.

## 1. Introduction

The common pathophysiological mechanism of acute coronary syndrome is sudden disruption of coronary arterial plaque due to rupture, fissuring, or superficial erosion leading to obstructive intracoronary thrombosis [1]. Acute ST-segment elevation myocardial infarction (STEMI) is characterized by complete thrombotic occlusion of a coronary artery [2]. Currently, primary percutaneous coronary intervention (PPCI) is the most effective treatment of STEMI. However, restoring the epicardial coronary is not always translated into optimum myocardial reperfusion, and this is a phenomenon at microvascular level called no-reflow

[3]. No-reflow is associated with mortality and other major adverse events [4]. The pathogenesis of no-reflow is multifactorial, including ischemic damage, reperfusion injury, distal embolization, and individual susceptibility [5]. Previous studies have found that higher thrombus burden in patients with STEMI is independently associated with distal embolization, no-reflow phenomenon, major adverse cardiac events, stent thrombosis, and death [6, 7]. Removal of the thrombus by manual thrombectomy before stent deployment has the potential of reducing distal embolization and improving microvascular perfusion [8]. The TAPAS (Thrombus Aspiration during Percutaneous Coronary Intervention

in Acute Myocardial Infarction Study) showed improved myocardial blush grade (MBG) and better clinical outcomes with the thrombus aspiration than conventional PCI [9]. However, the TASTE trial (Thrombus Aspiration in STEMI in Scandinavia) failed to show a significant benefit of routine thrombectomy in terms of early and medium-term mortality [10, 11]. In addition, the most recent and so far the largest trial showed that routine manual thrombectomy did not reduce the primary composite endpoint of cardiovascular death, recurrent MI, cardiogenic shock, or New York Heart Association class IV heart failure at 180 days, or the individual components of the primary endpoint, stent thrombosis, or target-vessel revascularization. Rates of TIMI 3 flow after PCI were the same in routine manual thrombectomy group and PCI alone treatment group and were similar for no-reflow rates on angiography. Subgroup patients with high thrombus burden and initial TIMI flow grade 0~1 also showed no benefits [12]. On the basis of the results of these studies, the prior class IIa recommendation for aspiration thrombectomy has been changed. Routine aspiration thrombectomy during primary PCI is now not recommended. Why these randomized trials showed limited clinical benefit of routine aspiration remains elusive. Furthermore, there are insufficient data to assess the potential benefit of a strategy of selective aspiration thrombectomy and to show who might benefit most from selective aspiration. It has been observed in a real world that patients with TIMI thrombus grade 4~5 or with initial TIMI flow 0~1 are more often treated with thrombus aspiration. Detecting of clinical predictors for insufficient myocardial reperfusion during PPCI will help patients with high thrombus burden to benefit most from thrombus aspiration, consequently improving the benefit of manual thrombus aspiration in these patients.

## 2. Methods

**2.1. Patient Population.** STEMI patients undergoing successful primary from January 2011 to December 2015 in Beijing Anzhen Hospital were initially retrospectively recruited in our study, and thrombus aspiration was performed before PPCI on all the patients. 202 patients with TIMI thrombus burden grade 4~5 were eventually included in our study. STEMI was defined as (1) typical ischemic chest pain with a duration time of more than 30 minutes (the pain could not be alleviated by resting or taking nitroglycerin); (2) ST-segment elevation >1mm in two consecutive leads or the onset of left bundle branch block; (3) detection of rise and/or fall of cardiac biomarker values (preferably troponin) with at least one value above the 99th percentile of the upper reference limit. Patients who presented with non-STEMI or had undergone a coronary artery bypass surgery were excluded.

**2.2. Angiographic Analysis.** All patients received 300 mg chewable aspirin and a 300 mg loading dose of clopidogrel on admission and 50–70 U/kg intravenous standard heparin before the procedure. Coronary angiography was performed according to the standard criteria. The thrombectomy was determined by the experienced cardiologist commonly according to the visual assessments for thrombus.

The administration of glycoprotein IIb/IIIa receptor inhibitor (Tirofiban) was left to the operator's discretion during PCI. The blood flow in the infarct-related artery (IRA) was graded according to the Thrombolysis in Myocardial Infarction (TIMI) grading system. Corrected TIMI frame counts (cTFC) values, the degree of stenosis, and TIMI thrombus burden grade were measured by quantitative coronary arteriography offline by two experienced interventional cardiologists who were blinded to the clinical data of the patients. The coronary angiograms were acquired at 15 frames/second with a digital angiographic system. Data were then converted to the most common filming speed of 30 frames/second by multiplying by a factor of two. cTFC was regarded as 100 for flows not reaching the distal reference point.

Insufficient reperfusion was diagnosed with a cTFC value > 40 in the IRA. A value  $\leq 40$  was taken to indicate sufficient reperfusion [13, 14]. Accordingly, the patients were subdivided into Group I (those with insufficient reperfusion) and Group II (those with sufficient reperfusion). Thrombus burden was scored in five degrees: G0 = no thrombus, G1 = possible thrombus, G2 = small [greatest dimension  $\leq 1/2$  vessel diameter (VD)], G3 = moderate ( $>1/2$  but  $< 2VD$ ), G4 = large ( $\geq 2VD$ ), and G5 = total occlusion. TIMI thrombus score  $\geq 4$  was defined as high-grade angiographic thrombus burden [15]. After the intervention, all patients were given clopidogrel 75 mg and aspirin 100 mg once daily for 12 months. Other routine medications were according to the guideline recently.

**2.3. Data Collected.** Variables including demographics, medical history, laboratory studies, and procedural characteristics were recorded by two independent researchers who were blinded to the study objectives.

**2.4. Statistical Analysis.** Continuous variables are expressed as mean  $\pm$  standard deviation. Normal distribution continuous variables were compared using Student's *t*-test, while Mann-Whitney *U* test was used for abnormal distribution. Categorical variables are expressed as number and percentage of patients. The chi-square test or Fish's exact test was used to analyze categorical variables. Univariate and multivariate analysis were performed to identify independent predictors of insufficient reperfusion phenomenon. In multivariable models, covariates included those with a *P* value  $< 0.1$  in the univariable analysis and those that were clinically relevant. Results were presented as adjusted OR with 95% CI. Any correlation between the data was tested by the Pearson correlation analysis. The receiver operating characteristic (ROC) was used to determine the relationship between time from symptom to PCI and post-PCI cTFC and to determine the correlation between postaspiration cTFC and post-PCI cTFC. Cut-off level of time from symptom to PCI was also calculated by ROC curve. Statistical analysis was made using SPSS 17.0. A *P* value  $< 0.05$  was considered statistically significant.

## 3. Results

**3.1. Baseline Clinical Characteristics.** The study population consisted of 202 STEMI patients (age  $56 \pm 11$  years, male

TABLE 1: Baseline characteristics.

Parameters	Group I (n = 31)	Group II (n = 171)	P value
Age (years, $\bar{X} \pm S$ )	59 $\pm$ 11	56 $\pm$ 11	0.108
Age $\geq$ 60 years	16 (51.6)	57 (33.3)	0.051
Gender, male, n (%)	28 (90.3)	149 (87.1)	0.773
Smoker, n (%)	18 (58.1)	115 (67.3)	0.321
Hypertension, n (%)	24 (77.4)	81 (47.4)	0.002*
Diabetes, n (%)	10 (32.3)	35 (20.5)	0.147
History of PCI, n (%)	1 (3.2)	9 (5.3)	1.000
SBP (mmHg, $\bar{X} \pm S$ )	116 $\pm$ 18	113 $\pm$ 15	0.437
DBP (mmHg, $\bar{X} \pm S$ )	73 $\pm$ 9	73 $\pm$ 10	0.771
Killip classification, n (%)			1.000
1~2	2 (6.5)	14 (8.2)	
3~4	29 (93.5)	157 (91.8)	
WBC ( $\times 10^9/L$ )	11.59 $\pm$ 2.77	11.31 $\pm$ 3.18	0.650
Red cell count ( $\times 10^{12}/L$ )	4.58 $\pm$ 0.69	4.64 $\pm$ 0.53	0.573
HBG (g/L)	140.41 $\pm$ 19.66	143.37 $\pm$ 13.90	0.310
Plt-count ( $\times 10^9/L$ )	193.94 $\pm$ 50.30	203.05 $\pm$ 51.12	0.361
PDW (%)	12.97 $\pm$ 2.52	13.19 $\pm$ 2.85	0.736
cTnI (ng/L)	85.93 $\pm$ 69.73	89.65 $\pm$ 69.64	0.975
ALT (U/L)	60.84 $\pm$ 32.33	56.28 $\pm$ 41.33	0.310
Scr ( $\mu\text{mmol/L}$ )	83.92 $\pm$ 24.26	77.51 $\pm$ 18.82	0.099
TG (mmol/L)	1.81 $\pm$ 0.80	1.65 $\pm$ 1.03	0.138
TCHO (mmol/L)	4.68 $\pm$ 0.99	4.59 $\pm$ 0.97	0.639
HDL-C (mmol/L)	1.04 $\pm$ 0.23	0.98 $\pm$ 0.22	0.227
LDL-C (mmol/L)	3.01 $\pm$ 0.89	3.01 $\pm$ 0.88	0.983
Admission GLU (mmol/L)	9.15 $\pm$ 4.90	7.87 $\pm$ 2.70	0.179

\*  $P < 0.01$ ; SBP, systolic blood pressure; DBP, diastolic blood pressure; HGB, hemoglobin, PDW, platelet distribution width; cTnI, cardiac troponin I; ALT, alanine transaminase; Scr, serum creatinine; TG, triglyceride; TCHO, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; GLU, glucose.

87.6%) with high thrombus burden undergoing manual thrombus aspiration and PPCI. The incidence of insufficient reperfusion was 15.3% ( $n = 31$ ). Compared to Group II, there were more patients older than 60 years in Group I (51.6% versus 33.3%), but the difference was statistically different. In Group I, 77.4% of the patients were with hypertension, while 47.4% of the patients were with hypertension in Group II. In respect to gender, smoking, diabetes, and history of PCI, there were no significant differences between Group I and Group II. In respect to SBP, DBP, Killip classification, and laboratory findings, the differences were not statistically different between the two groups (see Table 1).

**3.2. Percutaneous Intervention Findings.** Compared to Group II, Group I has a longer time from symptom to PCI, and the difference was statistically significant. The ROC curve was calculated for time from symptom and was illustrated in Figure 1. The area under the curve was 0.701 and showed a significant  $P$  value. The cut-off level of time from symptom was 4.8 hours with 83.9% sensitivity and 57.1% specificity. Compared with Group II, there were more patients with multivessel disease in Group I with a significant  $P$  value. The cTFC postaspiration in Group I was significantly higher than

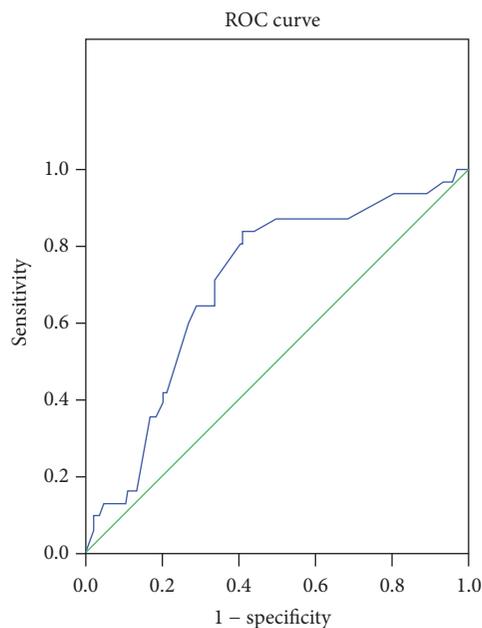


FIGURE 1: ROC analysis of time from symptom to PCI for insufficient reperfusion (AUC 0.701, 95% CI: 0.605–0.797,  $P = 0.000$ ).

TABLE 2: Percutaneous intervention findings of patients with insufficient reperfusion and sufficient reperfusion.

