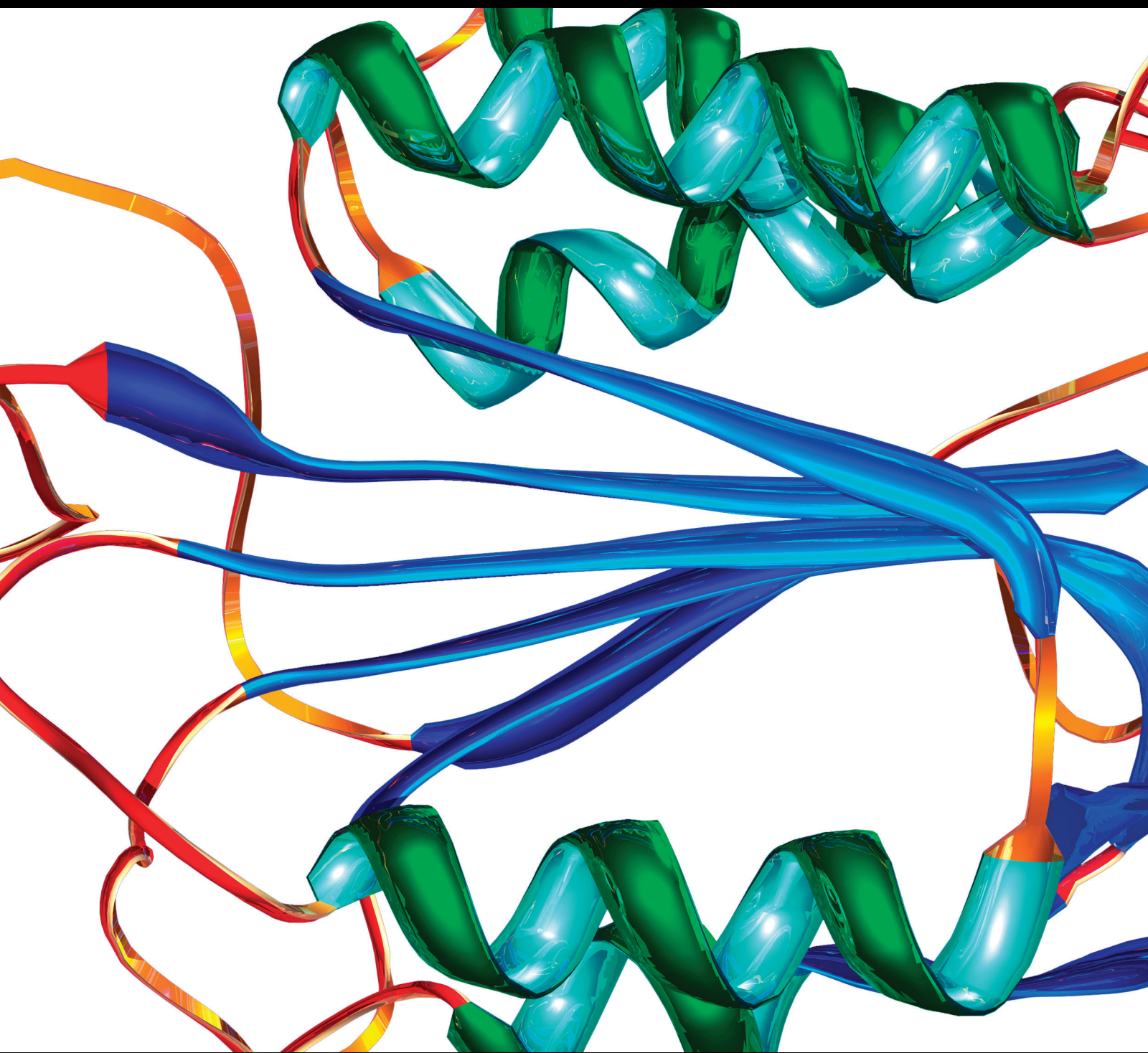
Disease Markers

Biomarkers of Cardiovascular Disease

Lead Guest Editor: Ying Huang
Guest Editors: Kailash Gulshan, Truc Nguyen, and Yuping Wu



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Editorial Board

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Editorial
Biomarkers of Cardiovascular Disease

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Cardiovascular disease (CVD) remains the leading cause of death globally. The identification of traditional risk factors such as age, hypercholesterolemia, hypertension, diabetes mellitus, and smoking has improved primary prevention of CVD. However, the overall mortality related to cardiovascular disease is still rising. Further scientific advances have led to the discovery of a broad range of novel biomarkers associated with cardiovascular risks, including B-type natriuretic peptide (BNP), N-terminal prohormone BNP (NT-proBNP), troponin, C-reactive protein (CRP), myeloperoxidase (MPO), lipoprotein-associated phospholipase A2, fibrinogen, TMAO, and cystatin C. Although these biomarkers have a prognostic value independent of the previous traditional risk factors, only a few have become important diagnostic tools in clinical practice. BNP and NT-proBNP have proven clinical utility in the diagnosis of heart failure and/or heart failure exacerbation. Troponin has been used as a cardiac biomarker for diagnosis and risk stratification of patients with suspected acute coronary syndrome (ACS). The blood levels of high sensitivity CRP (hs CRP) have been used to assess the risk of CVD, heart attack, and stroke. Circulating levels of MPO have been used to predict risks of coronary heart disease. However, so far, none of these biomarkers has significantly improved distinction between health and disease status. There remain tremendous challenges to scientists and clinicians in the discovery of novel biomarkers that may improve risk prediction of CVD, monitor disease progression, and potentially be used as therapeutic targets before clinical signs and symptoms appear.

This special issue contains nine papers, covering several aspects of biomarkers associated with cardiovascular disease. In six of the nine papers, the authors conduct small-scale retrospective cross-sectional or prospective clinical human studies to evaluate the clinical relevance of different biomarkers related to diverse disease conditions such as type 2 diabetes, acute ischemic stroke, heart failure, and aortic valve calcification. The biomarkers reported in this issue are miRNAs, cytokine IL-37, urinary NGAL, insulin resistance, metalloproteinase-8, metalloproteinase-1, parathyroid hormone, and lactate. Although respective authors have provided some evidence to show the promising prognostic value of these biomarkers, it is still unknown whether these markers will have a diagnostic impact or clinical implications. Also included in this special issue are three interesting review papers that discussed the effect of multiple vitamins, platelet miRNA expression profile, and ischemia-modified albumin on disease status.

In the paper "Plasma IL-37 Elevated in Patients with Chronic Heart Failure and Predicted Major Adverse Cardiac Events: A 1-Year Follow-Up Study," X. Shou et al. provided evidence that circulating level of anti-inflammatory cytokine IL-37 is associated with increased risks for chronic heart failure.

In the paper "Parathyroid Hormone Levels in the Prediction of Ischemic Stroke Risk," G. Çelik et al. performed a prospective study that examined parathyroid hormone (PTH) and/or 1,25-dihydroxyvitamin D (1, 25(OH)2 D) levels in 100 subjects who had acute ischemic stroke and

100 healthy control subjects. Their results suggested that PTH and 1,25(OH)2 D levels together may promote the determination of stroke risks.

In the paper "Handheld Capillary Blood Lactate Analyzer as an Accessible and Cost-Effective Prognostic Tool for the Assessment of Death and Heart Failure Occurrence during Long-Term Follow-Up," G. M. Kubiak et al. measured capillary blood lactate levels with a handheld analyzer in a total of 145 consecutive patients admitted to hospitals after acute myocardial infarction. The authors demonstrated that lactate levels may serve as a potential prognostic marker of late onset heart failure and death after acute myocardial infarction.

In the paper "Is Urinary NGAL Determination Useful for Monitoring Kidney Function and Assessment of Cardiovascular Disease? A 12-Month Observation of Patients with type 2 Diabetes," A Żyłka et al. assessed kidney function by monitoring the levels of urinary neutrophil gelatinase-associated lipocalin (uNGAL) in patients with type 2 diabetes mellitus (T2DM) after initiation of nephron-protective treatment in a one-year follow-up study. The authors found that better glycemic control in T2DM patients results in a significant decrease in uNGAL which is correlated with a significant improvement in renal function and the lowering of cardiovascular risk.

In the paper "Insulin Resistance in Adipose Tissue but Not in Liver Is Associated with Aortic Valve Calcification," the authors present a cross-sectional clinical study with 1201 study population participating in the Genetics of Atherosclerotic Disease study. They discovered that adipose tissue insulin resistance is positively associated with the prevalent risks for aortic valve calcification (OR: 2.40: 95% CI: 1.30–4.43). They also found no association between adipose tissue insulin resistance and coronary artery calcification.

The paper by J. Mieczkowska et al. showed that circulating levels of inflammatory biomarker metalloproteinase-8 (MMP-8) and tissue inhibitor of metalloproteinase-1 (TIMP-1) are negatively correlated with a heart rate in postmenopausal women during exercise treadmill testing.

The review paper by I. Mozos et al. discussed the latest studies related to the effect of multiple vitamins on arterial stiffness. They suggested that vitamins A, B12, D, K, C, and E may be potential biomarkers for arterial stiffness.

The review by M. Bijak et al. touches upon a recently discovered diagnostic value of profiling miRNAs in platelets of diseased versus heathy controls. This review summarized interesting and thought-provoking studies implicating the role of platelet miRNAs in various diseases. More specifically, the described studies highlighted the role of (1) miRNA-340/miRNA-624 in premature coronary artery disease patients, (2) miRNA150 in heart failure with atrial fibrillation, (3) miRNA-154/miRNA-329/miRNA-376 in sickle cell disease, and (4) miRNA-144/miRNA-223/miRNA-146a in type 2 diabetes patients with ischemic stroke.

In their review, I. Oran et al. proposed a revised concept of fatty acid-occupied albumin, rather than ischemia-modified albumin, as a biomarker of acute coronary syndrome.

This special issue is aimed to broaden our knowledge on biomarkers of cardiovascular disease.

Acknowledgments

We would like to thank the authors who contributed their science and interpretation of the current scientific data in this special issue. We also would like to thank the reviewers for their insightful comments on the papers.

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Research Article

Plasma IL-37 Elevated in Patients with Chronic Heart Failure and Predicted Major Adverse Cardiac Events: A 1-Year Follow-Up Study

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A great number of basic and clinical studies have demonstrated that inflammatory cytokines play an important role in the development and progression of chronic heart failure (CHF). However, there is limited information about the role of novel cytokine interleukin-37 (IL-37) in heart failure. We measured plasma IL-37 levels by enzyme-linked immunosorbent assay (ELISA) in 158 patients with chronic heart failure and 30 control subjects. Our results showed that plasma IL-37 levels were significantly elevated in patients with CHF compared with healthy controls (143.73 ± 26.83 pg/ml versus 45.2 ± 11.56 pg/ml, $P < 0.001$). Furthermore, plasma IL-37 levels were positively correlated with hs-CRP, hs-TnT, and NT-proBNP and negatively correlated with left ventricular ejection function (LVEF). 11 patients died of cardiovascular cause, and 27 HF patients were rehospitalized for worsening HF within 12 months. Multivariate Cox regression analysis showed that plasma IL-37 is an independent predictor of major adverse cardiac events (MACE). Furthermore, CHF patients with >99 pg/ml plasma IL-37 had significantly higher incidences of MACE within 12 months. Our data suggest that plasma IL-37 may play a role in the pathogenesis of CHF and may be a novel predictor of poor prognosis in HF patients.

1. Introduction

Chronic heart failure (CHF) is defined as impaired cardiac structure and/or function in ventricular filling or ejection that result in a complex set of clinical syndromes [1]. With the development of immunohistochemistry and molecular cell biology, the basic mechanism of heart failure has been proven to be due to cardiac remodeling characterized by necrosis and apoptosis of cardiomyocytes and progressive expansion of the ventricular cavity [2, 3].

A large number of studies have shown a close association between inflammation and cardiac remodeling. Inflammatory mediators, especially tumor necrosis factor- (TNF-) α, interleukin- (IL-) 6, IL-1β, and IL-18, impair cardiac function by promoting cardiomyocyte apoptosis, cardiac hypertrophy, inflammatory response, and matrix metalloproteinase-9 (MMP-9) activity; their plasma levels are increased in heart failure in association with disease severity [3–5]. Many groups therefore proposed inhibiting inflammation as a potent therapeutic target in heart failure [3, 5]. However, CHF is a more complicated process and there remain many unknowns regarding the relationship between inflammation and heart failure.

Interleukin-37 belongs to the IL-1 ligand family and is a newly identified anti-inflammatory cytokine [6]. IL-37 inhibits the secretion of proinflammatory cytokines such as IL-1β, IL-6, and TNF-α in peripheral blood monocytes, macrophages, dendritic cells, and epithelial cells, playing a critical role in innate immunity and adaptive immunity [7]. A great number of studies demonstrated that IL-37 is involved in the occurrence and development of chronic inflammation and autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, and diabetes [8–11]. In addition, evidence from clinical and animal studies has confirmed that

IL-37 not only participates in atherosclerotic disease but also has a close relationship with impaired heart function [12–14]. However, the level of plasma IL-37 in heart failure has yet been investigated. The goal of our study was to examine the plasma IL-37 level in patients with CHF and assess its relation to clinical parameters and biochemical laboratory data.

2. Materials and Methods

2.1. Study Population. A total of 158 patients were enrolled in this study. The diagnosis of CHF was based on typical symptoms and signs of heart failure and evidence of left ventricular enlargement and systolic functional impairment on echocardiography, according to the American College of Cardiology/American Heart Association guidelines [1]. Exclusion include patients with CHF secondary to specific aetiologies (e.g., malignant disease, chronic inflammatory disease, and/or infiltrative or congenital heart disease) and with end-stage renal failure (defined as estimated glomerular filtration rate $< 15\,\text{ml/min}\cdot\text{m}^2$). Thirty healthy individuals were selected as control subjects who matched with CHF patients in age, gender, and body mass index (BMI).

Every participant provided written informed consent, and the study was approved by the hospital ethical review board (Shaanxi Province People's Hospital and Center for Cardiovascular Diseases, China). All study procedures were in accordance with the ethical standards outlined in the Declaration of Helsinki of 1975, as revised in 1983.

2.2. Study Procedures. Once recruited, baseline assessments involve standardized history taking, physical examination, a resting 12-lead electrocardiogram, chest X-ray, blood sampling, and comprehensive transthoracic Doppler echocardiography using standardized equipment (Vivid ultrasound systems, General Electric, Milwaukee, WI, USA) complying with recommendations from the American Society of Echocardiography (2009). Coronary angiography was performed to define ischemic heart disease (IHD) and non-IHD as needed. All patients were followed up to 12 months from discharge to evaluate major adverse cardiac events (MACE), which was defined as first rehospitalization for CHF or death due to cardiovascular cause.

2.3. ELISA Detection of the Levels of Plasma IL-37. The level of plasma IL-37 (Adipogen AG, Liestal, Switzerland) was measured by an enzyme-linked immunosorbent assay (ELISA), following the manufacturer's instructions. The minimal detectable concentration of IL-37 by this assay is 10 pg/ml for IL-37. The ELISA intra-assay and inter-assay coefficients of variation are <5% and <10%, respectively. All of the samples were measured in duplicate.

2.4. Statistical Analysis. The SPSS 17.0 software package (SPSS, Chicago, IL, USA) was employed for statistical processing. Measurement data were presented as mean ± SD or median. Numeration data were presented as a constituent ratio. All continuous variables were tested for normal distribution and homogeneity for variance. Comparisons of CHF patients versus control subjects and the subgroup of CHF patients were performed using the two-tailed Student t-test. Coefficients of correlation (r) were calculated using Pearson's correlation coefficient. Hazard ratios (HR) and 95% confidence intervals (CI) were calculated for each factor with Cox proportional hazards analysis. To identify independent predictors of major adverse cardiac events, all baseline variables with $P < 0.05$ in the univariate analysis were entered into a multivariate model. In addition, differences in event-free survival by median of plasma IL-37 (IL-37 ≤ 99 pg/ml and IL-37 > 99 pg/ml) were examined using the Kaplan-Meier method and compared using a log-rank test. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Clinical Characteristics in Patients with CHF. The baseline clinical characteristics of patients with CHF are summarized in Table 1. The mean age of patients was 65.25 ± 9.63 years, and 67.72% were male. The mean BMI of patients was $23.33 \pm 2.09\,\text{kg/m}^2$. The proportion of patients with a diagnosis of ischemic heart disease (IHD), hypertension (HP), and diabetes mellitus (DM) was 56.33%, 46.20%, and 18.35%, respectively. The distribution of patients among the New York Heart Association (NYHA) cardiac function class included 62.03% from class II/III and 37.97% from class IV. The proportion of patients who were taking drugs was 88.61% of an angiotensin-converting enzyme inhibitor (ACEI)/angiotensin II receptor blocker (ARB), 83.54% of beta-blocker, 74.68% of loop diuretic, 44.30% of aldosterone antagonist, and 28.48% of digoxin. The mean left ventricular ejection function (LVEF) of CHF patients was 37.82 ± 4.90%. The concentration of plasma biomarkers hs-TnT, hs-CRP, and NT-proBNP was 37.82 ± 4.90 pg/ml, 3.62 ± 1.08 ng/ml, and 2043.59 ± 1094.89 pg/ml, respectively. In this study, the median length of follow-up was 109 days (range 35 to 365 days). No patient was lost to the follow-up.

3.2. Plasma IL-37 Elevated in CHF Patients. The mean plasma IL-37 level in patients with CHF was significantly elevated (143.73 ± 26.83 pg/ml) compared with that in control subjects (45.2 ± 11.56 pg/ml) ($P < 0.001$) (Figure 1(a)). In subgroup analyses of CHF patients, there was no significant difference between patients with IHD and without IHD, as well as patients with hypertension (HP) and with normal blood pressure (non-HP) (all $P > 0.05$) (Figures 1(b) and 1(c)). However, plasma IL-37 level in CHF patients with DM was significantly higher compared to that in patients without diabetes ($P < 0.01$) (Figure 1(d)).

3.3. Correlation of Plasma IL-37 with LVEF and Biomarkers. Next, we examined the correlation between plasma IL-37 and biomarkers of cardiac events, disease, and function and LVEF. As shown in Figure 2, plasma IL-37 positively correlated with hs-TnT (Figure 2(a)), hs-CRP (Figure 2(b)), and NT-proBNP (Figure 2(c)) (all $P < 0.001$). However, plasma IL-37 negatively correlated with LVEF (Figure 2(d)) ($P < 0.001$).

3.4. Higher Plasma IL-37 Is an Independent Predictor for MACE within 12 Months in CHF Patients. In this study, 38 major adverse cardiac events of 158 CHF patients were

Table 1: Baseline characteristics of patients with chronic heart failure.

Variables	CHF ($n = 158$)
Age (years)	65.25 ± 9.63
Male, *n* (%)	107 (67.72)
BMI (kg/m^2)	23.33 ± 2.09
IHD, *n* (%)	89 (56.33)
Hypertension, *n* (%)	73 (46.20)
DM, *n* (%)	29 (18.35)
NYHA class, *n* (%)	
II/III	98 (62.03)
IV	60 (37.97)
Medication, *n* (%)	
ACE-I/ARB	140 (88.61)
Beta-blocker	132 (83.54)
Loop diuretic	118 (74.68)
Aldosterone antagonist	70 (44.30)
Digoxin	45 (28.48)
LVEF (%)	37.82 ± 4.90
hs-TnT (pg/ml)	26.86 ± 9.19
hs-CRP (ng/ml)	3.62 ± 1.08
NT-proBNP (pg/ml)	2043.59 ± 1094.89

Values are mean ± standard deviation or proportions.

recorded within 12 months from discharge, including 11 patient deaths and 27 patient rehospitalizations for worsening HF. In the univariate Cox regression model, BMI, DM, LVEF, hs-TnT, hs-CRP, NT-proBNP, and IL-37 were associated with MACE in CHF patients (Table 2). When we performed Cox stepwise multivariate analysis including all variables with $P < 0.05$ on a univariate analysis, plasma IL-37 and NT-proBNP were significant predictors of MACE within 12 months of follow-up.

To determine the predictive value of the concentration of IL-37 on MACE, we divided CHF patients into a subgroup with IL-37 ≤ 99 pg/ml and >99 pg/ml by median levels of plasma IL-37. Kaplan-Meier curves and log-rank testing revealed that CHF patients with a higher concentration of plasma IL-37 (>99 pg/ml) had significantly higher MACE within 12 months from discharge (Figure 3) ($P < 0.001$).

4. Discussion

In the present study, our results showed that plasma IL-37 levels were significantly elevated in patients with CHF compared with healthy controls (143.73 ± 26.83 pg/ml versus 45.2 ± 11.56 pg/ml, $P < 0.001$). Furthermore, plasma IL-37 levels positively correlated with hs-CRP, hs-TnT, and NT-proBNP and negatively correlated with LVEF. 11 patients died of cardiovascular cause, and 27 patients were rehospitalized for worsening HF within 12 months. Multivariate Cox regression analysis showed that plasma IL-37 is an independent predictor of MACE in patients with CHF. Furthermore, CHF patients with >99 pg/ml plasma IL-37 had significantly higher incidences of MACE within 12 months. Our data suggest that plasma IL-37 might be involved in the pathogenesis of CHF and may be a novel predictor of poor prognosis in HF patients.

IL-37 is a novel homolog of the IL-1 cytokine family discovered by computational cloning and was originally designated as IL-1H4 in 2000 [15]. IL-37 is synthesized as a precursor molecule that needs to be cleaved by caspase-1 to generate mature IL-37 [16]. The production of IL-37 occurs at low levels in a physiological state and can be effectively induced in an inflammatory environment. Studies have shown that inflammatory stimulants such as IFN-γ, TNF-α, and lipopolysaccharide (LPS) promote the expression of IL-37 by peripheral blood mononuclear cells, dendritic cells, and epidermal cells. Recent studies confirmed that activated T lymphocytes also secrete IL-37 in an inducible manner [17]. Both endogenous and exogenous IL-37 have been shown to ameliorate inflammation and regulate immune disorder via inhibiting the production of inflammatory mediators including IFN-γ, TNF-α, IL-6, and IL-18 [6, 7].

Accumulating evidence shows that IL-37 plays a critical role in cardiovascular disease [7, 12–14]. Boraschi et al. first found high expression of IL-37 in atherosclerotic coronary and carotid artery plaques [7]. Ji et al. observed that circulating IL-37 levels are significantly increased and correlated with inflammatory markers and impaired left ventricular function in patients with acute coronary syndrome [12]. By using a myocardial ischemia/reperfusion injury model, Wu et al. found that exogenous IL-37 reduced infarct size, decreased cardiac troponin T levels, and improved cardiac function via suppressing the production of proinflammatory cytokines and chemokines and the infiltration of leukocyte [13]. Another study showed that IL-37 treatment can improve cardiac function through inhibiting the activation of NF-κB signaling pathway in a myocardial infarction model [14]. Coronary artery disease/myocardial infarction is one of the important causes of heart failure. In the present study, we are the first to demonstrate that plasma IL-37 becomes elevated in chronic heart failure patients, indicating a potential role of IL-37 in the development of heart failure.

Cardiac remodeling is the fundamental pathological process of heart failure. It is defined as structural and functional changes in the myocardium that result in left ventricular dilatation leading to heart failure [18]. In this study, we found that baseline IL-37 levels in patients with heart failure negatively correlate with LVEF while positively correlating with NT-proBNP and hs-TnT, the two most extensively studied biomarkers in evaluating the severity of cardiac function. These results are partly consistent with previous research that showed plasma IL-37 levels correlate with inflammatory markers and impaired left ventricular function in patients with acute coronary syndrome [12]. However, it is noted that our results found no significant differences in plasma IL-37 levels between the IHD and non-IHD subgroups. This observation seems contradictory with previous findings of elevated plasma IL-37 levels in patients with acute coronary syndrome. Because numerous research studies have confirmed the role of inflammation in acute coronary syndrome, the possible explanation for this contradiction is that IL-37 levels in plasma may correlate with the grade of inflammation

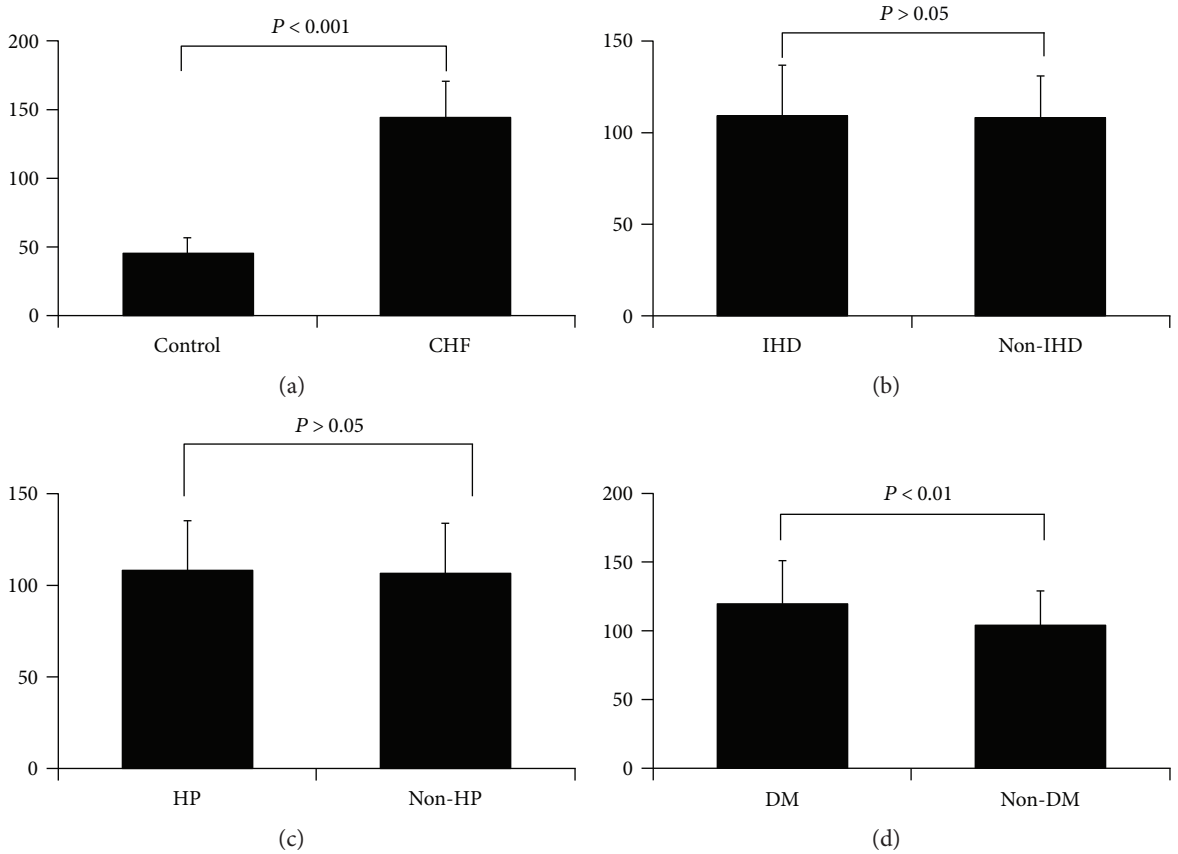

Figure 1: Elevated plasma IL-37 in chronic heart failure. (a) Plasma IL-37 in chronic heart failure (CHF) patients compared with control subjects (control); (b) plasma IL-37 levels in ischemic heart disease (IHD) subgroup and non-IHD subgroup; (c) plasma IL-37 levels in hypertension (HP) subgroup and nonhypertension subgroup (non-HP); (d) plasma IL-37 levels in diabetes mellitus (DM) subgroup and non-DM subgroup (non-DM).