Parameters	Group I (n = 31)	Group II (n = 171)	P value
Time from symptom to PCI (hour)	6.2 ± 2.3	4.7 ± 2.3	0.000*
Time from symptom to PCI (≥4.8 hours)	26 (83.9)	70 (40.9)	0.000*
Initial TIMI flow grade, n (%)			0.107
0-1	28 (90.3)	166 (97.1)	
≥2	3 (9.7)	5 (2.9)	
Multiple stents, n (%)			0.746
Yes	8 (25.8)	49 (28.7)	
No	23 (74.2)	122 (71.3)	
Multivessel disease, n (%)			0.038†
Yes	13 (41.9)	41 (24.0)	
No	18 (58.1)	130 (76.0)	
IRA, n (%)			0.183
LAD	10 (32.3)	71 (41.5)	
LCX	6 (19.4)	15 (8.8)	
RCA	15 (48.4)	85 (49.7)	
Infarct location, n (%)			0.363
Anterior wall	10 (32.3)	70 (40.9)	
Nonanterior wall	21 (67.7)	101 (59.1)	
Initial degree of luminal stenosis (%)	94.60 ± 17.63	98.10 ± 8.58	0.288
Initial cTFC	91.4 ± 22.8	94.3 ± 19.3	0.420
Initial cTFC > 40, n (%)	27 (87.1)	160 (94.1)	0.239
Degree of postaspiration luminal stenosis (%)	64.40 ± 22.94	67.66 ± 13.64	0.448
Postaspiration TIMI 0, n (%)	5 (16.1)	5 (2.9)	0.009*
Postaspiration cTFC	55.5 ± 24.7	30.4 ± 17.0	0.000*
Postaspiration cTFC > 40, n (%)	21 (67.7)	29 (17.0)	0.000*
GP IIb/IIIa inhibitor upfront used			
Intracoronary GPII b/IIIa inhibitor, n (%)	2 (6.5)	15 (8.8)	1.000
Intravenous GP IIb/IIIa inhibitor, n (%)	4 (12.9)	25 (14.6)	1.000

\*  $P < 0.01$ , †  $P < 0.05$ ; IRA, infarct-related artery; LAD, left anterior descending; LCX, left circumflex artery; RCA, right coronary artery.

TABLE 3: Correlation between post-PCI cTFC and other parameters.

Parameters	r	P value
Initial cTFC	0.015	0.832
Postaspiration cTFC	0.569	0.000*

\*  $P < 0.01$ .

that in Group II, and there were more patients with postaspiration cTFC > 40 in Group I. Compared to Group II, there were more patients with postaspiration TIMI flow grade 0 in Group I. There were no differences between the two groups in respect to initial cTFC value and initial degree of luminal stenosis. In respect to infarct-related artery, infarct location, and number of stents, no statistical differences were found between the two groups. There was also no difference between the two groups according to upfront intracoronary or intravenous administration of GP IIb/IIIa inhibitors (see Table 2).

**3.3. Correlation between Post-PCI cTFC and Other Parameters.** Correlation between post-PCI cTFC and other parameters measured by QCA was illustrated in Table 3. Significant positive correlation was demonstrated between postaspiration cTFC and post-PCI cTFC ( $r = 0.569$ ,  $P = 0.000$ ). There

was no significant correlation between post-PCI cTFC and initial cTFC ( $r = 0.015$ ,  $P = 0.832$ ).

ROC was calculated for initial degree of luminal stenosis, initial cTFC, postaspiration degree of luminal stenosis, and postaspiration cTFC. Only postaspiration cTFC was predictive of insufficient reperfusion (AUC 0.835,  $P = 0.000$ ) (Figure 2). Initial cTFC (AUC 0.447,  $P = 0.689$ ), initial degree of luminal stenosis (AUC 0.448,  $P = 0.355$ ), and postaspiration degree of luminal stenosis (AUC 0.471,  $P = 0.608$ ) were not correlated to insufficient reperfusion.

**3.4. Parameters after Aspiration in Patients with Initial Complete Occlusion of IRA.** Among patients with initially complete occlusion of IRA, postaspiration cTFC in Group I was higher than that in Group II. There were more patients with postaspiration cTFC > 40 and postaspiration TIMI flow grade 0 in patients with insufficient group (Table 4).

**3.5. Predictors for Insufficient Reperfusion in Patients with TIMI Thrombus 4~5 Grade.** At univariate analysis, hypertension, multivessel disease, time from symptom to PCI (≥4.8 hours), and postaspiration cTFC > 40 were found to be predictive of no-reflow and showed significant  $P$  values. At

TABLE 4: Parameters postaspiration in patients with initial complete occlusion of IRA.

Parameters	Insufficient reperfusion (n = 25)	Sufficient reperfusion (n = 156)	P value
Postaspiration cTFC	56.5 ± 24.8	30.5 ± 17.5	0.000*
Postaspiration cTFC > 40, n (%)	16 (64.0)	27 (17.3)	0.000*
Postaspiration TIMI flow grade 0, n (%)	4 (16.0)	5 (3.2)	0.022†

\* P < 0.01, † P < 0.05.

TABLE 5: Univariate and multivariate analysis in patients with TIMI thrombus 4~5 grade.

Parameters	Univariate analysis			Stepwise multivariate		
	OR	95% CI	P	OR	95% CI	P
Age ≥ 60 years	2.133	0.985–4.621	0.055	6.286	1.477–26.751	0.013†
Gender	1.378	0.386–4.917	0.621	4.227	0.610–29.289	0.144
Smoking	1.483	0.679–3.241	0.323	0.717	0.185–2.771	0.629
Diabetes	1.850	0.799–4.285	0.151	0.758	0.159–3.614	0.728
Hypertension	3.810	1.558–9.312	0.003*	7.235	1.864–28.076	0.004*
PCI history	1.667	0.204–13.643	0.634	6.661	0.248–179.053	0.259
Killip class ≥ 3	0.773	0.167–3.585	0.743	0.218	0.020–2.412	0.214
WBC	1.029	0.911–1.161	0.648	1.144	0.940–1.393	0.178
Admission GLU	1.108	0.999–1.229	0.052	1.115	0.889–1.400	0.346
Time from symptom to PCI (≥4.8 hours)	7.503	2.748–20.486	0.000*	37.926	6.241–230.46	0.000*
Multivessel disease	2.290	1.034–5.071	0.041†	2.173	0.493–9.574	0.305
Anterior wall infarction	0.687	0.305–1.548	0.365	1.000	0.308–3.247	1.000
Intracoronary GP IIb/IIIa inhibitor upfront used	0.717	0.156–3.305	0.670	0.044	0.003–0.716	0.028†
Intravenous GP IIb/IIIa inhibitor upfront used	0.865	0.279–2.685	0.802	3.359	0.491–22.984	0.217
Initial cTFC > 40	0.422	0.123–1.442	0.169	0.513	0.065–4.034	0.526
Degree of postaspiration luminal stenosis	0.987	0.965–1.010	0.280	0.973	0.935–1.012	0.166
Postaspiration cTFC > 40	10.283	4.384–24.116	0.000*	29.273	6.557–130.685	0.000*

\* P < 0.01, † P < 0.05.

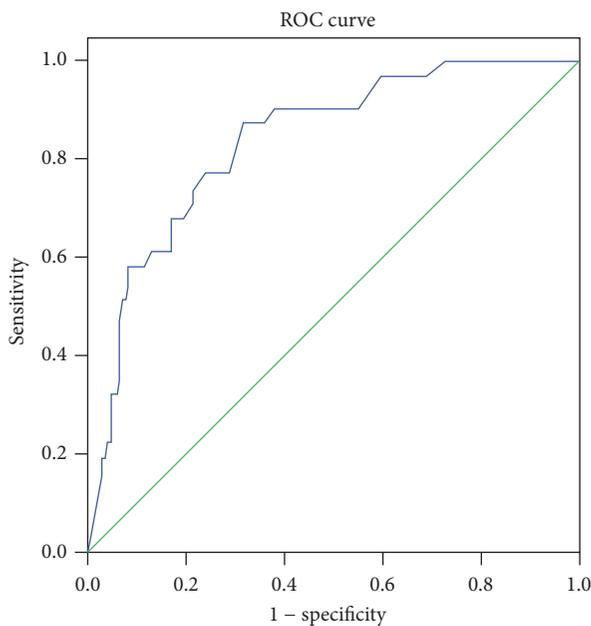


FIGURE 2: ROC analysis of postaspiration cTFC for insufficient reperfusion (AUC 0.837, 95% CI: 0.766–0.908, P = 0.000).

multivariate analysis, age ≥ 60 years [OR 6.286, 95% CI 1.477–26.751, P = 0.013], hypertension [OR 7.235, 95% CI 1.864–28.076, P = 0.004], time from symptom to PCI (≥4.8 hours) [OR 37.926, 95% CI 6.241–230.46, P = 0.000], and postaspiration cTFC > 40 [OR 29.273, 95% CI 6.557–130.685, P = 0.000] were independently predictive of insufficient reperfusion. Upfront intracoronary GP IIb/IIIa inhibitor administration was an independently positive predictor for sufficient reperfusion [OR 0.044, 95% CI 0.003–0.716, P = 0.028] (Table 5).

3.6. Predictors for Insufficient Reperfusion in Patients with Initial TIMI Flow Grade 0~1. Among the patients with initial TIMI flow grade 0~1, univariate analysis showed age ≥ 60 years, hypertension, admission GLU, time from symptom to PCI (≥4.8 hours), and postaspiration cTFC > 40 were negative predictors for insufficient reperfusion. At the multivariate analysis, age ≥ 60 years [OR 9.123, 95% CI 1.948–42.710, P = 0.005], hypertension [OR 10.762, 95% CI 2.384–48.582, P = 0.002], time from symptom to PCI (≥4.8 hours) [OR 43.917, 95% CI 6.554–294.287, P = 0.000], and postaspiration cTFC > 40 [OR 27.005, 95% CI 5.757–126.669, P = 0.000] were independent predictors for insufficient reperfusion (Table 6).

TABLE 6: Univariate and multivariate analysis in patients with initial TIMI flow grade 0~1.

Parameters	Univariate analysis			Stepwise multivariate		
	OR	95% CI	P	OR	95% CI	P
Age $\geq$ 60 years	2.329	1.036–5.234	0.041 <sup>†</sup>	9.123	1.948–42.710	0.005*
Gender	1.273	0.354–4.574	0.711	3.940	0.558–27.837	0.169
Smoking	1.306	0.573–2.978	0.526	0.515	0.123–2.146	0.362
Diabetes	1.773	0.738–4.259	0.200	0.613	0.122–3.085	0.552
Hypertension	5.066	1.838–13.964	0.002*	10.762	2.384–48.582	0.002*
PCI history	1.548	0.188–12.715	0.684	4.732	0.151–148.102	0.376
Killip class $\geq$ 3	0.835	0.179–3.891	0.819	0.390	0.040–3.761	0.415
WBC	1.031	0.909–1.169	0.638	1.133	0.925–1.388	0.229
Admission GLU	1.123	1.009–1.249	0.033 <sup>†</sup>	1.143	0.907–1.441	0.257
Time from symptom to PCI ( $\geq$ 4.8 hours)	6.467	2.343–17.847	0.000*	43.917	6.554–294.287	0.000*
Multivessel disease	1.973	0.855–4.553	0.111	1.831	0.382–8.770	0.449
Anterior wall infarction	0.666	0.284–1.560	0.349	0.862	0.260–2.862	0.809
Intracoronary GP IIb/IIIa inhibitor upfront used	0.835	0.179–3.891	0.819	0.150	0.013–1.736	0.129
Intravenous GP IIb/IIIa inhibitor upfront used	0.986	0.314–3.094	0.981	3.867	0.529–28.278	0.183
Degree of postaspiration luminal stenosis	0.990	0.966–1.014	0.407	0.973	0.935–1.012	0.166
cTFC > 40 after aspiration	10.405	4.268–23.365	0.000*	27.005	5.757–126.669	0.000*

\*  $P < 0.01$ ; <sup>†</sup>  $P < 0.05$ .

#### 4. Discussions

Early revascularization of infarct-related artery by primary PCI has become the most effective therapy in STEMI. Although PPCI has dramatically reduced the cardiovascular mortality, normal myocardial perfusion is not always restored [16]. Distal embolization of atherothrombotic material during primary percutaneous coronary intervention for STEMI is an important cause of unsuccessful reperfusion [6, 17]. A study showed that distal embolization was associated with a 5-fold increase in 5-year mortality [18].

Embolization of atherothrombotic material downstream in the IRA is related to microvascular obstruction [19]. Manual aspiration catheters are the most commonly used devices because they are easy and safe to use, even in the elderly, and effective manual aspiration of atherothrombotic material before balloon/stent inflation has the potential of decreasing the risks of no-reflow [20]. Previously, single-center randomized clinical trials have shown that manual thrombus aspiration is associated with improved angiographic and electrocardiographic outcomes [9, 21]. However, most recently randomized clinical trials showed limited clinical benefit of routine manual thrombectomy [10–12, 22]. Consequently, recommendation of routine manual thrombectomy was downgraded [23]. The reason why thrombus aspiration results in conflicting data on myocardial reperfusion, infarct size, and clinical outcomes remains incompletely clear. There were few current studies to assess who might benefit most from selective thrombectomy. Detecting clinical predictors for insufficient myocardial reperfusion will help patients with high thrombus burden benefit most from aspiration. In our study, we chose patients with TIMI thrombus grade 4~5 and 97% patients with initial TIMI flow grade 0~1.

According to our study, time from symptom to PCI was longer in patients with insufficient reflow. We took 4.8 hours

as a cut-off calculated by ROC curve. Patients with time from symptom to PCI  $\geq$  4.8 hours may have more frequency of insufficient myocardial reperfusion after PCI. Multivariate analysis showed time from symptom to PCI  $\geq$  4.8 hours was an independent predictor for insufficient reperfusion. Similar to the study of Yip et al. [24], the no-reflow was lower in those with reperfusion less than 4 hours among patients with AMI who had a high thrombus burden. The underlying mechanism is complex; prolonged ischemia leads to edema of distal capillary beds, swelling of myocardial cells, neutrophil plugging, alterations of capillary integrity, and disruption of microvascular bed, contributing to the pathogenesis of no-reflow [25].

The relationship between reperfusion time and impaired reperfusion could be also illustrated by the study of Kramer and his colleagues [26]. According to their study, fresh thrombus (<1 day) is defined as being composed of layered patterns of fibrin and intact platelets, erythrocytes, and granulocytes. Older thrombus (>1 day) consists of lytic (1 to 5 days) and/or organized (>5 days) thrombi. They previously showed that aspirate thrombus fragments may be >12 hours old in STEMI patients with an onset of symptoms of <12 hours. In about half of patients, the thrombus showed features of lytic changes (>24 hours to 5 days) and organization (>5 days). The total ischemia time was significantly longer in patient with older thrombus (4.1 versus 3.3 hours). The longer total ischemic time in the patients with older thrombus may diminish the potential benefit of thrombus removal in these patients [26]. The older erythrocyte-rich thrombi were moderate or large in size. Limited intracoronary material could be retrieved in these patients using thrombectomy devices [27]. With a longer duration to reperfusion, the rigid and older well-organized thrombi tend to fragment with balloon dilatation and may increase the risk of distal embolization during PPCI. Consequently, longer ischemic

time associated with older thrombus may be correlated to poor reperfusion and mortality in STEMI treated with aspiration and PPCI. According to TAPSE [9], the trial showed an improved myocardial reperfusion with treatment of aspiration, atherothrombotic material was retrieved in 73% of the patients who underwent thrombus aspiration, and the main constituent of the retrieved material was platelets. In this trial, aspiration was performed soon after the onset of symptoms in a large cohort of patients who were not selected on the basis of angiographic characteristics and were randomly assigned to a treatment group.

In our study, we detected that postaspiration cTFC value was a strong independent predictor for insufficient myocardial reperfusion in patients either with thrombus grade 4~5 or with initial TIMI flow grade 0~1. Furthermore, in patients with initial complete occlusion of IRA, insufficient reperfusion more frequently occurred when postaspiration cTFC values were  $> 40$ . This may be due to a variety of mechanisms. Mechanical resistance at the occlusion has a potential role of prevention of passage of the aspiration device through the IRA segment. In some STEMI patients, a high-grade, nonthrombotic, unstable atherosclerotic plaque causes the coronary obstruction [28]. Postaspiration cTFC value in these patients was often  $>40$ . In the procedure, some aspiration devices may cause physical damage to the vessel endothelium, which may create new plaque debris, thrombi, or distal embolization, leading to limited effectiveness of aspiration consequently [26].

Inadequate aspiration was a pivotal cause for unsatisfied postaspiration cTFC values. In respect to our study, there were more patients with TIMI flow grade 0 after aspiration in insufficient reperfusion group (16.1% versus 2.9%). In patients with initial complete occlusion of IRA, the percentages were 16.0% and 3.2%, respectively. The older thrombus associated with delayed reperfusion is one of the causes of inadequate aspiration as explained previously. According to our study, more patients with postaspiration cTFC values  $> 40$  had a reperfusion time  $\geq 4.8$  hours (58.0% versus 42%). The anatomy features of coronary should also be taken into account for inadequate aspiration. Limited intracoronary materials are retrieved in an instance of tortuous vessels. The selection of aspiration device and skills of the operators are pivotal to sufficient aspiration. TIMI flow grade  $\geq 2$  was a satisfied endpoint. In order to achieve an adequate aspiration, the device has to advance delicately over the thrombotic occlusion to perform continuous intracoronary blood suction. In the case of a large thrombus burden, repeated aspiration or use of a 7 Fr intracoronary manual thrombectomy device or rheolytic tools with greater suction force could be necessary [29, 30].

According to our analysis, patients with hypertension or over 60 years were at great risk for insufficient reperfusion after thrombectomy aspiration treatment. They could be explained by the remodeling of small intramyocardial vessels and interstitial fibrosis, leading to reduced tissue perfusion that easily happened in elderly or patients with hypertension [5]. Our study did not conclude the impact of diabetes mellitus on insufficient reperfusion. According to Iwakura et al. [31], compared to those without no-reflow phenomenon,

patients with AMI and no-reflow phenomenon have higher blood glucose levels on admission despite the similar frequency of diabetic mellitus and similar levels of hemoglobin A1C values of the two groups. The study of Ege et al. [32] also suggested that acute hyperglycemia in the acute phase of the MI has relationship with myocardial reperfusion after PCI, probably due to the augmented thrombus formation by hyperglycemia. Univariate analysis in our study showed that admission glucose negatively affected myocardial reperfusion among patients with initial TIMI flow grade 0~1; however, after adjustment for other confounders, the admission glucose was not significantly related to post-PCI cTFC, so a trial in larger sample size is needed in the future to demonstrate the effect of acute hyperglycemia on the myocardial reperfusion after PCI adjunct with thrombectomy aspiration.

Antithrombotic regimen is effective in reducing distal embolization. In a coincidence with the previous studies [33, 34], after multivariate adjustment in our study, upfront intracoronary administration of GP IIb/IIIa inhibitor showed positive effects on myocardial reperfusion after treatment with thrombectomy aspiration, whereas upfront intravenous administration of GP IIb/IIIa inhibitors showed no difference to the reperfusion.

## 5. Limitations

Several limitations of our study should be taken into account. Firstly, the anatomy features of coronary were not described in our study. Secondly our study was not powerful to adjust all the potential confounders, due to small sample size and limited data. Thirdly, elderly or patients with hypertension are susceptible to insufficient reflow; our study was not powerful to evaluate how the advanced age and hypertension impact insufficient reflow in patients treated with thrombectomy aspiration. Fourthly, in a prospective trial, De Vita et al. [35, 36] showed that increasing time to treatment was associated with a significantly decreased reperfusion rate in patients treated with PCI alone. Thrombectomy aspiration limited the adverse effects of increasing time to treatment. Our study only retrospectively observed the clinical predictors for insufficient reperfusion when patients with thrombus burden grade 4~5 were treated with PCI adjunct with thrombectomy aspiration in our center and did not analyze the impact of increasing time on PCI alone versus adjunct with thrombectomy aspiration. Thus, a larger sample, prospective, randomized controlled trial regarding the impact of treatment time on the efficiency of PCI alone versus thrombectomy aspiration is further needed in our further research.

## 6. Conclusion

Age over 60 years, hypertension, time from symptom to PCI ( $\geq 4.8$  hours), and postaspiration cTFC  $> 40$  were independently predictive of insufficient reperfusion in STEMI patients treated with adjunctive manual aspiration thrombectomy during PCI, whereas early intracoronary GP IIb/IIIa inhibitor Tirofiban administration improved the post-PCI cTFC. The results were consistent in patients with an initial TIMI flow grade 0~1. Identifying these risk factors will

improve the effectiveness of PCI adjunct with selective aspiration.

## Competing Interests

The authors declare that they have no competing interests.

## Authors' Contributions

Shuzheng Lyu contributed to the topic conception, manuscript revision, and the decision to submit for publication. Jinfan Tian participated in the study design, data collection, data analysis and interpretation, and writing of the manuscript. Yue Liu contributed to data analysis and interpretation and manuscript revision and is the co-first author. Xiantao Song, Min Zhang, Feng Xu, and Fei Yuan participated in the decision for treatment strategies and data collection and analysis.

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## Research Article

# High Systolic and Diastolic Blood Pressure Variability Is Correlated with the Occurrence of Peripheral Arterial Disease in the First Decade following a Diagnosis of Type 2 Diabetes Mellitus: A New Biomarker from Old Measurement

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**Background.** To assess whether the visit-to-visit variability in blood pressure (BP) is a risk factor of peripheral arterial disease (PAD) in patients with type 2 diabetes mellitus (T2DM) 10 years after diagnosis. **Methods.** The electronic medical records of 825 patients, who were diagnosed with type 2 diabetes mellitus (T2DM) during 2000–2002 and regularly followed for 10 years, were retrospectively reviewed. A total of 53,284 clinic visit records, including analysis of BP, BMI, serum glycohemoglobin, and lipid profile, were analyzed. **Results.** Patients were categorized into two groups according to their visit-to-visit variability in systolic and diastolic BP (SBP and DBP, resp.). The high-risk group included patients with high SBP and DBP visit-to-visit variability; this group had a 1.679-fold (95% CI: 1.141–2.472,  $P = 0.009$ ) increased risk of PAD compared with patients in the low-risk group. Cox regression analysis also demonstrated that the age at which the patients were diagnosed with T2DM, smoking status, and mean creatinine level was significantly associated with increased risk of PAD with a hazard ratio of 1.064 (95% CI: 1.043–1.084,  $P < 0.001$ ), 1.803 (95% CI: 1.160–2.804,  $P = 0.009$ ), and 1.208 (95% CI: 1.042–1.401,  $P = 0.012$ ), respectively. **Conclusions.** High SBP and DBP visit-to-visit variability is correlated with PAD in the first decade following a diagnosis of T2DM.

## 1. Introduction

In patients with diabetes mellitus, peripheral arterial disease (PAD) is a major risk factor for lower-extremity amputation [1]. However, it is difficult to determine the prevalence of PAD in patients with diabetes given its asymptomatic characteristic, the diverse screening modalities employed, and blunted pain sensation due to peripheral neuropathy, resulting in its underestimation [1]. The ankle-brachial index (ABI), the ratio of the systolic blood pressure (SBP) measured at the ankle to that measured at the brachial artery [2], has a sensitivity of 95% and specificity of almost 100% for PAD diagnosis, when validated against angiographically confirmed disease [3]. In diabetic patients older than 40 years of age examined using the ABI, the prevalence of PAD was 20% [4]. In contrast,

Hirsch et al. [5] reported a prevalence of PAD of 29% in diabetic patients older than 50 years of age.

Identifying the biomarkers for PAD is important for development of prevention modalities in diabetic patients, especially given that the 5-year cardiovascular event rate, including nonfatal myocardial infarction (MI) and stroke, for PAD patients with T2DM was 20% and the mortality rate was 30% [6]. Moreover, after adjusting for risk factors, patients with PAD had a twofold increased risk of MI, stroke, and mortality rate [7]. Moreover, 27% of patients with PAD demonstrate progression of symptoms with 4% experiencing limb loss after 5 years [8].

A diagnosis of PAD can identify patients who have higher risk of subsequent MI or stroke, and treating hypertension in PAD patients reduces the risk of MI, stroke, heart failure,

and death [9]. Diagnosis and treatment of symptomatic PAD patients with a supervised exercise program and cilostazol may improve the quality of life and prevent functional disability and limb loss as well [10]. Both the American Diabetes Association guidelines [11] and the seventh report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure [12] recommend using absolute BP as a therapeutic target to prevent clinical stroke and heart disease, as well as PAD with paucity of evidence [13]. However, no randomized prospective clinical trial has conclusively proven the benefits of treatment in individuals with stage 1 systolic hypertension [12].

Although mean BP values are largely considered the cause of the adverse cardiovascular consequences associated with hypertension, the possible role of increased BP variability has also been reported in observational studies and clinical trials [14–17]. Specifically, the visit-to-visit variability of systolic BP (SBP) had been shown to be a novel biomarker for the development of stroke and coronary artery diseases [14–19], the progression of a carotid artery stenosis and peripheral vascular disease [20–22], and the deterioration in renal function for stages 3–4 diabetic chronic kidney disease (CKD) patients [23]. However, little is known about the long-term association of BP visit-to-visit variability with PAD occurrence in diabetic patients with normal ABI at diagnosis.

The association between BP visit-to-visit variability and cardiovascular events generally considers BP measurements at a few time points and in a short to medium follow-up period, limiting the appreciation of the full impact of BP variability on PAD, especially for diabetic patients. Therefore, we evaluated the long-term relationship between BP visit-to-visit variability and the occurrence of PAD in patients from the beginning of their diagnosis with type 2 DM (T2DM).