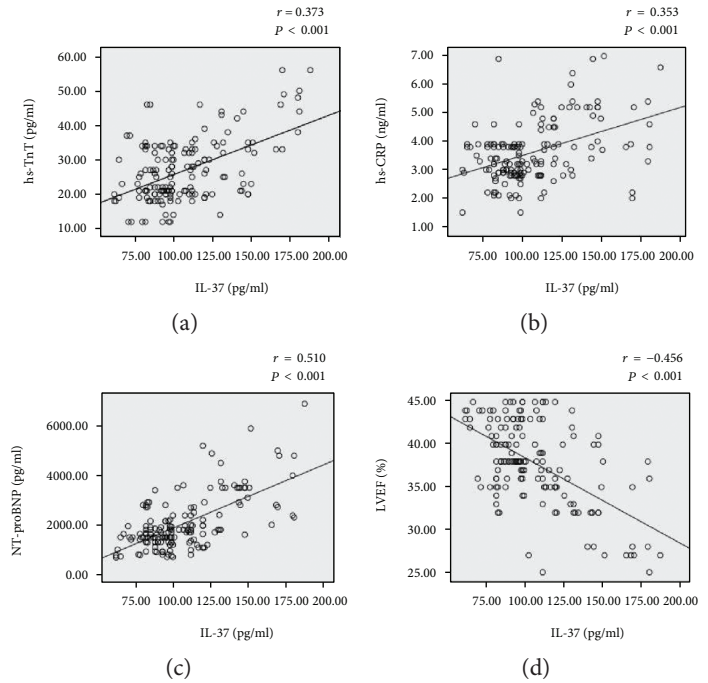

Figure 2: Correlation between plasma IL-37 and LVEF and biomarkers. (a), (b), (c) plasma IL-37 positively correlates with hs-TnT, hs-CRP, or NT-proBNP; (d) plasma IL-37 negatively correlates with LVEF.

Table 2: Cox regression analysis for major adverse cardiac events.

Variables	Univariate analysis Hazard ratio (95% CI)	*P* value	Multivariable analysis Hazard ratio (95% CI)	*P* value
Age (years)	0.974 (0.944–1.005)	0.098		
Male, *n* (%)	0.647 (0.342–1.225)	0.181		
BMI (kg/m^2)	1.179 (1.010–1.376)	0.037	1.301 (1.042–1.623)	0.020
IHD, *n* (%)	1.489 (0.774–2.865)	0.233		
Hypertension, *n* (%)	1.272 (0.679–2.384)	0.452		
DM, *n* (%)	2.797 (1.435–5452)	0.003	3.077 (1.435–5.452)	0.027
LVEF (%)	0.800 (0.749–0.854)	<0.001	0.925 (0.848–1.009)	0.077
hs-TnT (pg/ml)	1.097 (1.060–1.136)	<0.001		
hs-CRP (ng/ml)	1.982 (1.539–2.553)	<0.001		
NT-proBNP (pg/ml)	1.001 (1.001–1.001)	<0.001	1.001 (1.000–1.001)	<0.001
IL-37 (pg/ml)	1.053 (1.043–1.064)	<0.001	1.038 (1.017–1.059)	<0.001

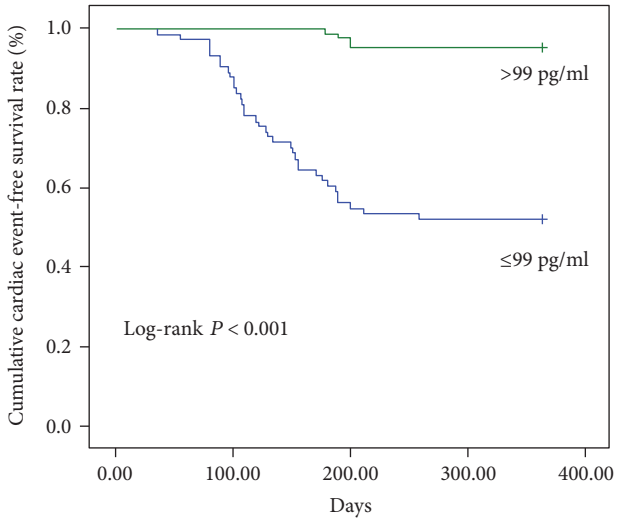

Figure 3: Kaplan-Meier curves demonstrating MACE in CHF patients during 12 months from discharge. Green line: CHF patients with lower concentration of plasma IL-37 (≤99 pg/ml); blue line: CHF patients with higher concentration of plasma IL-37 (>99 pg/ml). Log-rank test, $P < 0.001$.

rather than the disease status. This notion is further solidified by our results that plasma IL-37 levels positively correlate with hs-CRP, a well-known biomarker of inflammation [19]. Also, we did not observe significant differences in plasma IL-37 levels in the HP and non-HP subgroups. Interestingly, our study shows significantly increased IL-37 levels in the DM subgroup compared to that in the non-DM subgroup. Although Ballak et al. found that elevated IL-37 levels positively correlate with insulin sensitivity and a lower inflammatory status in human adipose tissue and that IL-37 ameliorates obesity-induced inflammation and insulin resistance in transgenic mice, the reasonable possibility for this discrepancy is that the DM status more closely correlates with the poor cardiac function in CHF patients [10, 20].

Finally, in this study, we demonstrated for the first time that plasma IL-37 is an independent predictor for MACE within 12 months. There are numerous reports that NT-proBNP and LVEF are predictors of poor outcomes in heart failure patients [21, 22]. Impaired LVEF is a hallmark of heart failure and reflects a fundamental weakness of the pump. It is no doubt that the lower the LVEF, the worse the prognosis of patients with heart failure. In this study, we showed that the higher concentration of plasma IL-37 (>99 pg/ml) is a significant predictor of recurrent hospitalizations for worsening HF and deaths due to a cardiovascular cause, independent of other clinical and laboratory variables. We believe that the increase in plasma IL-37 is associated with inflammation, cardiac remodeling, and acute cardiovascular events in heart failure patients. However, a direct causal relationship between an increase in plasma IL-37 and inflammation was not found in the study.

In conclusion, our study is the first to investigate the association between plasma IL-37 levels and chronic heart failure. We found that plasma IL-37 levels are significantly increased in patients with CHF and that the increase in plasma IL-37 levels is an unfavorable prognosis for patients with heart failure. However, there are some limitations in the present study. When performing the prognostic analysis, a sample population of 158 subjects is too small and a 12-month follow-up is too short, although statistical significance has been found. Therefore, a prospective trial consisting of a larger number of patients with heart failure and longer period of follow-up needs to be performed to clarify the significance of circulating IL-37 levels in heart failure. Furthermore, to fully assess the role of plasma IL-37 on cardiac inflammation and left ventricular remodeling, future studies are required to examine the underlying mechanism responsible for the increase of plasma IL-37 in patients with chronic heart failure.

Conflicts of Interest

The authors declare no conflicts of interests.

Authors' Contributions

Xiling Shou and Jing Lin contributed equally to this work.

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Review Article

Ischemia-Modified Albumin as a Marker of Acute Coronary Syndrome: The Case for Revising the Concept of "N-Terminal Modification" to "Fatty Acid Occupation" of Albumin

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Ischemia-modified albumin (IMA) is assumed "N-terminal modified" albumin which is generated immediately following myocardial ischemia. The diagnosis of IMA is based on reduced cobalt binding affinity to albumin which is attributed mainly to incapability of cobalt to bind at albumin's modified N-terminus. Although the albumin cobalt binding test was accepted as a potentially powerful marker for discriminating acute coronary syndrome from nonischemic chest pain, its usefulness has been brought into question in recent years. Patients with acutely ischemic myocardium exhibit a rapid increase in serum levels of fatty acids (FAs). Almost all released FAs are strongly bound to albumin which create conformational changes in the protein with resultant reduced cobalt binding affinity. There is a clear metabolic and temporal relationship between IMA measured via albumin cobalt binding testing and serum levels of FAs. In line with what has been suggested recently in the literature, we conclude that a shift from the concept of "N-terminal modified" to "FA-occupied" albumin is required, as this better describes IMA in patients with acute coronary syndrome. We also offer "oxidation modified albumin, OMA," which is conceptually different from the "FA-occupied" IMA, to describe modification of albumin in chronic disease associated with increased oxidative stress.

1. Introduction

Acute coronary syndrome is diagnosed biochemically by measuring myocardial proteins in serum originally found in cytoplasm, which appear in the blood not earlier than 4–6 hours after disruption of the myocardial cell membrane. These proteins include creatine kinase MB (CK-MB) and troponin. Biochemical markers that are sensitive and/or specific to ischemia prior to cell damage are therefore of great clinical importance. Such serum-based biochemical test was proposed by Bar-Or [1, 2]. The basic principle of this test involves the N-terminal region of human albumin and its inherent affinity for the cobalt metal ion (the so-called albumin cobalt binding, or ACB assay), the premise being that during myocardial ischemia, the albumin cobalt binding affinity is reduced due to an N-terminal modification of albumin [1, 2]. Note that N-terminal modified albumin has also been termed ischemia-modified albumin (IMA) since Bar-Or's first description.

2. Albumin Cobalt Binding (ACB) Assay

The ACB assay was approved by the FDA in 2003 as a method for identifying myocardial ischemia in patients admitted to the emergency department. In essence, the test involves adding cobalt chloride (approximately 1.5 equivalents per albumin molecule) to a serum sample, gently mixing, and then incubating to allow albumin cobalt binding. Dithiothreitol (DTT: a cobalt chelator) is added as a colorizing agent, and the brown color produced by the DTT-cobalt chelation (either free or unbound) is measured at 470 nm using a spectrophotometer. A serum-cobalt blank with no DTT is used for comparison, and the results are presented in absorbance units (ABSU). The ABSU data provide a measure

of the concentration of (free or unbound) chelated cobalt in the sample and reflect indirectly the level of IMA; that is, albumin that is incapable of binding cobalt due to what is referred to as "N-terminal modification."

Although the ACB assay has had FDA approval for more than a decade, the test has not achieved the expected clinical success. We have carried out a literature survey of reports accumulated over the 15 years following the first description of the concept of "N-terminal modification" of albumin to investigate the reasons for the limited reproducibility and accuracy of the ACB assay. We then go on to describe a new concept in the light of this knowledge.

3. Deficiencies of ACB Assay

The following list is based on literature reports to date and summarizes the main limitations of the ACB assay.

(1) Bar-Or's ACB assay was based on reduced cobalt binding affinity for albumin due to modification of the specific cobalt binding site (i.e., the N-terminal site). However, we now know that albumin has two additional cobalt binding sites, which have a higher affinity than the N-terminus (i.e., site A and site B), as well as an additional fourth binding site (i.e., Cyt-34) [3–6]. In the ACB assay, cobalt is exogenously added at a ratio of 1.5 equivalents per albumin molecule. If this cobalt binds to albumin, it is likely to bind predominantly at site A and/or site B. In other words, the ACB assay measures largely the level of binding of cobalt at site A and/or site B, rather than at the N-terminus; therefore, ischemia-induced N-terminal modification (if present) would lead to only an insignificant effect (or possibly no effect at all) on the total albumin cobalt binding capacity.

(2) Bar-Or's ACB assay was based on a protein structural modification (e.g., deletion of the amino acid sequence NH_2-Asp-Ala-His-Lys) on the albumin N-terminal; that is, so-called ischemia-modified albumin (IMA). However, structural modifications to the N-terminal have not been demonstrated in samples of patients following myocardial ischemia. For example, an analysis of the N-terminal amino acid sequence found a wild-type N-terminal sequence in six of seven patients with a positive ACB assay [7]. Bar-Or himself, the originator of the concept of "N-terminal modification" of albumin, was also unable to demonstrate truncated N-terminal IMA in patients with acute myocardial ischemia [8]. Therefore, and in contrast to Bar-Or's original assumption, IMA may not necessarily represent N-terminal modified albumin.

(3) Human albumin has a serum half-life of approximately 20 days, and so N-terminal modified IMA diagnosed via ACB assay should be detectable for several days following myocardial ischemia. However, studies have shown that the level of IMA increases within minutes of the onset of ischemia, remains elevated for 6–12 hours, and then returns to a normal level within 24 hours [9, 10]. The long half-life of albumin, together with the rapid normalization of modified albumin levels following ischemia, does not match the description of N-terminal modified IMA. Rapid clearance of N-terminal modified albumin from the circulation is a possible explanation for this [8]; however, there exists no credible evidence to support this hypothesis. Therefore, it appears that IMA may not represent N-terminal modified albumin.

(4) If the N-terminal site was responsible for the cobalt binding as measured by the ACB assay, we would expect a strong relationship between the enzyme-linked immunosorbent assay (ELISA) developed specifically to detect N-terminal modification of albumin and the classical ACB assay in clinical practice. However, Oh et al. [11] found no positive correlation between the ACB assay and the ELISA test for patients with either acute coronary syndrome or nonischemic chest pain. It therefore appears that the N-terminal site of albumin has only a limited (or even insignificant) role in the ACB assay.

(5) Domenicali et al. [12] reported posttranscriptional changes of serum albumin in healthy donors as well as in patients with stable cirrhosis and cirrhosis with acute worsening symptoms. They found that all three groups had the same fraction of N-terminal truncated albumin in their serum (approximately 2.5%). However, IMA measured by ACB assay was significantly higher in patients with acute-on-chronic liver failure [13]. This study does not support Domenicali's findings and therefore IMA detected by ACB assay might not be associated with an N-terminal modification only.