## 2. Materials and Methods

**2.1. Patients and Study Design.** We retrospectively collected the following 10-year measurements obtained at every outpatient clinic visit of 825 patients who were first diagnosed with T2DM during 2000–2002 at Chang Gung Memorial Hospital, Keelung: blood pressure, body weight, body height, and laboratory data. T2DM was diagnosed in accordance with the criteria of the American Diabetes Association [24]. Body mass index (BMI) was defined as weight (kilograms) divided by height (meters) squared. Patients were classified as nonsmokers, former smokers, or current smokers according to the electronic medical record. PAD was diagnosed on the basis of an ABI  $\leq 0.9$  [25]. Patients who developed PAD or cardiovascular disease (CVD), including coronary artery disease, MI, ischemic stroke, or transient ischemic attack [26], before being diagnosed with diabetes were excluded. Patients who never had an ABI assessment during the 10-year follow-up period, those without PAD who did not have complete ABI data in the 10th year of follow-up, and patients with less than 10-year follow-up were also excluded. Dyslipidemia was defined as without treatment, total cholesterol levels  $>200$  mg/dL, low-density lipoprotein cholesterol levels  $>100$  mg/dL, high-density lipoprotein cholesterol levels  $<50$  mg/dL in females and  $<40$  mg/dL in males, or

triglycerides  $>150$  mg/dL. Hypertension was defined as SBP  $\geq 130$  mmHg or diastolic blood pressure (DBP)  $\geq 80$  mmHg in diabetic patient before any treatment was initiated [12].

This study was conducted in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board of Chang Gung Memorial Hospital; informed consent was waived.

**2.2. Laboratory Assessments.** BP measurements at every visit were recorded throughout the follow-up period till PAD was diagnosed. All outpatient clinics used automated sphygmomanometers operated by trained medical assistants after 10–15 minutes resting, with repeated measurements performed as needed by physicians using aneroid sphygmomanometers [27]. Fasting serum total cholesterol, low-density lipoprotein, high-density lipoprotein, and triglyceride concentrations were assessed using standard enzymatic methods. Hemoglobin A<sub>1c</sub> was assayed using high-performance liquid chromatography and expressed with the unit defined by the National Glycohemoglobin Standardization Program.

**2.3. Definition of BP Visit-To-Visit Variability.** BP visit-to-visit variability and the coefficient of variation (standard deviation (SD) of mean BP divided by mean BP) of SBP and DBP were determined [28]. The BP instability index was expressed as the delta BP, which was defined as a difference between the maximum BP and the minimum BP, through all visits till PAD was diagnosed [29].

**2.4. Statistical Analysis.** Means and frequencies of potential confounding variables were calculated. The relationships between variability in SBP and DBP, as well as other variables, and PAD were examined by Pearson's correlation analyses. To examine the effects of various factors on the occurrence of PAD, the following factors were considered simultaneously as independent variables for Cox multiple regression analysis: age at DM diagnosis, sex, BMI, average SBP and DBP, SD of SBP and DBP, maximum of SBP and DBP, delta SBP, delta DBP, hemoglobin A<sub>1c</sub>, total cholesterol, triglyceride, smoking status, presence of CVD, hypertension, and dyslipidemia. All continuous variables are presented as the mean  $\pm$  SD or absolute number. A *P* value  $< 0.05$  was considered statistically significant. The area under each receiver operating curve and 95% confidence intervals (CI) were estimated to compare the relative ability of the SD of SBP and DBP to identify risk of peripheral arterial disease in diabetic patients. The optimal cut-point BP was calculated based on the Youden Index [30], which was calculated as sensitivity + specificity – 1 [31]. Multicollinearity was assessed using variance inflation factor (VIF) among average, SD, maximum, and grouping of VVV of SBP and DBP [32, 33]. The power calculation was performed with pass software. Multicollinearity was diagnosed with a VIF, one of the most common tools used by statisticians, of 5 and above [32, 33].

## 3. Results

Nine hundred and thirty-six patients were first diagnosed with DM at Chang Gung Memorial Hospital from 2000 to

2002. Sixty-nine patients who died or were lost to follow-up were excluded. Twenty-three patients without final ABI data and 19 patients who had PAD or CVD before their DM diagnosis were also excluded. The characteristics of the 825 patients enrolled in this study are shown in Table 1. The overall mean age at diagnosis with DM was  $53.6 \pm 10.5$  years. At baseline, the ABI for all patients was in the range of 0.9–1.3. The median observation period was  $148.1 \pm 16.0$  months. At the end of the observation period, the right and left leg ABI levels for all patients were  $1.02 \pm 0.24$  and  $1.02 \pm 0.42$ , respectively. There were 114 patients diagnosed with PAD during the 10-year follow-up with an average time of PAD diagnosis of  $116.7 \pm 12.8$  months after their DM diagnosis. The average SBP and DBP for all patients was  $136.8 \pm 10.2$  and  $73.5 \pm 6.4$  mmHg, respectively. The SD of the SBP and DBP for all patients was  $14.8 \pm 3.8$  and  $7.5 \pm 2.1$  mmHg, respectively.

Multivariate Cox regression analyses revealed that the SD of SBP was positively correlated with the occurrence of PAD ( $P = 0.037$ ; Table 2). However, the maximum of SBP and delta of SBP were not significantly correlated. The mean, SD, maximum, and delta of DBP were not significantly correlated with the occurrence of PAD. In addition to the SD of SBP, the age at DM diagnosis was positively correlated with the occurrence of renal function impairment ( $P < 0.001$ , HR = 1.064, 95% CI = 1.043–1.084). In addition, the occurrence of PAD was associated with mean creatinine level ( $P = 0.012$ ) and current smoking status ( $P = 0.009$ ). However, mean or SD of hemoglobin A<sub>1c</sub>, BMI, total cholesterol, low-density lipoprotein, high-density lipoprotein, and triglyceride were not independently correlated with the occurrence of PAD (Table 2).

We next categorized the patients into high- or low-risk groups on the basis of their SD of SBP or DBP. Cut-off points for the SD of SBP and DBP, where the sensitivity approximates specificity for the occurrence of PAD, are 16.3 and 7.6 mmHg, respectively. Patients with SD of SBP and DBP higher than the cut-off values ( $n = 199$ ) were placed in the high-risk group, and all the other patients ( $n = 626$ ) were in the low-risk group. The multicollinearity was assessed between VVV grouping and mean, SD, maximum, and delta of SBP and DBP. The VIFs of all these factors were less than 2 (Table 2), which represented the idea that the grouping according to the VVV was independently different factor among these parameters. The characteristics of both groups were shown in Table 3. The age at DM diagnosis, hypertension history, SD of BMI, average SBP and DBP, SD of SBP and DBP, delta SBP and DBP, mean and SD of hemoglobin A<sub>1c</sub>, mean total cholesterol, and mean and SD of creatinine level were significantly different between the low- and high-risk groups ( $P \leq 0.034$ ).

In the 10 years following their DM diagnosis, 50 patients (25.1%) in the high-risk group had PAD versus 64 patients (10.2%) in the low-risk group ( $P < 0.001$ ; Table 3). The PAD-free survival curve between patients in high- and low-risk groups was shown in Figure 1. In addition, the occurrence of CVD in the high-risk group was significantly higher than that of the low-risk group (25.1% versus 11.7%;  $P < 0.001$ ).

Cox multivariate regression analysis revealed that the risk of PAD increased by 1.064-fold as the age at diagnosis

TABLE 1: Characteristics of the study participants ( $n = 825$ ).

Characteristic	
Age at DM diagnosis (y)	$53.6 \pm 10.5$
Sex (male, %)	390 (47.3)
Smoking status (none/former/current)	628/49/148
Hypertension (%)	629 (76.2)
Hyperlipidemia (%)	793 (96.1)
Body mass index (kg/m <sup>2</sup> )	$26.8 \pm 3.9$
Number of measurements	$45.0 \pm 24.8$
Average SBP (mmHg)	$136.8 \pm 10.2$
Number of measurements	$63.4 \pm 29.4$
SD of SBP (mmHg)	$14.8 \pm 3.8$
Maximum of SBP (mmHg)	$174.5 \pm 17.5$
Delta SBP (mmHg)	$72.5 \pm 25.6$
Average DBP (mmHg)	$73.5 \pm 6.4$
SD of DBP (mmHg)	$7.5 \pm 2.1$
Maximum of DBP (mmHg)	$93.8 \pm 11.3$
Delta DBP (mmHg)	$38.2 \pm 14.6$
Hemoglobin A <sub>1c</sub> (%)	$7.6 \pm 1.0$
Number of measurements	$34.6 \pm 10.9$
Total cholesterol (mg/dL)	$193.5 \pm 28.6$
Number of measurements	$11.9 \pm 6.1$
High-density lipoprotein (mg/dL)	$38.5 \pm 10.6$
Low-density lipoprotein (mg/dL)	$118.5 \pm 20.0$
Triglyceride (mg/dL)	$150.1 \pm 112.1$
Initial creatinine (mg/dL)	$0.76 \pm 0.54$
Average creatinine (mg/dL)	$0.99 \pm 0.71$
Number of measurements	$17.9 \pm 6.7$
Clinical events during the 10-year follow-up	
PAD (%)	114 (13.8)
CVD <sup>a</sup> (%)	123 (14.9)
CAD or MI (%)	38 (4.6)
TIA or stroke (%)	88 (10.7)
Total follow-up period (months)	$148.1 \pm 16.0$

DM, diabetes mellitus; SBP, systolic blood pressure; DBP, diastolic blood pressure; SD, standard deviation; PAD, peripheral arterial disease; CVD, cerebrovascular disease; CAD, coronary artery disease; MI, myocardial infarction; TIA, transient ischemic attack; CKD, chronic kidney disease.

<sup>a</sup>Defined as cerebrovascular disease, neurodegenerative disease, and Parkinson's disease that required medical treatment and long-term follow-up.

increased by 1 year ( $P < 0.001$ , 95% CI 1.043–1.084; Table 4). High BP visit-to-visit variability also increased likelihood of PAD within 10 years of being diagnosed with DM by 1.679-fold ( $P = 0.009$ ). Current smoking status and elevation of mean creatinine level was also associated with increased risk of PAD (Table 4).

#### 4. Discussion

This study showed that patients with high visit-to-visit variability in both SBP and DBP were more frequently diagnosed with PAD in the first decade following diagnosis with DM. However, the mean and delta SBP/DBP, mean serum lipid profile, mean and SD of hemoglobin A<sub>1c</sub> concentration, and

TABLE 2: Multivariate Cox regression analyses of the factors associated with peripheral arterial disease ( $n = 825$ ) in the 10 years following a diagnosis of DM.

Independent variable	Hazard ratio	95% CI	VIF	P value
Sex (female = 0)	0.893	0.558–1.429		0.636
Age at DM diagnosis	1.069	1.043–1.095		< <b>0.001</b>
Nonsmoking				<b>0.026</b>
Former smoker	1.627	0.765–3.460		0.206
Current smoker	2.070	1.212–3.536		<b>0.008</b>
Hypertension	1.426	0.733–2.774		0.296
Dyslipidemia	4.124	0.543–31.319		0.171
Mean SBP	1.008	0.974–1.044	1.420	0.639
SD of SBP	1.088	0.970–1.220	1.816	<b>0.150</b>
Maximum of SBP	0.984	0.960–1.008	1.267	0.182
Delta of SBP	1.001	0.986–1.015	1.806	0.932
Mean DBP	0.996	0.936–1.060	1.460	0.899
SD of DBP	0.940	0.757–1.168	1.859	0.578
Maximum of DBP	1.015	0.982–1.049	1.267	0.364
Delta of DBP	0.997	0.971–1.023	1.779	0.809
Mean BMI	0.972	0.917–1.030		0.338
SD of BMI	1.251	0.868–1.802		0.230
Mean hemoglobin A <sub>1c</sub>	0.979	0.764–1.254		0.864
SD of hemoglobin A <sub>1c</sub>	0.979	0.565–1.695		0.940
Mean serum cholesterol	1.011	0.998–1.023		0.088
SD of serum cholesterol	0.994	0.983–1.005		0.258
Mean serum LDL	1.004	0.990–1.018		0.565
SD of serum LDL	0.988	0.976–1.001		0.079
Mean serum HDL	0.993	0.968–1.018		0.575
SD of serum HDL	1.022	0.980–1.067		0.309
Mean serum triglyceride	1.001	0.996–1.005		0.815
SD of serum triglyceride	1000	0.995–1.004		0.892
Mean creatinine	1.305	1.024–1.665		<b>0.032</b>
SD of creatinine	0.833	0.474–1.461		0.523

DM, diabetes mellitus; SBP, systolic blood pressure; DBP, diastolic blood pressure; SD, standard deviation; CV, coefficient of variation; BMI, body mass index; LDL, low density lipoprotein; HDL, high density lipoprotein; VIF, variance inflation factor.

BMI were not correlated with the occurrence of PAD. Our results also confirmed that old age at DM diagnosis, smoking, and renal function impairment would increase the risk of PAD in the first decade after DM was diagnosed. Cessation of smoking in patients with PAD could substantially reduce the risk of death, myocardial infarction, and amputation and increase the patency rate of lower-extremity angioplasty and surgical revascularization [25]. Two recent studies revealed that central obesity with elevated BMI was positively associated with ABI increase [34, 35]. However, the relationship between BMI and PAD could not be identified from our study.

Assessment of the effects of short-term variability of BP has traditionally dominated this field of research [36] and diminished the interest in long-term variability of BP, such as those occurring between days or months. However, recent studies have shown that long-term visit-to-visit variability of BP may have greater prognostic value than mean BP or short-term variability [19, 37, 38]. These studies recommended that

optimal antihypertension treatment included avoidance of inconsistent BP control and large BP visit-to-visit variability [36].

The effect of high BP variability on PAD occurrence has rarely been studied. Most prospective BP variability studies focused on the cardiovascular mortality and morbidities, including MI, stroke, and heart failure [17, 39–41]. These studies followed patients for a relatively short period of 1.5 to 7.8 y, and less than 10% of the study patients had DM [39–41]. DM is one of the strongest risk factors for critical limb ischemia and amputation, as well as incident PAD in population studies [42]. However, few studies have completely focused on the detrimental cardiovascular effect of BP variability in diabetic patients. Mancina et al. [43] revealed that DM did predict ABI decline over an average of 4.6 y of follow-up. Our study proved that high variability of BP is a risk factor for the occurrence of PAD in the 10 years following DM diagnosis. These diabetic patients with PAD are at high risk for adverse cardiovascular events unless

TABLE 3: Demographics and clinical characteristics of the low- and high-risk groups as determined by BP visit-to-visit variability.

	Low-risk group (n = 626)	High-risk group (n = 199)	P value
Age at DM diagnosis (y)	52.5 ± 10.3	57.2 ± 10.6	<0.001
Sex (male, %)	296 (47.3)	94 (47.2)	0.528
Smoking (none/former/current)	480/36/110	148/13/38	0.808
Hypertension (%)	449 (71.7)	180 (90.5)	<0.001
Hyperlipidemia (%)	600 (95.8)	193 (97.0)	0.313
Body mass index (kg/m <sup>2</sup> )	26.8 ± 3.9	27.0 ± 3.9	0.521
Number of measurements	44.8 ± 24.4	45.4 ± 26.0	0.772
SD of BMI (kg/m <sup>2</sup> )	1.0 ± 0.5	1.2 ± 0.6	<0.001
Average SBP (mmHg)	135.3 ± 9.6	141.3 ± 10.7	<0.001
Number of measurements	61.8 ± 27.6	68.6 ± 34.1	0.004
SD of SBP (mmHg)	13.3 ± 2.6	19.5 ± 3.3	<0.001
Maximum of SBP (mmHg)	170.1 ± 15.9	188.4 ± 15.1	<0.001
Delta SBP (mmHg)	65.1 ± 20.0	96.0 ± 27.1	<0.001
Average DBP (mmHg)	73.1 ± 6.0	74.7 ± 7.1	0.002
SD of DBP (mmHg)	6.8 ± 1.5	9.7 ± 2.3	<0.001
Maximum of DBP (mmHg)	91.3 ± 9.4	101.9 ± 12.9	<0.001
Delta DBP (mmHg)	34.4 ± 11.6	50.0 ± 16.7	<0.001
Hemoglobin A <sub>1c</sub> (%)	7.5 ± 1.0	7.7 ± 1.1	0.019
Number of measurements	35.4 ± 10.7	32.0 ± 11.3	<0.001
SD of Hemoglobin A <sub>1c</sub> (%)	0.9 ± 0.6	1.1 ± 0.5	<0.001
Total cholesterol (mg/dL)	192.3 ± 26.7	197.2 ± 33.7	0.034
Number of measurements	12.0 ± 5.8	11.3 ± 6.9	0.147
High-density lipoprotein (mg/dL)	38.5 ± 10.7	38.4 ± 10.5	0.877
Low-density lipoprotein (mg/dL)	118.6 ± 19.7	118.4 ± 21.0	0.877
Triglyceride (mg/dL)	147.0 ± 106.8	159.8 ± 127.0	0.1640
Average creatinine	0.89 ± 0.4	1.3 ± 1.2	<0.001
Number of measurements	13.6 ± 5.1	15.8 ± 9.5	<0.001
SD of creatinine	0.1 ± 0.3	0.3 ± 0.4	<0.001
Clinical events during the 10-year follow-up period			
PAD (%)	64 (10.2)	50 (25.1)	<0.001
Interval from DM diagnosis (y)	8.1 ± 2.0	7.7 ± 2.5	0.403
CVD <sup>a</sup> (%)	73 (11.7)	50 (25.1)	<0.001
Interval from DM diagnosis (y)	5.4 ± 2.8	4.9 ± 3.5	0.388
CAD or MI (%)	24 (3.8)	14 (7.0)	0.051
Interval from DM diagnosis (y)	5.4 ± 2.7	3.7 ± 3.3	0.094
TIA or stroke (%)	51 (8.1)	37 (18.6)	<0.001
Interval from DM diagnosis (y)	5.3 ± 2.9	5.4 ± 3.4	0.903
Recurrent TIA or stroke events	0.2 ± 0.5	0.4 ± 0.8	0.152
Total follow-up (months)	148.6 ± 15.2	146.4 ± 18.4	0.098

DM, diabetes mellitus; SBP, systolic blood pressure; DBP, diastolic blood pressure; SD, standard deviation; PAD, peripheral arterial disease; CVD, cerebrovascular disease; CAD, coronary artery disease; MI, myocardial infarction; TIA, transient ischemic attack; CKD, chronic kidney disease.

<sup>a</sup>Defined as cerebrovascular disease, neurodegenerative disease, and parkinsonism that required medical treatment and long-term follow-up.

the PAD is recognized. From the results of the present study, diabetic patients with high BP visit-to-visit variability had a 1.679-fold increased risk of the occurrence of PAD. For the first time, our results demonstrated that a visit-to-visit variability VVV of SBP > 16.3 mmHg with a visit-to-visit variability of DBP > 7.6 mmHg significantly increased the risk of PAD in the first decade following a diagnosis with DM. The adverse consequences of high BP variability on

the cardiovascular system might result from the traumatic effect of large blood pressure oscillations enhancing the intravascular pressures on the vessel wall, promoting tissue growth and atherosclerosis [44].

Office BP as the measurement of variability of BP may be limited due to white coat hypertension although this issue is controversial. A meta-analysis that included 7961 untreated participants reported that the cardiovascular risk

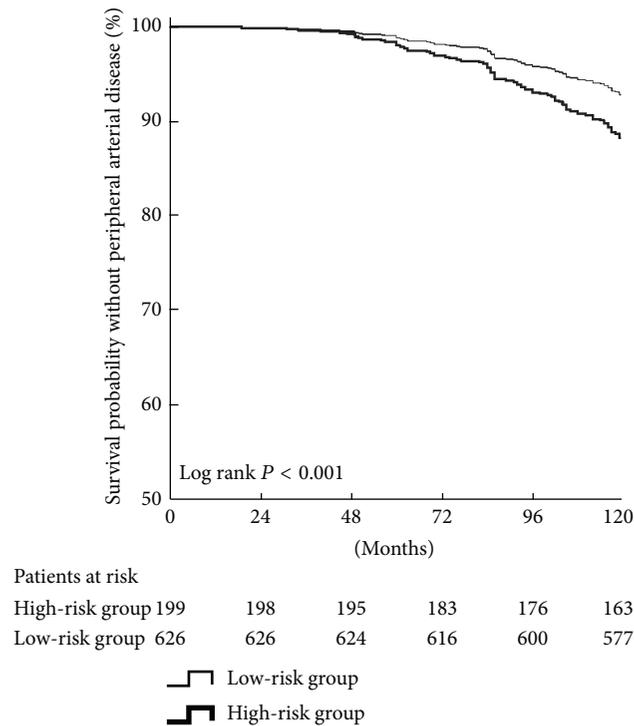


FIGURE 1: Kaplan-Meier plot of peripheral arterial disease occurrence over 10 years following a diagnosis of type 2 diabetes. Patients were grouped into high- (high BP visit-to-visit variability) and low-risk groups.

TABLE 4: Multivariate Cox regression analysis of factors associated with the occurrence of peripheral arterial disease.

	Hazard ratio	95% CI	$P$ value
Age (+1 y)	1.064	1.043–1.084	<0.001
Nonsmoking	1		
Former smoking	1.645	0.771–2.783	0.244
Current smoking	1.803	1.160–2.804	0.009
High SBP and DBP visit-to-visit variability	1.679	1.141–2.472	0.009
Mean Creatinine (mg/dL)	1.208	1.042–1.401	0.012

DM, diabetes mellitus; SBP, systolic blood pressure; DBP, diastolic blood pressure; CI, confidence interval.

was not significantly different between white coat hypertension and normotension [45]. Another study from the International Database of HOme blood pressure in relation to Cardiovascular Outcome, which followed 6458 patients for a median of 8.3 y, reported that the cardiovascular risk for the white coat hypertension did not differ from that of the treated participants [46]. However, another large meta-analysis of 29,100 patients comparing white coat hypertension participants with sustained hypertension participants found that CVD mortality (4.2%) was significantly lower in white coat hypertension participants (6.6%; OR = 0.47, 95% CI 0.35–0.64;  $P < 0.001$ ) than those with sustained hypertension participants [46]. Thus, when office BP is used as the measurement of BP variability, white coat hypertension is not a factor that induces bias.

The retrospective nature of the present study and its sample size are two of the limitations of the present study. The possibility of type 2 error exists. Another limitation is the medication record through the 10-year follow-up period. Antihypertensive drug use in each patient was not consistent throughout the 10-year follow-up period. Thus, it is very difficult to clarify the effect of BP visit-to-visit variability amelioration by each category of antihypertensive drug, such as calcium channel blockers, which had a controversial effect on blunting the amplitude of BP visit-to-visit variability [23]. The other limitation of the present study is the absence of antihypertensive prescription fill data and information regarding patients' adherence to treatment regimens. However, low antihypertensive medication adherence explained only a small proportion of BP visit-to-visit variability [38], which implied that the absence of medication adherence data does not have a major impact on the results of the present study.

Despite the aforementioned limitations, this study has several strengths, including the long follow-up period to assess the occurrence of PAD and the available information on demographic, clinical, and long-term BP data. In addition, the use of an electronic medical record database provided real-world evidence on the status of hypertension control in the first decade following a diabetes diagnosis and minimizes selection bias related to self-selection into the study. Prospective studies are in need in verifying the high variability of SBP visit-to-visit as early biomarker for detection of PAD in the first decade following the diagnosis of T2DM. More research is needed to fully understand the association between BP

visit-to-visit variability and risk of vascular events in diabetic patients, and large-scale pooled analyses of multiple cohorts will be required [19].

## 5. Conclusion

The present study showed that, in diabetic patients with initially normal ABI values, high BP visit-to-visit variability was a significant early biomarker for detection of PAD in the first decade following the diagnosis of T2DM.

## Competing Interests

There are no competing interests to declare.

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## Research Article

# Elevated Levels of Plasma Superoxide Dismutases 1 and 2 in Patients with Coronary Artery Disease

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**Aims.** To measure plasma levels of superoxide dismutases 1, 2, and 3 (SOD1, 2, 3) and determine whether SODs can function as biomarkers for coronary artery disease (CAD). **Patients & Methods.** Patient groups were as follows: patients with stable angina pectoris (SAP,  $n = 33$ ), patients with acute coronary syndrome (ACS,  $n = 49$ ), and controls ( $n = 42$ ). Protein quantification was done using ELISA. **Results.** The concentrations of plasma SOD1 and SOD2 were higher in CAD than in healthy controls. No difference in SOD3 levels between CAD and control groups was found. Limited correlations were found between SODs and gender, age, and severity of coronary artery stenosis. **Conclusions.** Plasma levels of SOD1 and SOD2 were elevated in patients with CAD and might serve as surrogate biomarkers for CAD.

## 1. Introduction

Cardiovascular diseases are the most common cause of death worldwide [1, 2]. In 2010, coronary artery disease (CAD) accounted for one in six deaths in the United States, accounting for 379,559 deaths. It is estimated that 620,000 Americans will suffer a first coronary attack each year and another 295,000 will have a repeat attack [1]. In China, approximately 290 million people had cardiovascular disease in 2010 [3]. Cardiovascular disease in China accounts for 3.5 million deaths each year, accounting for 41% of the total number of deaths per year, and this number is continuously increasing [3]. Currently, 94.9 per 100,000 deaths are estimated to be associated with coronary heart disease in urban areas and 71.3 per 100,000 in rural areas. The annual mortality due to CAD increased from 95.3 in 100,000 in 1999 to 103.4 in 100,000 in 2008 [4]. The known general risk factors for CAD are smoking, hypertension, diabetes mellitus, obesity, metabolic syndrome, physical inactivity, and hyperlipidemia [5]. However, these conventional risk factors reflect only 80–90% of CAD cases. Despite significant improvements in the control of these traditional risk factors in the Chinese

population, the incidence of CAD remains high, resulting in serious problems such as substantial medical expense, disability, and even death. Thus, the need for new indicators to assess cardiovascular risk is urgent.