(6) In Bar-Or's ACB assay, the cobalt chelator DTT was used as a colorizing agent. DTT is also a known thiol-based protein disulfide (S-S) reducing agent, leading to disulfide-bond cleavage and subsequent unfolding of the molecule (i.e., denaturation), when used at concentrations in the range 1–10 mM [14]. In a typical ACB assay, the concentration of DTT is 1.6 mM. Since DTT incubation with subsequent spectroscopic reading ends up within 2 minutes during ACB assay, the denaturation, if present, might be limited in magnitude. It is worth noting, however, that protein denaturation may result in displacement of cobalt from the albumin, leading to reduced cobalt binding capacity, thus impairing the results of the ACB assay.

(7) Bar-Or [8] himself questioned the original concept of the ACB assay, based on observations (some of which are listed above) that were not easily explained by the concept of N-terminal modification of albumin. The authors preincubated serum samples with an excess of cobalt, allowing for full saturation of the albumin, and then filtered the albumin to exclude other serum-born proteins capable of binding to

cobalt. They washed the remaining albumin solution several times to exclude unbound cobalt and added DTT to the sample. Interestingly, the brown coloration was still present, leading to speculation that DTT-albumin complexes were associated with displacement of cobalt from albumin during the ACB assay [8]. This observation further supports the idea that, as discussed above, the assay DTT itself may denature the albumin in the sample, resulting in displacement of cobalt from the albumin, reducing the cobalt binding capacity of the albumin, and hence impairing the results of the ACB assay.

4. Interaction between Fatty Acids and Albumin

Human serum contains a mixture of at least six FAs, as follows (with approximate percentages): oleic acid (38%), palmitic acid (25%), linoleic acid (22%), stearic acid (10%), arachidonic acid (3%), and linolenic acid (2%). Almost all blood FAs are strongly bound to albumin and are transported throughout the body in this form, whereas only a very small percentage (less than 1/100,000) is present in the unbound form, so-called unbound or free FAs [15]. Human serum albumin has at least seven binding sites, with varying affinities for medium- and long-chain FAs [16]. Under normal physiological conditions, on average 0.1–2 molecules of FA are bound to each albumin molecule; however, the molar ratio of FAs to albumin can reach up to 6-7 during fasting or extreme exercise or in patients with liver and cardiovascular diseases (e.g., acute myocardial ischemia) [17, 18]. The numbering of these seven sites (FA sites 1–7) in the crystal structure of albumin is arbitrary and not based on the affinity for FA molecules. Among the seven binding sites on albumin, sites FA2, FA4, and FA5 have been identified as having the highest affinity for FAs [19]. Although the binding pockets appear well adapted to accommodate FA molecules, they are not specific to any particular FAs and thus are capable of binding to other ligand molecules. Fujiwara and Amisaki [20] have recently summarized the list of competing ligands that share common binding site with FAs.

5. Fatty Acid as a Marker of Myocardial Ischemia

Myocardial ischemia leads to a hyperadrenergic state within minutes of the onset of chest pain, which results in the breakdown of tissue and plasma phospholipids, as well as triglycerides, resulting in increased plasma concentration of free FAs. Patients with acutely ischemic myocardium exhibit a rapid increase in serum levels of free FAs, which can exceed normal average values at the time of admission by a factor of 3–10 [22–27]. Measurement of serum levels of free FAs in the diagnosis of acute myocardial ischemia has been the subject of a number of patent applications [28–30]. Serum levels of free FAs have also been reported to increase within 30 minutes of coronary balloon angioplasty (a well-known in vivo model for transient myocardial ischemia caused by balloon inflation) and a mean 5-fold increase in FA levels has been reported [31]. A recent multicenter study investigated the utility of measuring levels of free FAs compared with other available clinical tests (i.e., amino terminal pro-B-type natriuretic peptide, IMA, heart fatty acid binding protein, classical troponin T, and high-sensitive troponin I) [32] and found that free FA had the highest overall sensitivity (75%), specificity (72%), and negative predictive values (92%) for discriminating acute coronary syndrome from nonischemic chest pain in patients admitted to the emergency department. Thus, current data, although limited, suggest that monitoring of levels of free FAs in patients presenting with chest pain may provide an early indication of myocardial ischemia that is able to discriminate between acute coronary syndrome and nonischemic chest pain.

6. Link between the ACB Assay and Free FA

The first study into the effects of FAs on metal binding to albumin appeared in 2003. A British team found that cadmium binding was dramatically affected by high FA loading of albumin [33]. Mothes and Faller [3] speculated a putative relationship between FA binding to albumin site A and increased levels of free cobalt (i.e., increased IMA) in the ACB assay for patients with myocardial ischemia. Bhagavan et al. [34] reported an in vitro study, using pooled sera, and found a significant positive correlation between free FA and IMA measured by ACB assay; that is, the addition of FAs to the sample resulted in increased IMA levels (i.e., decreased albumin cobalt binding) with the same ratio. Lately, the same team from UK has made substantial progress in understanding the impact of FAs on the binding of albumin to metal ions (i.e., cobalt) and demonstrated allosteric inhibition of cobalt binding with albumin due to FAs. They concluded that IMA may correspond to albumin with increased levels of bound FAs [5, 35, 36].

A number of clinical studies have also discussed the possible links between serum levels of free FAs and the IMA results. Amirtharaj et al. [37] reported an inversely proportional relationship between the albumin cobalt binding capacity and FA level in patients with increased free-FA levels due to fatty liver disease. Bhagavan et al. [34] found an increase in IMA (lower cobalt binding capacity) in the sera of patients with acute myocardial infarction and discussed a plausible relationship between increased levels of free FA and increased IMA as measured by ACB assay. Jalan et al. [13] demonstrated a strong correlation between FAs bound to the FA1 and/or FA2 sites of albumin and reduced cobalt binding capacity and speculated overlap between cobalt binding sites and the FA1 and/or FA2 sites of albumin.

It is unsurprising to see such parallelism between IMA and albumin sites occupied by (bound) FAs, since all cobalt binding sites, except the N-terminal (including site A and site B), share common/neighbour albumin binding sites with FAs. It therefore appears reasonable to suppose that reduced cobalt binding to albumin, which is termed IMA, is actually due to occupation of the metal binding regions at site A and/or site B by bound FAs. The N-terminal site of albumin

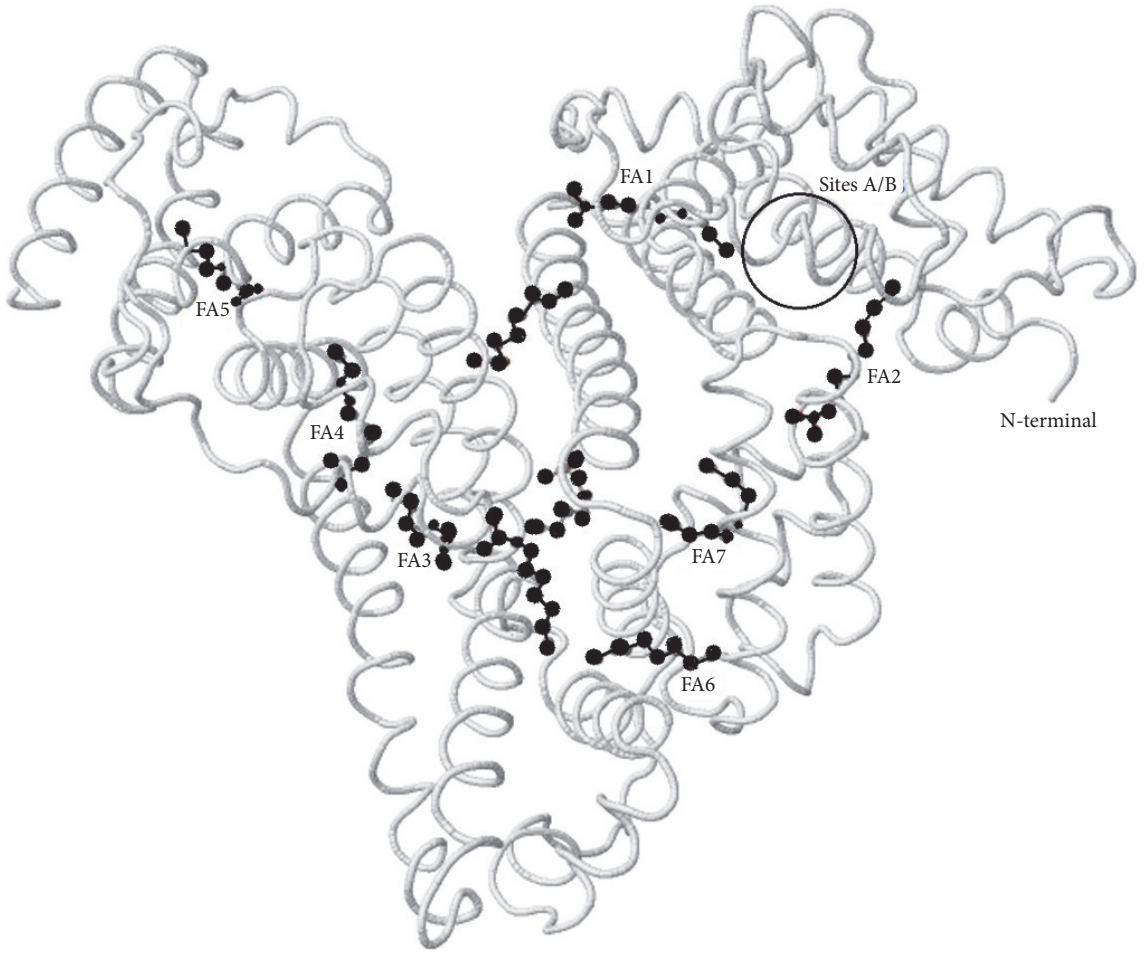

Figure 1: Location of the seven major FA-binding sites of human serum albumin, in relation to the main cobalt binding sites (i.e., N-terminal, site A, and site B). FA2 is one of the high affinity sites for FAs and communicates allosterically with cobalt binding to sites A/B. N-terminus never involves FA binding, and thus continues cobalt binding in the presence of "FA-occupied albumin." Image was generated using Protein Data Bank (PDB) ID: 1E7E [21] and modified according to Lu et al. [5].

does not bind to FAs [16, 19, 20, 36] and so "FA occupation" does not nullify completely the total cobalt binding capacity of albumin due to the (unoccupied) N-terminal site, further supporting the general theory of "albumin occupied by FAs" (Figure 1).

7. Evidence in Support of "FA Occupation of Albumin"

Elevated levels of free and bound FAs are known to be associated with a number of clinical conditions in addition to myocardial ischemia, including metabolic syndrome, nonalcoholic fatty liver, obesity, cancer, diseases with chronic inflammation, diabetes mellitus, hypertension, cardiovascular disease, stroke, and Alzheimer's disease [38, 39]. Noncardiac sources of increased IMA are associated with ischemic stroke, intracranial haemorrhage, mesenteric ischemia, skeletal muscle ischemia, peripheral atherosclerosis, hyperlipidemia, obesity, metabolic syndrome, hepatosteatosis, preeclampsia, foetal distress, diabetes mellitus, advanced renal disease, and liver cirrhosis [40, 41].

Here we assume, in line with the recent literature, that IMA is in fact "FA-occupied albumin" in patients with acute myocardial ischemia. The preceding section discusses substantial direct and indirect evidence from in vitro and clinical studies, which support the "occupation" concept; however, some additional data are required to support this theory. Some such evidence is discussed below.

(1) Other diseases, or clinical or laboratory conditions whereby albumin is "enveloped" by ligands or adducts, further support the "FA-occupied albumin" concept. One such example is poorly controlled diabetes, whereby the nonenzymatic covalent attachment of glucose molecules to albumin, as well as the subsequent oxidation, gives rise to advanced glycation end-products that bind to the albumin surface (also termed "glycated albumin"). The N-terminus itself, 59 lysine and 24 arginine residues on the albumin molecule, acts as potential sites for the formation of glycation products, which impair the ligand binding functions of the protein [42]. If the glycated albumin

was occupied by irreversibly bound glycation end-products, we would expect higher levels of IMA due to the inability of cobalt to bind to these (blocked) sites. Indeed, diabetics exhibit higher levels of IMA than control subjects; there is a significant correlation between IMA and HbA_{1c}, and patients with poor glycaemia control have higher IMA levels in comparison with those with good glycaemia control [43, 44]. Baraka-Vidot et al. [45] recently reported an in vitro model of glycated albumin, showing a positive correlation between the extent of glycation of albumin and the IMA levels measured by ACB assay (note that there were no other effects that could have potentially led to modifications of albumin other than glycation). Additionally, if the glycated albumins were fully occupied by irreversibly bound glycation end-products, then we would expect higher levels of free FAs, together with lower albumin FA-binding capacity because of the inability of the FAs to bind to the (irreversibly blocked) sites on the albumin. Unsurprisingly, glycated albumin has been shown to exhibit a diminished albumin FA-binding capacity [46, 47]. Thus, the impaired function of glycated albumin further supports the "FA occupation" concept.

(2) Human serum albumin is the most abundant circulating protein in the plasma and exhibits important antioxidant activities. Similar to the mechanism for generating "glycated albumin," the presence of long-standing oxidative stress may result in irreversible adducts on the protein, generating "oxidized albumin," in particular, thiol oxidation at Cys34 and carbonylation of several amino acids including proline, arginine, lysine, threonine, tyrosine, and methionine [48]. Oxidative damage to albumin impairs the functioning of ligand binding sites [49] and is associated with chronic diseases including diabetes mellitus, nephrotic syndrome, advanced liver diseases, chronic kidney disease, cardiovascular diseases and ageing, Alzheimer's disease, rheumatologic diseases, and cancers associated with increased oxidative stress [48]. If the oxidized albumins were occupied by irreversibly attached oxidation end-products that impair the ligand binding capacity, we would expect to find elevated levels of IMA in such chronic diseases. Anyway, as mentioned above, chronic diseases are associated with increased IMA levels [40, 41]. Thus, the impaired functioning of oxidized albumin also supports the "occupation" concept.

(3) If we assume that IMA is actually "FA-occupied" rather than "N-terminal modified" albumin in patients with acute myocardial ischemia, then we would expect the diagnostic accuracy of tests that measure serum levels of FAs to be higher than that of IMA measured by ACB assay. Furthermore, we would also expect tests that measure serum levels of FAs to be useful for discriminating acute coronary syndrome from nonischemic chest pain. Some recent studies with relatively large (>300 patients) sample sizes have reported negative results, calling into question the use of IMA measured by ACB assay as a marker for acute coronary syndrome [32, 50, 51]. Overall, the sensitivity of IMA measured by ACB assay was in the range 60–98%, with specificity in the range 35–94%, and with a negative predictive value of 60–90%. Today, it is generally accepted that the test is useful for ruling out acute coronary syndrome in clinical conditions, with negative troponin and negative ECG [52]. Studies on the use of serum levels of free FAs as a marker for myocardial ischemia are scarce and were discussed in the previous two sections. Although the level of free FAs appears to be more valuable than IMA for identifying acute myocardial ischemia (there are, at least, no studies reporting even insignificant increases in levels of free FAs, in contrast to the reports on IMA), there has been only one study comparing its merit with that of other markers of ischemia [32]. This was a multicenter study, with a total of 318 patients, which evaluated the power of free-FA levels, along with other available clinical tests (i.e., amino terminal pro-B-type natriuretic peptide, IMA, heart fatty acid binding protein, classical troponin T, and high-sensitive troponin I). They found that the free-FA level had the highest overall sensitivity (75%), specificity (72%), and negative predictive value (92%) for discriminating acute coronary syndrome from nonischemic chest pain in patients admitted to the emergency department. In summary, although further study is required, it appears that levels of free FAs have better discriminating power than IMA measured by ACB assay in patients presenting at the emergency department with acute chest pain and suspected acute coronary syndrome. This further supports the concept of "FA occupation."

(4) If IMA is in fact "FA-occupied" rather than "N-terminal modified" albumin and if the ACB assay actually measures the cobalt binding capacity of albumin (which is occupied by FAs in patients with acute myocardial ischemia), then we would expect a strong temporal relationship between IMA and levels of free FAs. Indeed, both IMA and level of free FAs increase within minutes from the onset of ischemia (including balloon-induced myocardial ischemia during percutaneous coronary intervention) [31, 53], remain elevated for 6–12 hours, and then return to normal within 24 hours [27, 40]. This relationship also supports the concept of "FA occupation."

8. Why Should We Make Changes to the Concept of IMA?

As discussed above, there is no positive correlation between the ELISA test developed specifically to detect N-terminal modification of albumin and the classical ACB assay [11]; therefore, the N-terminal site of albumin has no significance with respect to the results of the ACB assay for patients

with suspected acute coronary syndrome. Furthermore, if we assume that IMA is "FA-occupied" albumin rather than "N-terminal modified" albumin in patients with acute myocardial ischemia, then we conclude that IMA measured via an ELISA assay is useless for discriminating acute coronary syndrome from nonischemic chest pain. Researchers dealing with IMA-guided diagnosis of acute myocardial ischemia must take this into consideration and should continue to use the classical ACB assay in preference to an ELISA assay to detect IMA which is actually not "N-terminal modified" but "FA-occupied" albumin.

Researchers dealing with chronic diseases associated with "glycated/oxidized" albumin may continue to use both the classical ACB assay and ELISA tests for determining IMA levels, since irreversible modification of the N-terminus (as well as many other moieties of albumin, including cobalt binding sites) is common in such circumstances, making both ACB and ELISA tests useful. Alzheimer's disease is one such well-defined example of chronic disease for which long-standing oxidative stress is significant in the pathogenesis, leading to enhanced protein oxidation, DNA oxidation, lipid peroxidation, advanced glycation end-products, and carbonylated proteins. As expected, IMA levels measured by both classical ACB assay [54] and ELISA [55] were significantly higher in Alzheimer's patients and correlate well with other oxidative stress markers.

A marker like IMA that is sensitive and/or specific to myocardial ischemia prior to cell damage may be enormously valuable to the emergency physician assessing chest pain patients. We still require, however, a better understanding of this marker before it is ready for prime time use. All researchers interested in IMA should be aware of what exactly the IMA is, as well as the difference between the results of the ACB assay and ELISA testing, and what the results of the tests actually mean. We must therefore revise the concept of IMA.

9. Conclusion

Despite its deficiencies, the ACB assay for IMA levels may still be useful in emergency settings to discriminate acute coronary syndrome; however, the ELISA test should not be used for this purpose. We conclude that a conceptual change from "N-terminal modified" to "FA-occupied" albumin is required to better delineate IMA in patients with acute coronary syndrome. In that respect, we would like to offer using the new term "oxidation modified albumin" (OMA) instead of well-known "ischemia-modified albumin" to better delineate modified albumin in patients with chronic disease associated with increased oxidative stress. Thus, the new nomenclature may be useful in differentiating albumin modification occurring in acute coronary syndrome with myocardial ischemia from chronic disease with increased oxidative stress. The myocardial ischemia generates "reversible" "ischemia-modified albumin, IMA" secondary to FA occupation of albumin, while the oxidative stress generates "irreversible" "oxidation modified albumin, OMA" secondary to oxidation adducts on albumin.

Competing Interests

The authors report no conflict of interests.

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Review Article

Crosstalk between Vitamins A, B12, D, K, C, and E Status and Arterial Stiffness

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Arterial stiffness is associated with cardiovascular risk, morbidity, and mortality. The present paper reviews the main vitamins related to arterial stiffness and enabling destiffening, their mechanisms of action, providing a brief description of the latest studies in the area, and their implications for primary cardiovascular prevention, clinical practice, and therapy. Despite inconsistent evidence for destiffening induced by vitamin supplementation in several randomized clinical trials, positive results were obtained in specific populations. The main mechanisms are related to antiatherogenic effects, improvement of endothelial function (vitamins A, C, D, and E) and metabolic profile (vitamins A, B12, C, D, and K), inhibition of the renin-angiotensin-aldosterone system (vitamin D), anti-inflammatory (vitamins A, D, E, and K) and antioxidant effects (vitamins A, C, and E), decrease of homocysteine level (vitamin B12), and reversing calcification of arteries (vitamin K). Vitamins A, B12, C, D, E, and K status is important in evaluating cardiovascular risk, and vitamin supplementation may be an effective, individualized, and inexpensive destiffening therapy.

1. Introduction

Cardiovascular diseases are the main cause of mortality worldwide and prophylactic measures deserve special attention. Arterial stiffness, one of the earliest detectable signs of structural and functional changes of the vessel wall [1], is associated with cardiovascular risk, morbidity and mortality, atherosclerosis and arteriosclerosis, aging, and several chronic disorders. Measurement of pulse wave velocity (PWV) is a simple, noninvasive, validated, the most used, and reproducible method to assess arterial stiffness [2]. A recently published meta-analysis, including 17,635 participants, demonstrated that an increase of PWV of 1 m/s is associated with a 7% increased risk of subsequent cardiovascular events, concluding that aortic PWV enables identification of high cardiovascular risk subjects, that might benefit from more aggressive risk factor management [3]. Augmentation index, a measure of peripheral arterial reflective properties, is also a complex and indirect marker of arterial stiffening [4, 5]. A 10% increase in the augmentation index was associated with 31.8% increased risk of cardiac events [6].

Aging and several disorders cause degenerative changes of the vessel wall of large arteries, related to the rupture of the elastic fibers, impaired cross-linking of extracellular matrix components, accumulation of collagen, fibrosis and necrosis of muscle fibers, inflammation, and calcification, leading to their stiffening [7, 8]. Vascular calcification, calcium phosphate complexes deposition in the arterial wall, is an active process, enabled by several mechanisms, and leads also to loss of arterial wall elasticity and an increased PWV, related to vascular remodeling, organ damage, and overall morbidity and moratlity [9, 10]. It can be present as medial calcification (Monckeberg's medial sclerosis, prevalent among patients with diabetes and renal and hyperparathyroid disorders) or intimal calcification (on the surface of the atherosclerotic plaque) [11]. Hypertension, inflammation, oxidized low density lipoproteins, and a high calcium-phosphorus ion product enable transformation of vascular smooth muscle

cells into osteocyte-like cells [12, 13]. Functional deterioration of the arteries involves reduced bioavailability of NO and endothelial dysfunction [1]. Reduction of arterial compliance causes a faster reflection of the systolic wave from the peripheral arteries to the heart, increasing the central aortic pressure and causing myocardial hypertrophy and ischemia [1].

Destiffening is a challenge of cardiovascular prevention and deserves special attention. Diet is a modifiable cardiovascular risk factor [14] and Mediterranean diet and nutrients with antioxidant and anti-inflammatory properties may improve vascular function, despite contradictory findings of randomized trials [15–17].

There is a worldwide trend toward nutritional insufficiency [18], related to aging of the population, with changes of anatomy and function of the kidney. Socioeconomic improvement has been associated with nutritional changes and increased prevalence of cardiometabolic disorders [19]. Dietary factors may accelerate or slow the evolution of cardiovascular disorders. Dietary vitamin supplements, easily accepted and often used by Western populations, may provide potential benefits and harms related to their use [20].

Considering the worldwide trend toward nutritional insufficiency, it was the aim of the present paper to review the main vitamins related to arterial stiffness and enabling destiffening, their mechanisms of action, including a brief description of the latest studies in the area, and their implications for primary cardiovascular prevention, clinical practice, and therapy.

2. Vitamin D

Vitamin D, "the sunshine vitamin," is known especially for promoting calcium deposition in bones. Vitamin D receptors have been found in several other tissues, including vascular smooth muscle, endothelial cells, and cardiomyocytes [27]. Vitamin D deficiency is very common, due to indoor lifestyle, sun avoidance strategies, air pollution, and smoking, is often unrecognized or untreated, and is associated with increased all-cause mortality and cardiovascular event rate [28] and linked to major cardiometabolic risk factors, including obesity, hypertension, and diabetes mellitus [50]. Unfortunately, it is also very common during perinatal period, linked not just with poor bone development, but also with heart disease, type 1 diabetes mellitus, and cancer [51, 52]. Vitamin D deficiency *activates the renin-angiotensin-aldosterone system*, and the increased vascular tone, due to release of angiotensin II, and arterial stiffness precede the development of hypertension [53]. Vitamin D has also pleiotropic effects on the *immune system* and *suppresses the low grade inflammation in the cardiovascular system*, downregulating Th1 activity and dendritic cell maturation, inhibiting production of cytokines, and upregulating Th17 regulatory activity and modulating macrophage activity [28, 54, 55].

Usually *25 hydroxy vitamin D* and not 1,25 dihyrdoxy vitamin D is assessed. 25 hydroxy vitamin D is the nutritional parameter of vitamin D status, the primary circulating and storage form of vitamin D in the human body, a reliable, available marker of low vitamin D levels, easy to administer, with few side effects, and able to bind to vitamin D receptors [5, 28]. In the Whitehall study, the optimal concentration of 25(OH) vitamin D was 80–90 mmol/l and a linear, inverse association of vitamin D level with both vascular and nonvascular mortality was reported [56].

The role of vitamin D deficiency in vascular disease is an emerging issue [18]. Several studies revealed associations between vitamin D level and PWV [5, 57–60], but other authors found no significant association between vitamin D concentrations and markers of subclinical atherosclerosis [61, 62]. The effect of vitamin D supplementation on arterial stiffness showed also conflicting results, either beneficial or not (Table 1). Daily 2,000 IU vitamin D supplementation for 16 weeks resulted in a significant decrease in carotid-femoral PWV in 25 normotensive black boys and girls [33]. A significant decrease of arterial stiffness was also noticed after a single dose of 300,000 IU of cholecalciferol in children with chronic kidney disease [22]. Zaleski et al. reported a decrease of arterial stiffness with high-dose vitamin D supplementation for 6 months, and no effect on blood pressure [24]. The pleiotropic beneficial effects of vitamin D on arterial stiffness were suggested to be *dose-dependent*, which could explain the conflicting results of different studies [24]. McGreevy et al. found a significant decrease in augmentation index after 100,000 IU of vitamin D3 (the high-dose group) for 8 weeks, in older adults with vitamin D deficiency [25]. Forouhi et al. showed a modest reduction in PWV after D2 and D3 supplementation [21]. Ryu et al. found no beneficial effect of high-dose vitamin D supplementation on cardiovascular risk factors, PWV, or augmentation index in diabetic patients [27] and the same lack of change was reported by Chitalia et al. in predialysis chronic kidney disease patients [28]. Six months of vitamin D supplementation did not decrease central blood pressure parameters or arterial stiffness in healthy postmenopausal native American women [26]. A recent meta-analysis of randomized controlled trials concluded inconsistent evidence for destiffening induced by vitamin D supplementation, attributable to the heterogenity of the included studies [63].

Vascular calcification in the coronary or peripheral arteries is a powerful predictor of cardiovascular morbidity and mortality, linked to PWV [10], especially in hemodialysis patients [64]. High concentrations of vitamin D enable increased gastrointestinal calcium absorption, resulting in higher circulating calcium concentrations and increasing the number and size of calcification foci, resulting in vascular calcification, especially in atherosclerotic plaques [9, 62, 65]. Sachs et al. observed associations of lower concentration of vitamin D metabolites with reduced coronary artery calcium prevalence and severity, measured by computed tomography, in patients with type 1 diabetes mellitus [61]. A mouse model of chronic kidney disease revealed protective effect against aortic calcification at low vitamin D3 doses, sufficient to correct secondary hyperparathyroidism (higher doses stimulated aortic calcification) [66].

The main pathophysiological vasculoprotective mechanisms by which vitamin D supplementation reduces arterial stiffness include decrease of the renin-angiotensin-aldosterone system activity, suppression of endothelin-induced

Table 1: Vitamin D supplementation and arterial stiffness studies.