Free radical reactions play an important role in the onset and development of many human diseases [6], including diabetes [7], ischemic injury, stroke, and heart disease [8–11]. Previous research suggests that oxidative stress may contribute to the development of CAD [12, 13]. Oxygen free radicals promote low-density lipoprotein peroxidation, increase the number of foam cells, cause vascular endothelial cell injury, and induce expression of proinflammatory cytokines in endothelia. As a result, the blood vessels thicken, ultimately resulting in stenosis of the arteries [14]. Cells possess efficient antioxidant defense systems to protect them from oxidative damage. These are mainly composed of enzymes such as superoxide dismutases (SODs) and glutathione peroxidase (GPX), which scavenge the reactive oxygen species (ROS) produced by cellular metabolism. The process of coronary atherosclerosis is affected by these important enzymes [15]. SOD, which belongs to an enzyme family that is widely found in human cells and body fluids, protects cells against

potential cytotoxicity by catalytically scavenging harmful superoxide radicals ( $O_2^-$ ). SOD achieves this by catalyzing the dismutation of highly reactive  $O_2^-$  to  $O_2$  and  $H_2O_2$ , a less reactive ROS [16]. Early research showed that SOD is an important marker of lipid peroxidation and of the progression of atherosclerosis correlated with oxidative stress [17]. There are three types of SODs. SOD1, the major intracellular SOD, is mainly localized to the cytoplasm but is also found in nuclei, lysosomes, and peroxisomes. SOD1 requires copper/zinc metal ions as cofactors and is present in many kinds of cells [18]. SOD2 is associated with magnesium and is localized to the mitochondrial matrix. SOD3 is a secretory form found in the vascular extracellular space and is also associated with copper/zinc. It is highly expressed in certain tissues, such as heart, lung, and blood vessels [19].

GPX-1 and SOD have both been described as protective factors in preventing cardiovascular events in patients with CAD [12]. However, some studies have placed in question the effect of SOD activity in CAD [13, 20, 21]. Gupta et al. reported that SOD activity decreased with the development of CAD [21]. Pytel et al. reported that SOD activity in patients with CAD decreased by 17% compared with healthy controls [15]. Zengin et al. reported that elevated SOD1 activity was associated with poor prognosis in patients with CAD [13]. However, to date, few reports have measured plasma concentrations of SODs in patients with CAD or attempted to correlate SOD levels with different types of CAD. In this study, we investigated the plasma levels of SODs in patients with stable angina pectoris (SAP), patients with acute coronary syndrome (ACS), and normal control subjects. We aimed to determine whether the plasma levels of SODs can be used as biomarkers for CAD.

## 2. Methods

**2.1. Subjects.** Participants with CAD who underwent coronary angiography at the Department of Cardiology, Dongyang Peoples' Hospital, between November 2014 and November 2015 and had at least one stenosis of  $\geq 50\%$  in a major coronary artery were enrolled in the study. These participants were divided into a SAP group (19 males and 14 females) and an ACS group (27 males and 22 females) according to clinical manifestation. Age-matched healthy controls (23 males and 19 females) were also recruited. The age of the subjects ranged from 44 to 82 years for controls (mean  $\pm$  SD =  $66.6 \pm 7.3$  years), 44 to 81 years for the SAP group ( $66.3 \pm 8.7$  years), and 38 to 85 years for the ACS group ( $67.6 \pm 11.6$  years). All participants were Han Chinese. Informed consent was obtained from each participant, and all procedures were approved by the Institutional Review Board of Affiliated Dongyang Hospital of Wenzhou Medical University. Patients with severe infection, surgery, trauma within the previous month, known cancer, or severe liver or renal insufficiency were excluded.

**2.2. Coronary Angiography.** Coronary angiography was performed in all patients using transradial approaches with left and right Judkins catheters. The major coronary vessels included the left main artery (LM), left circumflex artery

(LCX), left anterior descending artery (LAD), and right coronary artery (RCA). Two experienced observers quantified the severity of coronary atherosclerosis for each patient using the Gensini score, an assessment for predicting the likelihood of death or cardiovascular events [22]. The severity score is based on the degree of stenosis:  $\leq 25\%$  (score = 1), 26–49% (score = 2), 50–75% (score = 4), 76–90% (score = 8), 91–99% (score = 16), and 100% (score = 32). In addition, each score was adjusted using artery-specific multipliers described by Gensini [22]: LM =  $\times 5$ ; proximal segment of LAD and proximal segment of LCX =  $\times 2.5$ ; middle segment of LAD =  $\times 1.5$ ; distal segment of LAD, the RCA, the posterolateral artery, and the obtuse marginal artery =  $\times 1$ ; and all other areas =  $\times 0.5$ .

**2.3. Measurement of Plasma SOD Levels.** Venous blood (10 mL) was collected from patients with SAP and ACS the morning after coronary angiography; samples were also collected from controls at the time of enrollment. All participants were free of acute infection and stress at the time of collection. Serum collected using lithium heparin tubes was harvested by centrifugation at  $3000 \times g$  for 10 min, divided into aliquots, and frozen at  $-80^\circ C$  until use. Plasma concentrations of SOD1, SOD2, and SOD3 were determined using commercially available ELISA kits (human SOD1, Cloud-Clone, Houston, TX, USA; human SOD2, Abnova, Taipei, Taiwan; and human SOD3, MDBio, Qingdao, China).

**2.4. Statistical Analysis.** All data were analyzed using Prism v5.0 (GraphPad Software, La Jolla, CA, USA). Continuous variables were expressed as the mean  $\pm$  SD (standard deviation). Plasma SOD levels among the SAP, ACS, and control groups were compared using one-way analysis of variance. The correlation between ages, severity of coronary atherosclerosis, and SOD concentration was assessed using Pearson correlation analysis. Diagnostic accuracies of SOD concentrations were calculated using a receiver operating characteristic (ROC) curve, and diagnosis performance was measured as the area under the ROC curve.  $p$  values  $< 0.05$  were considered statistically significant.

## 3. Results

**3.1. Baseline Characteristics.** Clinical characteristics of CAD patients and controls are shown in Table 1. The groups were matched for gender, age, body mass index, smoking habit, hypertension, and diabetes. Compared with the control group, the SAP and ACS groups had significantly higher mean concentrations of total cholesterol (TC, by 10.9% and 20.8%, resp.) and lower HDL cholesterol (HDL-C, by 24.8% and 27.2%, resp.). In addition, ACS patients had higher LDL cholesterol (LDL-C, 28.3% increase) and higher Hs-crp (134.7% increase).

**3.2. Comparison of SOD Levels.** In the control group, the mean concentrations of plasma SOD1 and SOD2 were  $0.73 \pm 0.22$  mg/mL and  $64.9 \pm 18.5$  ng/mL, while the medians were 0.71 mg/mL and 65.2 ng/mL, respectively (Figure 1). Compared with control, SOD1 and SOD2 concentrations in the

TABLE 1: Characteristics of coronary artery disease (CAD) patients and controls<sup>a</sup>.

Characteristic <sup>b</sup>	Control ( <i>n</i> = 42)	SAP ( <i>n</i> = 33)	ACS ( <i>n</i> = 49)	<i>p</i> values <sup>c</sup>
Age (years)	66.6 ± 7.3	66.3 ± 8.7	67.6 ± 11.6	0.852; 0.627; 0.569
Male, <i>n</i> (%)	23 (54.8)	19 (57.6)	27 (55.1)	0.811; 0.974; 0.827
Diabetes, <i>n</i> (%)	7 (16.7)	6 (18.2)	8 (16.3)	0.866; 0.966; 0.829
Hypertension, <i>n</i> (%)	24 (57.1)	18 (54.5)	30 (61.2)	0.825; 0.697; 0.825
BMI (kg/m <sup>2</sup> )	25.4 ± 2.4	23.7 ± 2.4	23.2 ± 3.6	0.129; 0.159; 0.482
Smoking habit, <i>n</i> (%)	23 (54.8)	16 (48.5)	25 (51.0)	0.595; 0.725; 0.482
Hs-crp (mmol/L)	1.21 ± 0.49	1.72 ± 0.96	2.84 ± 2.15	0.104; <b>0.016</b> ; <b>0.011</b>
TC (mmol/L)	4.13 ± 0.89	4.58 ± 1.04	4.99 ± 1.05	<b>0.040</b> ; <b>0.001</b> ; 0.117
TG (mmol/L)	1.77 ± 0.80	2.11 ± 1.42	1.63 ± 1.01	0.228; 0.495; 0.079
LDL-C (mmol/L)	2.30 ± 0.82	2.52 ± 0.92	2.95 ± 1.03	0.261; <b>0.002</b> ; 0.075
HDL-C (mmol/L)	1.31 ± 0.27	1.05 ± 0.22	1.03 ± 0.23	< <b>0.001</b> ; < <b>0.001</b> ; 0.70

<sup>a</sup>BMI: body mass index; HDL-C: high-density lipoprotein cholesterol; Hs-crp: high-sensitivity C-reactive protein; LDL-C: low-density lipoprotein cholesterol; TC: total cholesterol; and TG: triglycerides.

<sup>b</sup>Values for age, BMI, Hs-crp, and lipid analysis represent the mean ± SD.

<sup>c</sup>Order of *p* values: SAP versus ctrl; ACS versus ctrl; and SAP versus ACS. Bold indicates significant differences (*p* < 0.05).

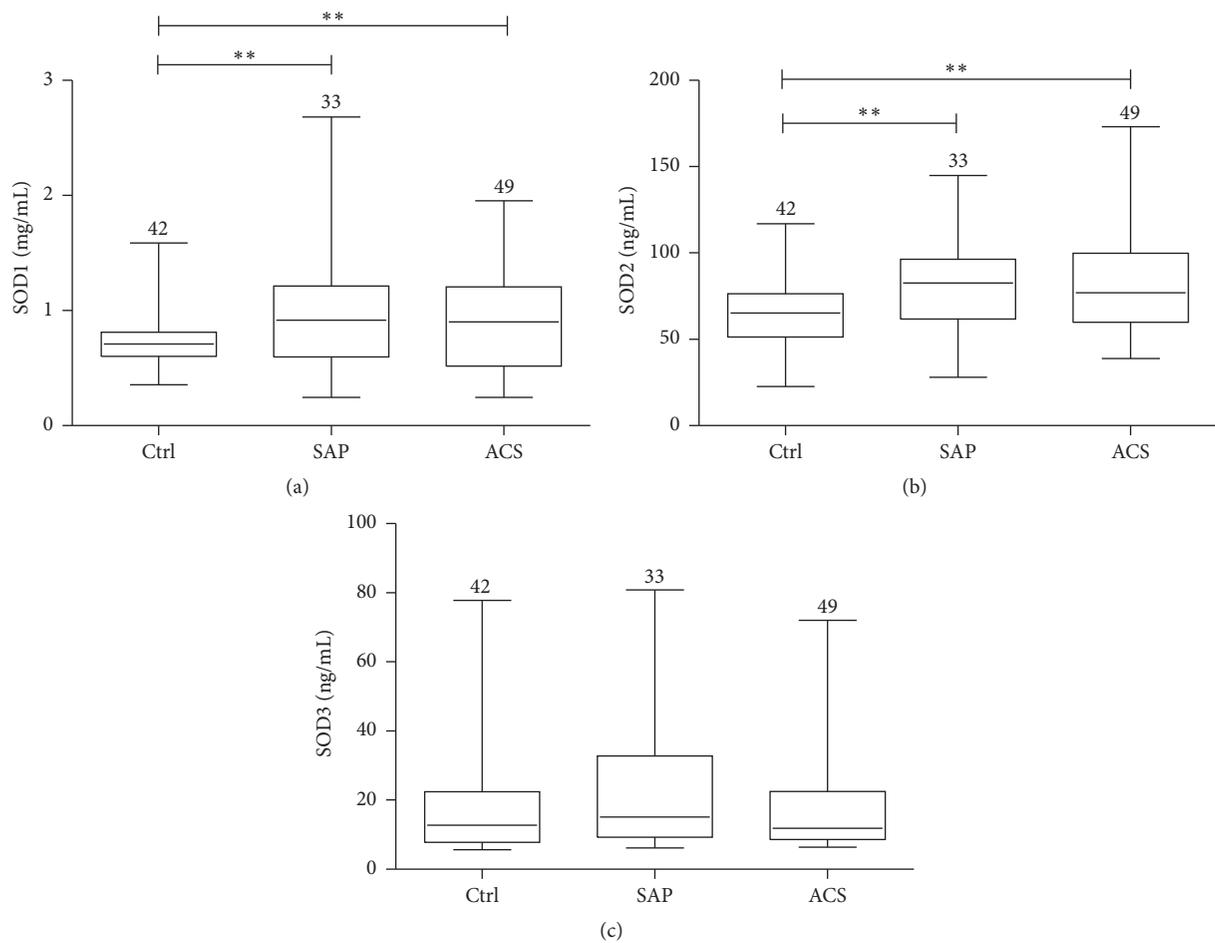


FIGURE 1: Concentrations of plasma SOD1, SOD2 and, SOD3 in healthy controls (Ctrl), patients with SAP, and patients with ACS. Values for *n* are shown above each error bar. The middle lines mean median values. \*\* *p* < 0.01.