Authors	Year of publication	Vitamin D dose	Follow- up	Participants	Results
Forouhi et al. [21]	2016	100,000 IU/month vitamin D2 or D3	4 months	340 nondiabetic patients with hyperglycemia or positive diabetes risk score	Modest reduction in PWV
Aytaç et al. [22]	2016	Single dose of 300,000 oral cholecalciferol	12 weeks	41 children with chronic kidney disease	Significant lower arterial stiffness
Munisamy et al. [23]	2016	0.25 μg alfacalcidol/day	6 months	28 type 2 diabetic nephropathy patients	Decrease of arterial stiffness
Zaleski et al. [24]	2015	4,000 IU/day versus 400 IU/day	6 months	40 vitamin D deficient adults with prehypertension	High dose of vitamin D lower arterial stiffness
McGreevy et al. [25]	2015	50,000 IU or 100,000 IU D3	8 weeks	119 vitamin D deficient subjects	Significant decrease in augmentation index in the high dose group
Gepner et al. [26]	2015	400 IU or 2,500 IU vitamin D	6 months	98 healthy postmenopausal native American women	No decrease of arterial stiffness
Ryu et al. [27]	2014	Cholecalciferol 2.000 IU/day	24 weeks	40 type 2 diabetes	No beneficial effect on arterial stiffness, cardiovascular risk, insulin resistance
Chitalia et al. [28]	2014	300,000 IU cholecalciferol at baseline and 8 weeks	16 weeks	26 nondiabetic patients with chronic kidney disease	Improvement of endothelial function, no change of arterial stiffness
Martins et al. [29]	2014	100,000 IU monthly	3 months	130 overweight and obese African Americans with elevated blood pressure	Decreased level of inflammatory and oxidative stress mediators of arterial stiffness, but not a decrease of arterial stiffness
Levin et al. [30]	2014	5,000 IU 25 vitamin D and 0.5 μg 1,25 vitamin D 3 times/week in oral suspension for 6 months	9 months	128 stable chronic kidney disease patients	No results published yet
Mose et al. [31]	2014	3,000 IU cholecalciferol/day	6 months	50 chronic dialysis patients	No decrease of 24-hour blood pressure, arterial stiffness or cardiac function
Klop et al. [32]	2014	100,000 IU vitamin D3	Single dose	6 men and 6 women	Reduction of augmentation index, reduced postprandial leukocyte activation
Stricker et al. [4]	2012	100,000 IU vitamin D3	Single dose	62 elderly patients with peripheral arterial disease and low vitamin D levels	No influence on endothelial function, arterial stiffness, coagulation and inflammation parameters
Dong et al. [33]	2010	2,000 IU/day	16 weeks	25 Normotensive black boys and girls	Significant decrease in carotid-femoral PWV

vascular smooth muscle cell proliferation, renoprotective effects, effects on calcium metabolism and PTH level, counterbalance of inflammation and oxidative stress, and improvement of carbohydrate metabolism and insulin sensitivity [24, 29, 33, 67, 68]. Fibroblast growth factor 23 (FGF-23), a circulating peptid secreted by bone cells, known for inducing phosphaturia, lowering 1,25 dihydroxy-vitamin D, and suppressing PTH secretion, a new marker of inflammation, insulin resistance, and visceral fat accumulation, has been also associated with cardiovascular events and arterial stiffness [50, 69]. FGF-23 acts on the vascular function through its coreceptor Klotho, which increases nitric oxide availability and it has also been related to the presence of vascular calcifications [50, 70]. Low levels of vitamin D were associated with increased activity of matrix metalloproteinases and C reactive protein, correctable by supplementation [71].

The inconsistencies of vitamin D supplementation trials could be attributable to heterogeneity in vitamin D dosage, compounds and baseline concentration, study duration, design, population and follow-up, lack of a control group with normal vitamin D level, biases due to different comorbidities, therapy known to affect arterial stiffness, and several other confounding factors [24, 72]. Further large, longer duration, controlled randomized studies are required in order to demonstrate a causal relationship between vitamin D supplementation and decrease of arterial stiffness and to understand the importance of maintaining vitamin D sufficiency. There is no consensus on optimal levels of vitamin D in order to provide a beneficial cardiovascular effect, and this will be the aim of future clinical trials.

3. Vitamin K

Vitamin K, a lipid-soluble vitamin, is an essential micronutrient. It includes vitamins K1, K2, and K3. Phylloquinone (vitamin K1, phytonadione) is found especially in dark-green leafy vegetables and seeds, and menaquinone-7 (Vitamin K2), formed mostly by bacteria, may be obtained from meat, eggs, and fermented cheese [11, 73]. Vitamin K3 (menadione) is a synthetic vitamin K.

Vitamin K is essential for blood coagulation, but vitamin K insufficiency was associated also with an increased risk of cardiovascular events in healthy middle-aged people, type 2 diabetes, and end-stage renal disease patients [34]. Observational studies revealed a lower prevalence of arterial calcification and cardiovascular mortality in subjects with a high intake of menaquinones (vitamin K2) and no effect for phylloquinone (vitamin K1) [35, 74, 75]. An accelerated progression of aortic stiffness, associated with the use of warfarin, has been demonstrated in a study including 18 hemodialysis patients [76]. Vitamin K antagonists inhibit the recycling of vitamin K in the epoxide cycle, reduce carboxylation of coagulation factors, and cause calcifications in several arteries and valves [11]. Progression of aortic stiffness was related to the severity of vitamin K deficiency, assessed using circulating PIVKA-II, the undercarboxylated level of prothrombin and a sensitive subclinical vitamin K deficiency marker [76]. Matrix Gla-protein (MGP) is an inhibitor of soft tissue calcification; in its mature, active form it contains five Gla-residues (resulting from vitamin K-dependent gamma-carboxylation of the respective Glu-residues) and three phosphoserine residues [11, 76, 77]. The inactive form, desphospho-uncarboxylated MGP (dp-ucMGP) is regarded as a sensitive marker for vascular vitamin K status and increased levels of plasma dp-ucMGP are associated with increased cardiovascular and all-cause mortality [11, 35, 78]. As calcification develops, MGP is upregulated in vascular smooth muscle cells, as a negative feed-back mechanism [11, 79]. Pivin et al. found a positive association between PWV and inactive MGP, before and after adjustment for common cardiovascular risk factors and renal function [80]. Mayer Jr. et al. confirmed the relationship between inactive MGP and aortic stiffness, after adjusting for all potential confounders, but not with stiffness of muscular-type arteries [81]. MGP levels were not predictors for carotid-femoral pulse wave velocity in renal transplant recipients [82]. *Vitamin K-dependent proteins,* requiring carboxylation to become biologically active, contribute to thrombus formation, vascular calcification, vascular stiffness, and ischemic cardiovascular events [11, 83].

A possible role of vitamin K in *suppressing chronic inflammation* and *reversing calcification of arteries* was suggested [34]. Observational studies have shown an inverse association between vitamin K status and inflammatory markers, such as interleukin-6 and C reactive protein [35, 84]. Vitamin K might suppress inflammation by decreasing expression of genes for cytokines [85]. Warfarin may impair pulse pressure in patients with a history of hypertension and higher cardiovascular risk [86], contributing to progression of arterial stiffness. Vitamin K2 has also the ability to *improve lipid profile* by increasing HDL cholesterol and decreasing total cholesterol [74].

Induction of type 1 diabetes mellitus, in rats with streptozotocin, resulted in augmentation of arterial stiffness, increase of aortic and femoral calcifications, and reduction of γ-carboxylated MGP (the active form of MGP) [8]. Reduced MGP is involved in the early development of medial artery calcification (MAC) in diabetes and the deposition of hydroxyapatite crystals along the large arteries, resulting in accelerated arterial stiffness [8].

A correlation between PWV and aortic calcium content has been previously demonstrated in a warfarin-vitamin K rat model of MAC [7]. Increased vitamin K2 intake has been associated with decreased arterial calcium deposition and the ability to reverse vascular calcification in animal models [10]. Vaccaro and Huffman found an inadequate vitamin K1 intake in older adults, especially in Hispanic and Black Americans, and vitamin K1 was an independent predictor of high arterial pulse pressure [87].

Daily supplementation with oral vitamin K2 for 6 months caused a modest, nonsignificant improvement in PWV in older patients with vascular disease [34] (Table 2). Long-term supplementation with 180 μg menaquinone/day for three years decreased arterial stiffness in healthy postmenopausal women, especially in women with a high arterial stiffness [35]. Spronk et al. fed warfarin-treated rats diets containing vitamins K1 or K2 and found that just menaquinone inhibits warfarin-induced arterial calcification, explained by its more

Table 2: Effect of vitamin K supplementation and arterial stiffness in human subjects.

Authors	Year of publication	Follow- up	Dose of vitamin K	Study population	Results
Fulton et al. [34]	2016	6 months	Oral 100 mcg vitamin K2	Participants aged ≤70 years, with a history of vascular disease	A modest nonsignificant decrease in pulse wave velocity (PWV)
Knapen et al. [35]	2015	3 years	180 μg menaquinone	120 healthy postmenopausal women	carotid-femoral PWV and the stiffness index β decreased
Vossen et al. [36]	2015	24 months	360 microgram menaquinone-7 (MK-7)	Patients with coronary artery disease	Difference in coronary artery calcification score between MK-7 and control group

effective utilization in the aorta [88]. The VitaK-CAC trial will explore the effect of menaquinone-7 supplementation on progression of coronary artery calcification in a randomized trial [36].

Concluding, vitamin K, especially K2, enables destiffening by impairing and reversing calcification of arteries, suppressing the inflammatory reaction in the vascular wall and improving the lipid profile. Additional intake of vitamin K2 does not increase procoagulant activity or thrombosis risk because the blood coagulation factors are fully carboxylated [35].

4. Vitamin E

Vitamin E, the most abundant fat soluble *antioxidant* in the human organism, is found in high concentrations in palm oil, rice ban, and oily plants [41, 89] and includes tocopherols and tocotrienols. Plasma concentrations of vitamin E depend on the amount of plasma lipids and LDL cholesterol, considering that the latter is the main plasma carrier of tocopherol [90]. There is no generally accepted recommendation for an adequate intake of vitamin E [89]. Concentrations of vitamin E are influenced by age, lifestyle factors, such as obesity, smoking, alcohol consumption, fat malabsorption, interindividual differences in vitamin E metabolism, and interaction with pharmaceuticals (vitamin K, statins) [89].

Dietary intake of alpha-tocopherol, the main component of vitamin E, may reduce the cardiovascular risk [91, 92]. The antiatherogenic role of vitamin E has been suggested by its ability to *decrease LDL oxidation* [91, 93], *quench free radicals*, *inhibit protein kinase C* (PKC), *inhibit expression of adhesion molecules* and monocyte transmigration [47, 94], and *impair vascular smooth muscle cell proliferation* [93]. PKC is a key mediator of the vasoconstrictor response to oxidative stress [95], and the inhibition of PKC is another mechanism enabling improvement of vascular endothelial function besides the antioxidant action [47]. Vitamin E may become *prooxidant* at high doses, in the absence of an effective cooxidant, enabling production of alpha-tocopheroxyl radical, which can be inhibited by coantioxidants such as vitamin C [46, 96, 97]. Vitamin C reverses the prooxidant state of vitamin E, supporting the combined administration of vitamins C and E [98, 99]. But vitamin C can become prooxidant as well [98]. Human studies did not confirm a reduction of oxidative stress biomarkers due to combined administration of vitamins E and C, and the synergistic effects of the two vitamins disappeared under anaerobic conditions and became prooxidant [99]. Vitamin E has also *anticoagulant* properties by interfering with vitamin K-dependent clotting mechanisms not related to its antioxidant effect [89].

Conflicting results have been obtained regarding effectiveness of vitamin E in reducing atherosclerosis progression or lipid lowering effect [41]. Vitamins E and C *increased the collagen content of the arterial wall* and reduced vascular metalloproteinase-1, stabilizing the atherosclerotic plaque in a porcine model of atherosclerosis [100]. Antioxidant vitamins inactivate free radicals, increase plasma and tissue antioxidant defense, *reduce inflammation*, inhibit DNA oxidation by H_2O_2 in human lymphocytes, and *restore endothelial function* [16, 101, 102].

Hampson et al. found significant associations between PWV and alpha-tocopherol/gamma-tocopherol ratio, but not with alpha-tocopherol and gamma-tocopherol, in a cross-sectional study, including 278 postmenopausal women, in a multilinear regression model, adjusted for lipids, age, and blood pressure, emphasizing the importance of the balance between the two vitamin E isomers in maintaining arterial compliance [103].

Short term supplementation with 1,000 IU of vitamin E improved endothelial function but not systemic arterial compliance in young patients with type 1 diabetes mellitus [47]. The improvement in endothelial vascular function was related to a reduction of LDL oxidation [47], and there is evidence that oxidized LDL might inactivate endothelial cell derived relaxing factor [104]. The endothelium, through the generation of vasoactive mediators (NO and endothelin), might influence arterial stiffness [105]. Wigg et al. demonstrated improved endothelium-dependent and independent vasodilation in mesenteric arteries from diabetic rats, associated with PKC, after vitamin E, independent of advanced glycation end-product accumulation [106]. In the femoral artery, vitamin E prevented the wall stiffening associated with diabetes [106]. The benefit may be due to a direct effect of vitamin E on smooth muscle function as a consequence of inhibition of PKC-β2 isoform and due to improved NO availability in the smooth muscle [106]. Vitamin E supplementation significantly improved the endothelial function, despite

heterogeneity of the studies, especially in study participants with a lower baseline plasma vitamin E concentration (less than 20 μM), and did not depend on age, vitamin E dose, and duration of supplementation [99].