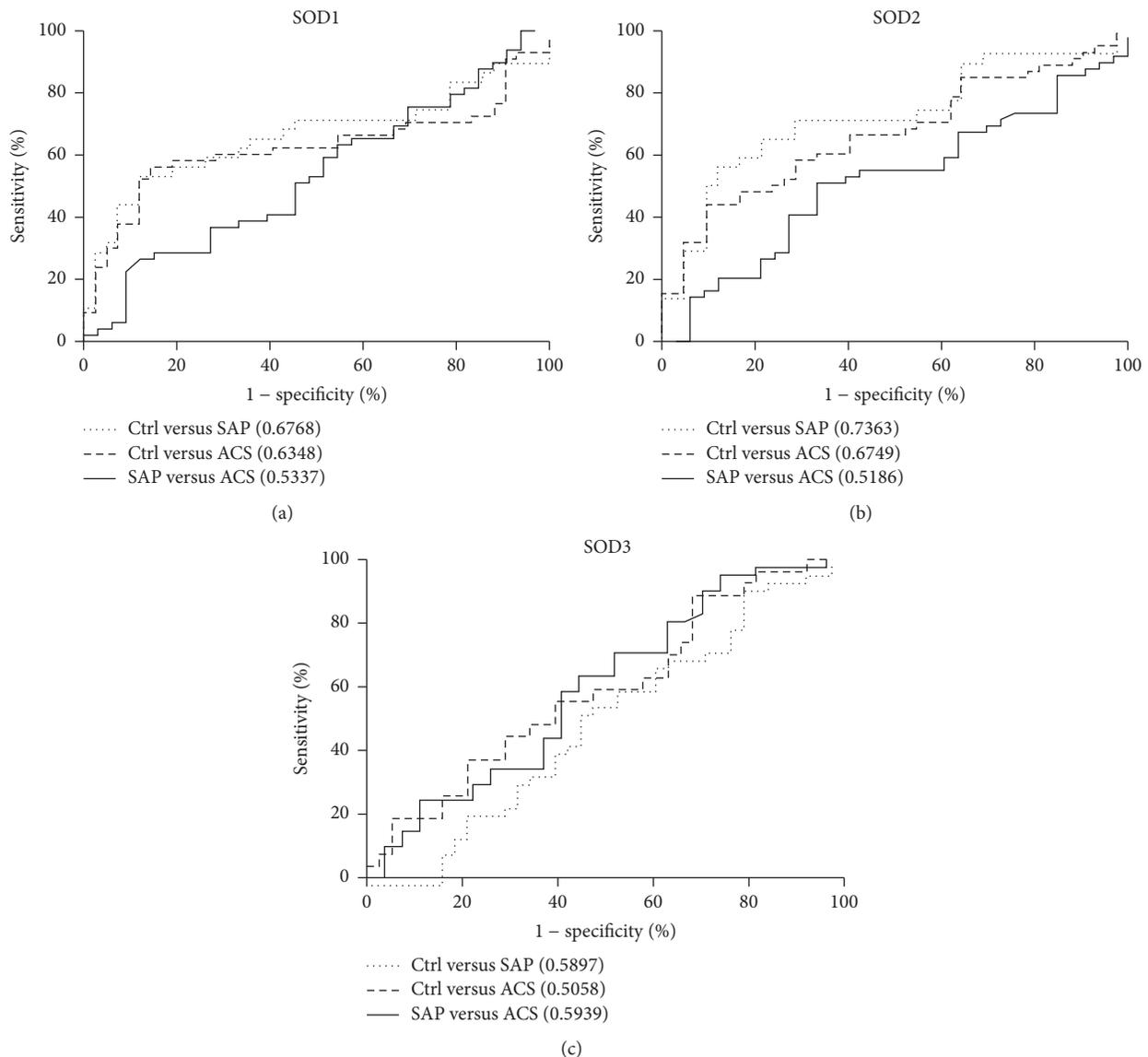


FIGURE 2: ROC curves of SODs for the diagnosis of CAD. The area under the curve is shown in parentheses for each curve.

SAP group were elevated by 37.6% ( $1.0 \pm 0.53$  mg/mL, median = 0.92 mg/mL, and  $p = 0.003$ ) and 28.3% ( $83.2 \pm 27.2$  ng/mL, median = 82.5 ng/mL, and  $p = 0.001$ ), respectively. Similarly, relative to control, SOD1 and SOD2 concentrations were elevated in the ACS group by 28.1% ( $0.93 \pm 0.45$  mg/mL, median = 0.90 mg/mL, and  $p = 0.008$ ) and 30.8% ( $85.0 \pm 34.6$  ng/mL, median = 76.9 ng/mL, and  $p = 0.001$ ). The concentrations of these two proteins were not significantly different between the SAP and ACS groups ( $p = 0.53$  for SOD1 and  $p = 0.81$  for SOD2). For SOD3, there were no significant differences among the SAP, ACS, and control groups ( $22.6 \pm 18.3$ ,  $16.1 \pm 11.9$ , and  $18.3 \pm 15.5$  ng/mL, while the medians were 15.1, 11.9, and 12.8 ng/mL, resp.;  $p > 0.05$ ). These results suggest that plasma concentrations of SOD1 and SOD2 but not SOD3 maybe useful as biomarkers for diagnosis of CAD.

**3.3. Performance of SODs in the Prediction of CAD.** We distinguished between patients with SAP or ACS and controls

by measuring the area under receiver operating characteristic (ROC) curves. At a cut-off value of 0.8962 mg/mL, SOD1 exhibited the best performance with 54.55% sensitivity and 88.10% specificity and an area under the curve (AUC) of 0.6768 for SAP versus control (Figure 2(a)). In the comparison between ACS and control, SOD1 showed 57.14% sensitivity and 85.71% specificity with an AUC of 0.6348 and a cut-off value of 0.8725 mg/mL. SOD1 showed no difference between the SAP and ACS groups. In the comparison between SAP and control, SOD2 showed 57.58% sensitivity and 88.10% specificity with an AUC of 0.7363 at a cut-off value of 80.49 ng/mL (Figure 2(b)). In the comparison between ACS and control, SOD2 showed 44.90% sensitivity and 90.48% specificity with an AUC of 0.6749 at a cut-off value of 82.80 ng/mL. There was no difference between these two patient groups. SOD3 levels showed no significant differences among the three subject groups (Figure 2(c)). Based on this analysis, the plasma level of SOD2 performed best as a biomarker for the diagnosis of CAD.

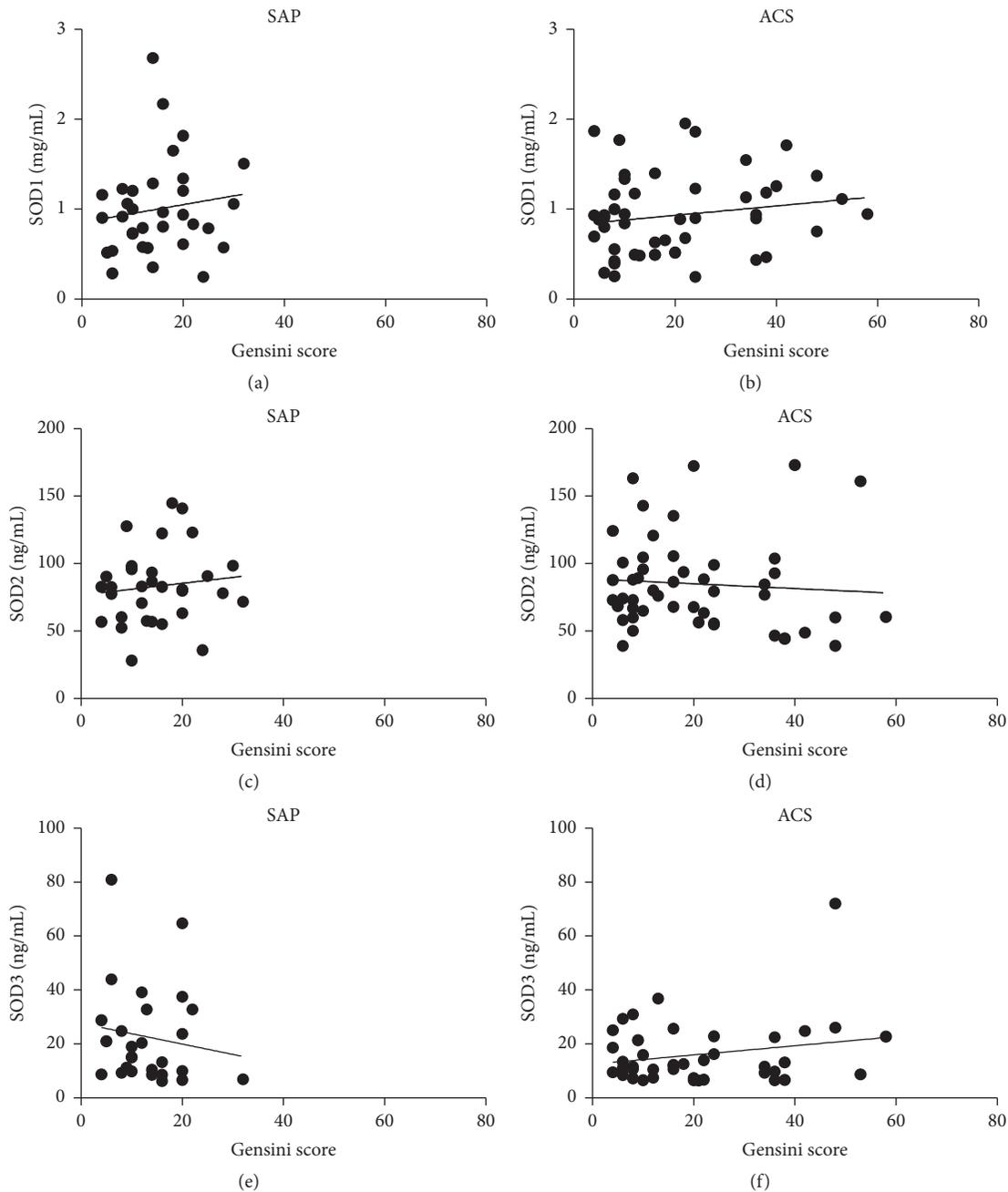


FIGURE 3: Correlations between Gensini scores and SOD levels in patients with SAP or ACS.

**3.4. Correlation between Severity of Coronary Artery Stenosis and SOD Levels.** The severity of coronary artery stenosis for each patient with CAD was determined using the Gensini score [22], and the correlation between these scores and plasma SOD levels was analyzed (Figure 3). There was no significant correlation between concentrations of plasma SODs and severity scores in patients with SAP (SOD1:  $r = 0.14$  and  $p = 0.44$ ; SOD2:  $r = 0.12$  and  $p = 0.50$ ; and SOD3:  $r = 0.04$  and  $p = 0.84$ ) or ACS (SOD1:  $r = 0.17$  and  $p = 0.24$ ; SOD2:  $r = 0.07$  and  $p = 0.61$ ; and SOD3:  $r = 0.14$  and  $p = 0.38$ ).

**3.5. Correlation between SODs and Gender or Age.** The concentrations of SOD1 and SOD2 were observed to be significantly higher in male patients with CAD than in male controls ( $p < 0.05$ ; Figures 4(a) and 4(b)). The level of SOD2 in female patients with SAP was significantly higher compared with female controls ( $p < 0.05$ ), but the average levels of SOD1 were not different in females across the three groups. The concentration of SOD3 was not significantly different among the three groups for either gender (Figure 4(c)). In addition, levels of SODs were not significantly correlated with age (Figure 5) among patients with SAP (SOD1:  $r = 0.06$  and  $p = 0.73$ ; SOD2:  $r = 0.16$  and  $p = 0.38$ ; and SOD3:

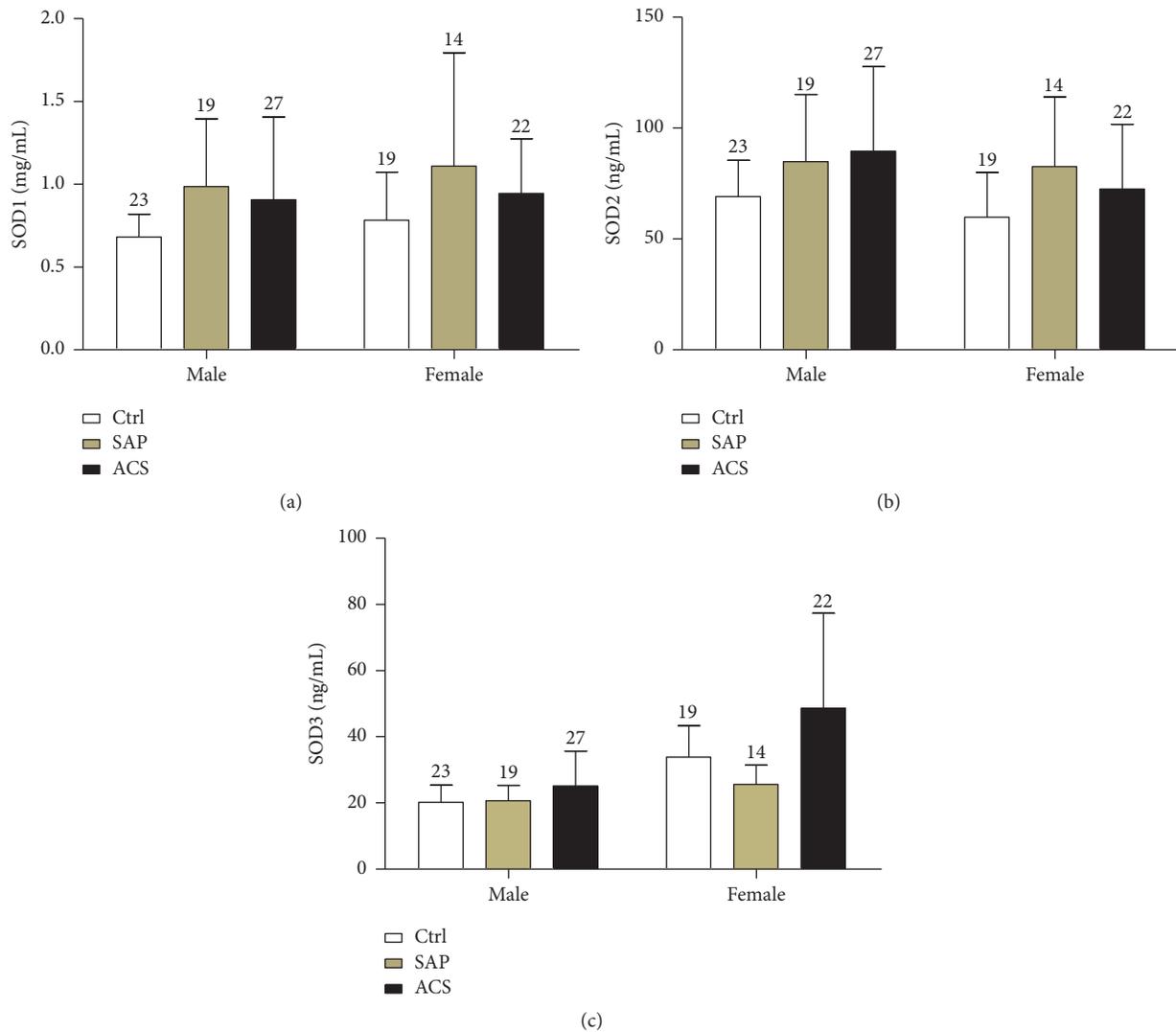


FIGURE 4: Levels of SODs by gender in controls and patients with SAP or ACS. Values for *n* are shown above each error bar.

$r = 0.02$  and  $p = 0.97$ ), patients with ACS (SOD1:  $r = 0.08$  and  $p = 0.59$ ; SOD2:  $r = 0.18$  and  $p = 0.22$ ; and SOD3:  $r = 0.25$  and  $p = 0.19$ ), or control subjects (SOD1:  $r = 0.09$  and  $p = 0.56$ ; SOD2:  $r = 0.11$  and  $p = 0.49$ ; and SOD3:  $r = 0.19$  and  $p = 0.25$ ).

#### 4. Discussion

SOD catalyzes the dismutation of  $O_2^{\bullet-}$  to  $H_2O_2$ , which can be reduced to  $H_2O$  by catalase and GPX. By inhibiting the effects of oxidative alterations induced by  $O_2^{\bullet-}$ , SOD is believed to prevent atherogenesis and associated cellular responses such as apoptosis, hypertrophy, and cell migration [23–25]. These results indicate the potential protective role of SODs against atherosclerosis. Elevated SOD1 activity confers protection against acute or chronic oxidative injury, including atherosclerosis [26, 27]. Gupta et al. also reported that the enzymatic activity of SOD decreases with the development of CAD [21]. In contrast, a pathological role for SOD1 was reported in which an increase of SOD1 activity enhances

oxidative injury by increasing the rate of formation of distal oxidants and elevating  $H_2O_2$  to a toxic level [28, 29]. SOD2 can regulate mitochondrial ROS and control endothelial dysfunction and apoptosis, leading to the development of atherosclerosis [30]. However, previous research reported controversial findings for the effect of SOD activity relative to CAD. Gupta et al. and Pytel et al. reported that the activity of SOD decreases in CAD [15, 21], whereas Zengin et al. drew the opposite conclusion [13]. These inconsistencies may be due to the possibility that high SOD activity enhances oxidative injury by increasing the rate of formation of distal oxidants. It has been suggested that the effects of SOD are dose dependent and are characterized by a bell-shaped curve [31, 32]. In the present study, plasma levels of SOD1 and SOD2 in patients with CAD (both SAP and ACS) were higher than those in healthy controls, which is consistent with a previous study [13], assuming that higher plasma SOD levels correlate with higher SOD activity. The regulation of  $O_2^{\bullet-}$  and  $H_2O_2$  is complex and depends on both the equilibrium and pathophysiologic changes within a cell. When this balance

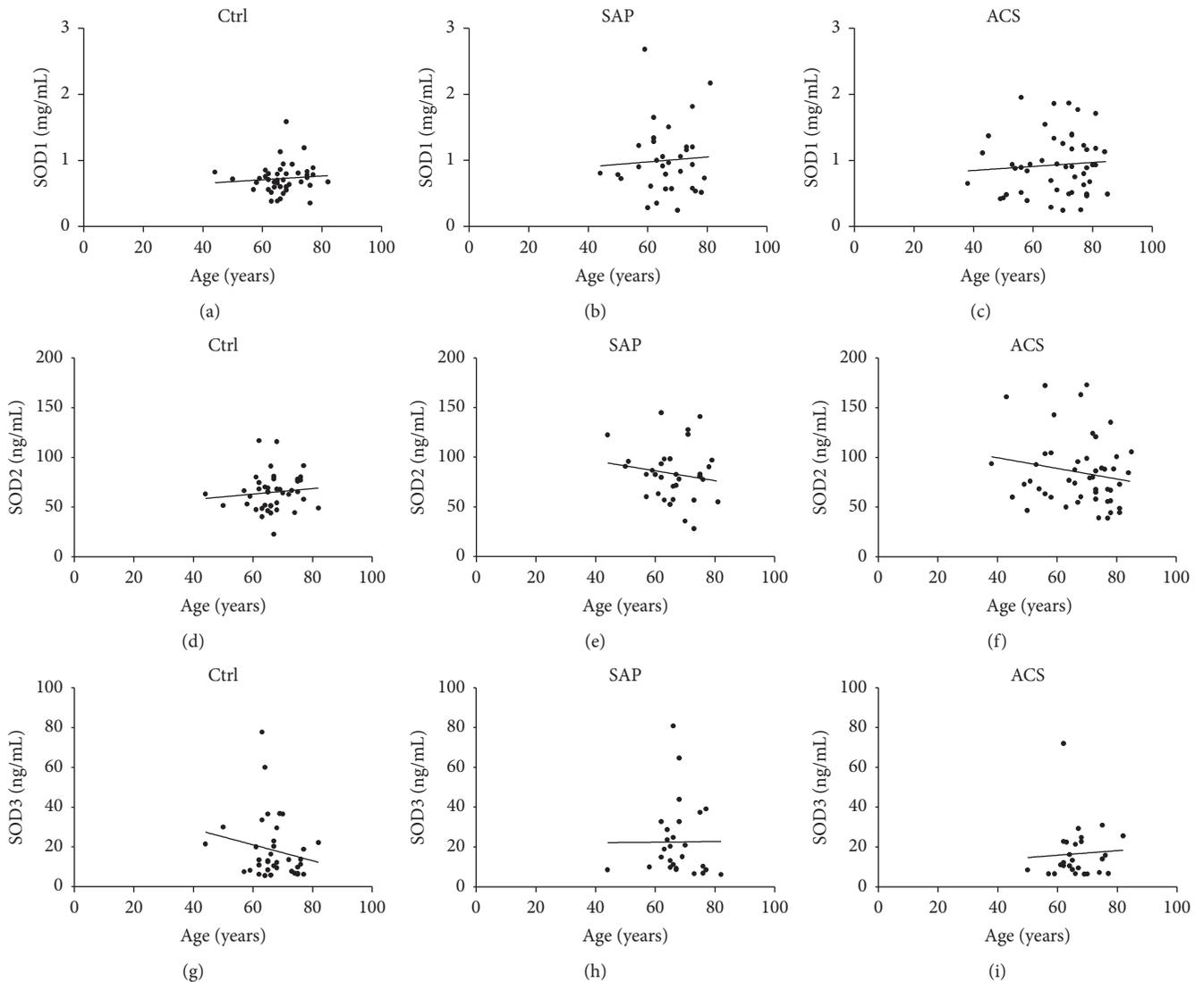


FIGURE 5: Correlations between age and SOD levels in controls and patients with SAP or ACS.

is broken, multiple biochemical reactions can be affected, potentially resulting in damage to the cell as a result of relatively high SOD activity. Some reports suggest that SOD3 may play a role in atherosclerosis, but its functional significance is unclear. Wang et al. reported that decreasing plasma SOD3 levels are associated with increasing history of myocardial infarction [33]. Sentman et al. concluded that SOD3 might have little effect on the development of atherosclerosis [34]. Consistent with this latter report, we found that the plasma level of SOD3 in patients with CAD was not significantly different from that in control subjects, suggesting that SOD3 might do little in the development of atherosclerosis.

The present study found no significant difference between patients with SAP and those with ACS for plasma levels of any of the three SODs. We found that Gensini scores of patients with CAD were not significantly correlated with plasma levels of SODs, regardless of the kind of CAD (SAP or ACS). A possible explanation for these results is that the increase in SODs may be compensated by a common mechanism, such

as oxidative stress [26, 27]. These results suggest that the plasma levels of SODs in CAD patients may not be affected by coronary plaque formation, coronary atherosclerosis rupture, or thrombosis.

We collected subjects with a smoking habit and compare the SOD levels among three groups. SOD1 and SOD2 concentrations in the SAP and ACS group were elevated as compared with control. Similarly, no significant difference of SOD1 and SOD2 was observed between SAP and ACS groups. There was no significant difference of SOD3 among the three groups. To compare the smoking effects, we compare the SOD levels in controls with/without a smoking habit. The mean concentration of plasma SOD1 with nonsmoking was minorly elevated; meanwhile no significant difference for SOD2 and SOD3 levels between groups with or without smoking was found. We also compared SOD levels in subjects with diabetes and hypertension among three groups. The levels of SOD1, SOD2, and SOD3 in the three groups with diabetes were not significantly changed. However, SOD1 and

SOD2 concentrations in the SAP and ACS subjects with hypertension were elevated as compared with control. SOD3 showed no difference. Although we cannot conclude the disease effects toward SOD levels, similarly, with overall comparison, the SOD1 and SOD2 were expressed higher in plasma of CAD patients as compared with control in the smoking or hypertension groups and SOD3 showed no significant difference in control, SAP, and ACS groups, excluding the diabetes patients. This issue could be analyzed in the future.

Among the three SODs, SOD2 was most promising as a biomarker for predicting CAD, with an AUC of 0.74 for SAP versus control and 0.67 for ACS versus control. Although this prediction level does not meet current standards for Good Medical Practice, this study provides valuable information about the circulating concentrations of SODs and the potential effects of SODs on CAD.

## 5. Limitation

The following limitations to the study should be considered. First, the measurement of SOD levels was performed at only one time point and thus cannot provide information on changes in SOD levels in individual patients. Second, evaluation of the percentage of coronary artery stenosis was subjective; however, it was assessed independently by two experienced cardiologists. Third, the subjects took some medications, such as atorvastatin, aspirin, clopidogrel, calcium channel blocker, angiotensin converting enzyme inhibitor (ACEI), angiotensin receptor antagonist (ARB), and beta blocker. It is reported that statins, ACEI, ARB, and beta blocker may affect oxidative stress [35–37]. The medication effects to influence the levels of SODs were not considered in the experimental design. Like our analyses of medication use described above, the SOD levels cannot be summed up either.

## 6. Conclusion

Plasma levels of SOD1 and SOD2 were elevated in patients with CAD. These enzyme levels may be useful in the future as biomarkers for diagnosis of CAD.

## Competing Interests

The authors declare that there are no competing interests concerning this study.

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