Rasool et al. used low, medium, and high doses of vitamin E as self-emulsifying tocotrienol rich vitamin E, for 2 months, in 36 healthy men and reported a significant reduction of PWV and augmentation index for the groups 100 and 200 mg, and no change in blood pressure, serum total, and LDL cholesterol [41]. A systematic review and meta-analysis revealed a small protective effect of antioxidant vitamins (vitamins C, E, A, and beta-carotene) on arterial stiffness, especially in younger healthy participants compared to those with cardiometabolic disease, more important in subjects with lower baseline plasma level of vitamins C and E [102]. The beneficial effects of the combined treatment with antioxidant vitamins C and E are due to the reduction of the damaging effects of free radicals on the vascular components and the anti-inflammatory effect, increasing the bioavailability of NO, improving endothelium-dependent vasodilation, and decreasing arterial stiffness and protecting the integrity of the vascular wall [43, 102]. The rate constant of the reaction of antioxidant vitamins and superoxide is lower than that of the reaction between NO and superoxide, which makes the antioxidant vitamins ineffective in protecting NO from free radical inactivation [99]. Additionally, systemic chronic inflammation may accelerate aging due to reactive oxygen species-mediated exacerbation of telomere dysfunction and cell senescence [107]. Natural antioxidants such as vitamins C and E, the beneficial components of fruits and vegetables, may exert toxic prooxidant activities at higher doses or under certain conditions [98], vitamin E being able to depress myocardial function [108]. Alpha-tocopherol may suppress other fat soluble, more powerful antioxidants such as gamma-tocopherol, increasing vulnerability to oxidative damage [109]. Miller III et al. concluded, in a meta-analysis, that there is a *dose-dependent* relationship between vitamin E supplementation and all-cause mortality and that high doses of vitamin E (≥400 IU) may increase all-cause mortality and should be avoided [97]. All-cause mortality progressively increased for doses exceeding 150 IU/day, substantially lower than the tolerable upper intake level for vitamin E (1,500 IU natural or 1,100 IU of synthetic vitamin E) [97]. No relation was observed between brachial-ankle PWV and alpha-tocopherol in a study including 178 Japanese male workers [110].

Shargorodsky et al. demonstrated that combined antioxidant supplementation with vitamins C and E, coenzyme Q10, and selenium improves glucose and lipid metabolism, blood pressure, and arterial compliance in patients with at least 2 cardiovascular risk factors [16].

In the study of Veringa et al., 93 patients with chronic kidney disease received pravastatin, vitamin E, and homocysteine lowering therapy, resulting in significant improvement of arterial compliance and distensibility, but the effect of vitamin E is not clear because it was combined with pravastatin [40].

Vucinovic et al. demonstrated that the acute intake of an antioxidant cocktail, including vitamins C and E, preserved bioavailability of NO and vascular function against hyperoxia-induced oxidative stress [37] (Table 3). Combined supplementation of vitamins C and E was ineffective in improving endothelial function in 14 randomized trials, including 597 participants, regardless of age, duration, dose, or baseline plasma concentration of vitamins [99]. Park et al. demonstrated that higher intake of beta-carotene, vitamins C, E, and folate may protect individuals genetically vulnerable to stiffening of the arteries, in a study including 3,198 healthy men and women from the Korea Multirural communities, quantifiyng dietary intakes by a food frequency questionnaire [111].

Concluding, vitamin E supplementation is supposed to decrease arterial stiffness due to its antiatherogenic and antioxidant effects and the ability to restore endothelial function, but its prooxidant effect must be considered at higher doses. Meta-analyses failed to confirm the role of antioxidant supplementation in primary and secondary cardiovascular prevention and recommend combined use of antioxidants [112]. Several limitations have been noticed in studies evaluating the effect of antioxidant vitamins on arterial stiffness and cardiovascular risk, such as a small sample size, heterogenous study populations, lack of objective criteria to include participants as healthy, differences in definition criteria of patients who are potential candidates for antioxidant therapy, different type and dosage of antioxidants, low correlations between dietary vitamins C and E intake, and plasma levels, reflecting the innacuracy of dietary questionnaires in the assessment of intake, individual variations in vitamin absorption and metabolism, missing plasma levels of antioxidants, and data regarding treatment compliance [16, 102, 108]. When excluding small studies (with less than 20 participants), antioxidant vitamin supplementation in larger studies significantly decreased arterial stiffness [102].

5. Vitamin C

Ascorbic acid is the cofactor of hydroxyproline synthesis, stabilizing the triple helix structure of *collagen*. Vitamin C is also a potent water-soluble *antioxidant*, enabling scavenging of superoxide anions and other reactive oxygen species [38], and prevents LDL oxidation, through recycling of alpha-tocopherol or by directly scavenging free radicals [46]. Arterial stiffness is impaired by oxidative stress and negative correlations were obtained between PWV and superoxide dismutase level [113]. PWV was dependent on the free radical/antioxidant Redox balance and nitric oxide bioavailability in patients with chronic obstructive pulmonary disease, and PWV increased after an antioxidant cocktail including vitamins C and E and alpha-lipoic acid [39] (Table 3). Results of antioxidant using trials, as an intervention in cardiovascular disease, have been mixed, but it is important to mention that most trials used a single antioxidant. Kelly et al. reported no effect of a single dose of oral vitamin C on augmentation index and several markers of oxidative stress, including DNA base oxidation products, in 26 healthy volunteers [42]. On the other hand, Katayama et al. suggested that oral vitamin C administration prevents smoking-induced acceleration in arterial stiffness through *reducing endothelial dysfunction*, but

Table 3: Effect of vitamin E and C supplementation and arterial stiffness in human subjects.

Authors	Year of publication	Follow-up	Dose of vitamin E and C	Study population	Results
Vucinovic et al. [37]	2015	1 week	600 IU vitamin E 1,000 mg vitamin C 600 mg alpha-lipoic acid	12 healthy males	Hyperoxia resulted in increased augmentation index and lipid peroxides and decreased nitrite in placebo; not in the antioxidant group
Hildreth et al. [38]	2014	—	Infusion of 7.5 g ascorbic acid	97 healthy women (premenopausal, perimenopausal and postmenopausal)	Improvement of arterial compliance in late perimenopausal and postmenopausal women
Ives et al. [39]	2014	90 minutes	Oral antioxidant cocktail (1) Dose: 300 mg alpha lipoic acid, 500 mg vitamin C, 200 IU vitamin E (2) Dose: the same doses of alpha lipoic acid and vitamin C, 400 IU vitamin E	30 patients with chronic obstructive pulmonary disease	Vascular dysfunction mediated by an altered redox balance can be mitigated by an oral antioxidant; the antioxidant cocktail improved also PWV
Veringa et al. [40]	2012	18 months	Pravastatin supplemented with vitamin E after 6 months and homocysteine lowering therapy after other 6 months	93 chronic kidney disease patients	Significant improvement of compliance and distensibility in the common carotid and femoral artery
Shargorodsky et al. [16]	2010	6 months	1,000 mg vitamin C, 400 IU vitamin E, 120 mg co-enzyme Q, 200 mcg selenium	70 patients with multiple cardiovascular risk factors (at least 2)	Significant increase of large and small vessel elasticity
Rasool et al. [41]	2008	2 months	50, 100 and 200 mg/day tocotrienol rich vitamin E	36 healthy men	Improvement of arterial compliance after 100 and 200 mg/day tocotrienol rich vitamin E; NO effect on serum lipids
Kelly et al. [42]	2008	8 hours	Oral dose of 2 g vitamin C	26 healthy human volunteers	No effect on augmentation index and markers of oxidative stress
Plantinga et al. [43]	2007	8 weeks	400 IU vitamin E 1 g vitamin C	30 male with essential hypertension	Beneficial effects on endothelium-dependent vasodilation and arterial stiffness
Katayama et al. [44]	2004	2 hours	Single dose 2 g vitamin C before smoking	17 healthy male volunteers	Significant reduction of smoking-induced elevation of brachial-ankle PWV
Mullan et al. [45]	2004	120 minutes	2 g i.v. ascorbic acid	12 healthy men	Pretreatment with ascorbic acid prevented the hyperglycemia induced increase of the central aortic pulse pressure and blood pressure
Mullan et al. [46]	2002	4 weeks	Oral 500 mg ascorbic acid/day	30 patients with type 2 diabetes mellitus	lowered blood pressure, decreased arterial stiffness
Skyrme-Jones et al. [47]	2000	3 months	1,000 IU/day oral vitamin E	41 young diabetic subjects (type 1 diabetes mellitus)	Improvement of endothelial vasodilation; no effect on systemic arterial compliance

does not influence heart rate and blood pressure [44]. The long-term effect in smokers is not beneficial on endothelial function [114] and smoking negatively influenced vitamin C level [115]. The use of an antioxidant cocktail, containing both water and fat soluble vitamins, seems to be more beneficial for vascular stiffness [39]. Acute administration of vitamin C has also been previously reported to lower augmentation index in healthy volunteers [116].

Peripheral and central hemodynamic changes noticed in acute systemic hyperglycemia, may be prevented or attenuated by pretreatment with a 2 g intravenous bolus of ascorbic acid according to a study including 12 healthy men [45]. Ascorbic acid may reverse impaired endothelium-dependent NO-mediated vasodilation in several conditions, including acute hyperglycemia and chronic renal failure [45, 117]. The main mechanisms of *NO increase* include reduced NO degradation by free radicals considering ascorbic acid, as an extremely potent free radical scavenger, increase of endothelial NO synthase activity, increase in the intracellular content of tetrahydrobiopterin, reduced insulin resistance, or smooth muscle sensitivity to NO [45, 46, 116, 118, 119]. Diabetes mellitus is associated with endothelial dysfunction due to several factors, including hyperglycemia, insulin resistance, hyperlipidemia, oxidized LDL and ascorbic acid deficiency (due to an impaired vitamin C recycling), and arterial stiffness [46]. Oral administration of 500 mg ascorbic acid/day, for 4 weeks, reduced arterial stiffness in diabetic patients, suggesting a functional change, probably due to increased NO [46].

Vitamins C and E increased the collagen content of the arterial wall and reduced vascular metalloproteinase-1, which explains their role in the *structural remodeling of the vessel wall* and stabilizing the atherosclerotic plaque [100].

Stiffening of large arteries increases progressively during menopause, mediated by estrogen deficiency, oxidative stress, and reduction of NO bioavailability [38]. Infusion of supraphysiological doses of ascorbic acid increased carotid artery compliance in late perimenopausal and postmenopausal women, but not in premenopausal women; the carotid artery compliance was not restored to premenopausal levels [38]. Late perimenopause is associated with changes in lipid metabolism and cardiovascular risk factors, known amplifiers of oxidative stress and arterial stiffening [38, 120]. Incomplete suppression of reactive oxygen species (ROS) by ascorbic acid and involvement of other sources of ROS, such as peroxynitrite, could explain why artery compliance was not restored to premenopausal levels [38].

High doses of vitamin C may have a *prooxidant* effect [98] and may impair arterial stiffness. On the other hand, only supraphysiological concentrations of ascorbate may prevent the interaction of superoxide and nitric oxide [46, 121]. Higher vitamin C levels were associated with lower levels of *inflammatory markers*, fasting blood glucose, and improved endothelial function [108, 122]. Supplementation with vitamin C alone improved endothelial function, especially in study participants older than 56 years; no significant modifying effects of the dose or duration of vitamin C supplementation on endothelial function were found [99]. Older people are more likely to have inadequate micronutrient intakes and absorption and greater oxidative stress due to age-related mitochondrial dysfunction, with a greater benefit from vitamin C supplementation [99].

Concluding, vitamin C supplementation may reduce arterial stiffness by stabilizing the atherosclerotic plaque and its antoxidant and anti-inflammatory effect and by improving endothelial function.

6. Vitamin A

Vitamin A includes several organic compounds, such as retinol, retinoic acid, and carotenoids (lycopene, lutein/zeaxanthin, beta-carotene, alpha-carotene, and beta-cryptoxanthin). Besides vitamin A importance in good vision and immune system, it is included in the group of *antioxidant* vitamins [102]. High serum concentrations of carotenoids, abundant in many fruits and vegetables, associated with the Mediterranean diet, may be protective against early atherosclerosis, by *inhibiting LDL oxidation* [123, 124]. High serum concentrations of lycopene and alpha- and beta-carotene were associated with reduced intima-media thickness progression during 7-year follow-up. Vessel walls of carotid arteries were more elastic in subjects whose diets were rich in carotenoids according to the ARIC study [125]. Rissanen et al. found also low plasma lycopene levels associated with early atherosclerosis, manifested as increased intima-media thickness of the common carotid artery wall, in 520 middle-aged men and women living in eastern Finland [126].

Due to the antioxidant activity that attenuates the inflammatory atherosclerotic process, the carotenoids *delay vascular aging* due to several mechanisms: their antioxidant activity (that attenuates the inflammatory atherosclerotic process), the ability to *increase bioavailability of NO*, the *improvement of the metabolic profile*, and their LDL lowering effect [127].

Free radicals, resulting from smoking, deplete serum carotenoid levels, especially alpha- and beta-carotene, lutein/zeaxanthin, and beta-cryptoxanthin [115]. Vitamin A supplementation may slow progression of atherosclerosis, by reducing the production of the *inflammatory cytokine* IL-17 and retinoid-related orphan receptor-c gene expression, the main transcriptor factor that controls Th17 cells differentiation [128].

In other words, vitamin A supplementation enables destiffening due to its antioxidant and anti-inflammatory effect and by improving endothelial function and metabolic profile. Carotenoid utilization failed to decrease the rate of major cardiovascular events in randomized trials, and their role in secondary cardiovascular prevention is not clear [127], but further follow-up studies are needed in order to confirm its importance.

7. Vitamin B12

Vitamin B12 (cobalamin) is an essential, water-soluble nutrient, involved in DNA synthesis [19]. Vitamin B12 deficiency, very prevalent in Europe, is caused mainly by vegetarianism and is known to be associated with megaloblastic anemia and neuropathy. Metformin therapy, a first line therapy in type 2

Table 4: Effect of vitamin B12 supplementation on arterial stiffness in human subjects.

Authors	Year of publication	Follow-up	Dose of vitamin B12	Study population	Results
Van Dijk et al. [48]	2015	2 years	500 μg B12 vitamin	569 hyperhomocystenemic elderly	No effect on PWV or carotid intima-media thickness
Koyama et al. [49]	2010	3 weeks	500 mug methylcobalamin and 15 mg/day folate 3 times weekly	20 patients undergoing hemodialysis	Decreased arterial stiffness

diabetes mellitus, reduces the circulating B12 levels by 25% [129].

Several studies investigated the effect of vitamin B12 level and supplementation and cardiovascular health and arterial stiffness. Previous results suggested also associations between vitamin B12 level and *adverse serum lipid profiles* in patients with type 2 diabetes mellitus, especially with triglycerides and cholesterol/HDL ratio, due to inhibition of carnitine palmitoyl transferase, the rate-limiting enzyme of fatty acid oxidation [129]. Vitamin B12 deficiency caused also *elevation of homocysteine*, a risk factor for cardiovascular disease, by inhibiting its conversion to methionine [19]. It has been hypothesized that low B12 vitamin increases cardiovascular risk, partly through direct effects [130].

Su et al. found no significant differences of arterial function parameters between postmenopausal vegetarians and omnivores [131]. Just brachial artery resistance was lower in vegetarians [131].

Van Dijk et al. did not report any effect of vitamin B12 and folic acid supplementation on PWV or carotid intima-media thickness in hyperhomocysteinemic elderly patients [48] (Table 4).

Koyama et al. found decreased arterial stiffness, associated with decreased serum asymmetric dimethylarginine in 20 patients undergoing hemodialysis after supplementation with folate and methylcobalamin [49]. Vitamin B12 levels were marginally associated with PWV in 86 patients with diabetes mellitus [132].

Vitamin B12 supplementation enables destiffening by improving the lipid profile and reducing the homocysteine level. A systematic review of cohort studies concluded that current data do not support vitamin B12 supplementation to reduce cardiovascular risk [130]. Further long-term follow-up studies should focus on specific populations in order to confirm destiffening through vitamin B12 supplementation, not just in patients undergoing hemodialysis.

8. Conclusions

There is a complex relationship between vitamin status and arterial stiffness, and each vitamin has specific effects on the vascular wall. Vitamin supplementation may be an effective and inexpensive adjunctive therapy in several conditions associated with increased arterial stiffness and they should be implemmented in patients' diet, considering individual vitamin status.

Vitamin D deficiency, involved in the pathophysiology of cardiovascular disease, may be an important therapeutic target. Despite heterogeneity and conflicting results of trials on vitamin D supplementation, arterial stiffness was significantly decreased in children with chronic kidney disease, black adolescents, adults with vitamin D deficiency with or without prehypertension, nondiabetic patients with hyperglycemia or positive diabetes score, and type 2 diabetic patients with nephropathy. Further large, randomized, evidence based, follow-up studies, including subjects with several other disorders, will demonstrate if vitamin D level is a marker of subclinical atherosclerosis, an effective target in cardiovascular prevention, therapy, destiffening, and vascular protection, or just a marker of poor health status, and which is the most effective form and level of vitamin D.

Vitamin K was beneficial in decreasing arterial stiffness in healthy postmenopausal women, patients with a history of vascular or coronary artery disease, vitamin E in subjects with type 1 diabetes mellitus and chronic kidney disease, and vitamin C in smokers, late perimenopausal, and postmenopausal women, and patients with type 2 diabetes mellitus. The combination including vitamin C and E could play an important role in cardiovascular disease prevention in young participants with lower baseline plasma levels, resulting in decreased arterial stiffness in patients with chronic obstructive pulmonary disease and essential hypertension. Further studies are needed in order to explore the effect of vitamin A supplementation on arterial stiffness, considering the antioxidant effect of vitamin A, its effect on endothelial function, metabolic profile, and its anti-inflammatory effect. Vitamin B12 supplementation was demonstrated to reduce arterial stiffness in patients undergoing hemodialysis.

The divergent results and mismatch between epidemiological and interventional studies warrant further investigation, but vitamins A, B12, D, K, C, and E may be markers of arterial stiffness and cardiovascular health. Cardiovascular prevention guidelines should consider and include trials with positive results. Vitamin K2 and low dose vitamin D have promising potential for prevention of vascular calcification. The potential public health importance of vitamin level and supplementation remains to be further tested in stratified intervention studies, and future research should focus on optimal vitamin levels and identifying patients who would benefit most from vitamin supplementation in order to enable individualized therapy, a personalised approach, and early interventions in primary, but also secondary prevention of cardiovascular disease.

Competing Interests

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

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Research Article

Parathyroid Hormone Levels in the Prediction of Ischemic Stroke Risk

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Objective. It was examined whether PTH and 25-dihydroxyvitamin D (25(OH)D) levels, together or separately, are indicators of the risk of stroke. *Materials and Methods*. This prospective study was performed at two centers. In the study, 100 patients diagnosed with acute ischemic stroke and 100 control individuals in the same age range were examined. In addition to neurological examination, cranial imaging, extensive routine blood chemistry, PTH, and 25(OH)D levels were evaluated in all cases. Stroke risk factors were determined. Logistic regression was used for statistical analysis. *Results*. A total of 60 patients and 79 control individuals were included in the study. Different estimation models were designed in order to examine the relationship between PTH and 25(OH)D levels with stroke. According to modeling results, it was determined that the most effective predictor for risk of stroke was 25(OH)D levels, followed by hypertension and PTH levels, respectively. *Conclusion*. PTH and 25(OH)D levels together can make important contributions to determination of stroke risk, and further investigations are needed to understand this relationship more fully.

1. Introduction

The key role of parathyroid hormone (PTH) in bone health and homeostasis is well known. However, recent studies have indicated that PTH has various effects on other organs and tissues like 25(OH)D. PTH shows the effect of PTH receptors in tissues through which receptors are expressed in smooth muscle cells on the vascular wall, endothelium, and myocardium [1]. Its level is frequently increased by 25(OH)D (25-dihydroxyvitamin D) deficiency or, to a lesser extent, chronic renal failure. However, some studies have demonstrated that elevated PTH levels are common even in healthy people with neither 25(OH)D deficiency nor chronic renal failure [2]. Elevated PTH levels raise blood pressure and cardiac contractility, resulting in fibrosis, apoptosis, and hypertrophy in cardiomyocytes of the left ventricle and vascular smooth muscle cells [3]. Several recent studies have demonstrated that it is associated with various cardiovascular conditions such as endothelial dysfunction, vascular stiffness, and calcification [4], increased aortic pulse pressure [5], reduced great artery elasticity [6], coronary microvascular dysfunction, and hypertension [7]. Furthermore, it has also been shown that increased PTH levels may affect the cardiovascular system by stimulating cytokine release from lymphocytes and vascular smooth muscle cells [1, 8]. It has been argued that PTH confers a risk for cardiovascular disease even at normal or slightly elevated levels and in the absence of mineral metabolism disorders. Since all these data suggest that increased PTH levels may increase risk for cerebrovascular disease, the aim of this study was to investigate the correlation between PTH levels and stroke and to study 25(OH)D because of its close correlation with PTH.

Table 1: Age distribution in the patient and control groups.

	Group	n	Mean	Std. deviation	Std. error mean	Test	p
Age	Patient	60	61.2833	6.55897	.84676	$t = -0.759$	0.449
	Control	79	62.2152	7.59668	.85469		

2. Materials and Methods

This prospective study was conducted in two separate centers between 2010 and 2014. The study group included 100 patients admitted to emergency department or outpatient clinics for symptoms of acute stroke and were diagnosed with acute ischemic stroke using cranial imaging methods. The control group consisted of 100 age-matched subjects with completely normal MRI cranial examination results, no history of stroke or transient ischemic attack, and no signs or symptoms of cerebrovascular disease. In both groups, subjects using supplemental calcium or vitamin D were excluded, as well as those with chronic renal failure, chronic liver disease, or osteoporosis.

In addition to obtaining a detailed medical history and performing a thorough neurological examination, either cranial magnetic resonance imaging (MRI) or computerized tomography (CT) was obtained for differential diagnosis at admission in all subjects. Age, gender, arterial blood pressure, and electrocardiography were recorded for all subjects in both groups. Additionally, comprehensive blood biochemistry and levels of 25(OH)D and PTH were measured in all subjects. Risk factors for stroke (hypertension (HT), diabetes mellitus (DM), atrial fibrillation (AF), coronary artery disease (CAD), history of myocardial infarction (MI), tobacco or alcohol use, and obesity) were recorded. Blood samples were obtained by venipuncture after an overnight fast and placed into tubes that were protected from sunlight. Sera were separated and stored at −80°C within 30 min of collection. Serum PTH levels were determined by a chemiluminescent microparticle immunoassay (CMIA) method in an Abbott Architect i2000 analyzer using the Abbott Architect Intact PTH assay kit; the reference range was set at 10–65 pg/mL. The same method was also used to determine 25(OH)D levels. The reference range for 25(OH)D was set at 10–55 μg/L.

This research project was approved by the local ethics committee.

2.1. Statistical Analysis. Since the data regarding ages and PTH levels was normally distributed, a two-sample independent t-test was used to compare the two groups (patient and control groups). 25(OH)D levels were not normally distributed, and thus the nonparametric Mann–Whitney U test was used in order to compare the two groups. Gender distribution between the two groups was evaluated using the chi-square test. Logistic regression analysis was used in order to properly estimate the stroke patients and healthy individuals ($n = 139$). Three appropriate logistic regression models were designed using efficient and significant predictors. In the first model, 25(OH)D and PTH levels were used for estimation of stroke risk. In the second model cardiac risk factors (AF, CAD, and MI) were used in addition to 25(OH)D and PTH levels for stroke risk estimation. In the third model, 25(OH)D and PTH levels as well as all cardiovascular risk factors were evaluated. For these models, the formula of hazard ratio (HR) was used. For example, HR for Model 1.3 is

$$\pi\left(\mathrm{OH}_{25}; \mathrm{PTH}\right) = 1 - \frac{e^{-0.757+0.113(\mathrm{OH}_{25})-0.013(\mathrm{PTH})}}{1+e^{-0.757+0.113(\mathrm{OH}_{25})-0.013(\mathrm{PTH})}}. \quad (1)$$

If 25(OH)D and PTH levels are 11.9 and 164.7 in the patient, respectively, ischemic stroke risk is estimated as 83.4%.

$$\begin{aligned}\pi\left(11.90; 164.7\right) &= 1 - \frac{e^{-0.757+0.113(11.9)-0.013(164.7)}}{1+e^{-0.757+0.113(11.9)-0.013(164.7)}} \\ &= 1 - 0.1659 = 83.4\%.\end{aligned} \quad (2)$$

If 25(OH)D and PTH levels are 19.20 and 60.3 in the patient, respectively, ischemic stroke risk is estimated as 35.3%.

$$\begin{aligned}\pi\left(19.20; 60.30\right) &= 1 - \frac{e^{-0.757+0.113(19.20)-0.013(60.30)}}{1+e^{-0.757+0.113(19.20)-0.013(60.30)}} \\ &= 1 - 0.6463 = 35.3\%.\end{aligned} \quad (3)$$

The study power for 25(OH)D measurements was 92%, using a standard deviation of 8.05 and difference value of 5, with 60 patients in each group. For PTH measurements, the study power was 79% using a standard deviation of 38.91, difference value of 20, and 59 patients in each group. Minitab Release 14.0 and SPSS 15.0 for Windows (SPSS, Inc., Chicago, IL) statistical programs were used for statistical analyses.

3. Results

This study included a total of 200 subjects: 100 patients with acute stroke and 100 control subjects. Subjects with problematic blood sampling, storage, or analysis for PTH and 25(OH)D levels were excluded from the study. Subjects with extreme measurement values who created heterogeneity in the distribution of both groups and those who were difficult to age-match between the groups (the extremely old or young) were excluded. Obese subjects and those with habits of tobacco or alcohol use were also excluded due to the small number and unequal distribution of these individuals across the groups. After the completion of the above exclusion procedures, data from a total of 60 patients and 79 control subjects remained in the data set used for 25(OH)D analysis. PTH analysis was performed after excluding 1 more subject with PTH levels of approximately 500 pg/mL.

No significant difference was found between the mean age and gender distribution of both groups (Tables 1 and 2).

Table 2: Gender distribution in the patient and control groups.

		Gender		Total
		Female	Male	
Patient	Count	25	35	60
	% within group	41.7%	58.3%	100.0%
Control	Count	32	47	79
	% within group	40.5%	59.5%	100.0%
Total	Count	57	82	139
	% within group	41.0%	59.0%	100.0%

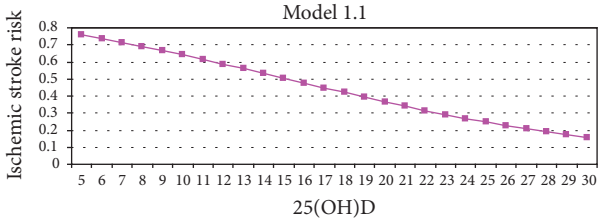

Figure 1

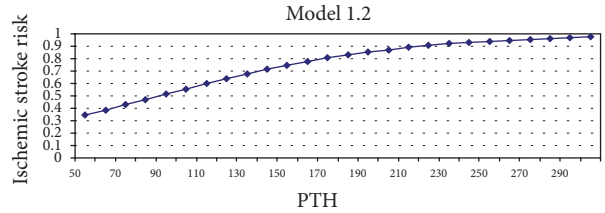

Figure 2

As shown in Table 3, the mean 25(OH)D level was significantly lower in the patient group (15.7±4.27) compared to the control group (20.1 ± 8.05) ($Z = 3.147$, $p = 0.002$). In contrast to the vitamin D level, the PTH level was significantly higher in the patient group (82.83 ± 38.91) compared to the control group (64.74 ± 28.80) ($t = -2.998$, $p = 0.002$). These two comparisons were strongly significant.

Different prediction models were used to examine the association between stroke and PTH, 25(0H)D levels. First, it was determined whether PTH and 25(OH)D could be used as markers for predicting stroke risk (Table 4). The abilities of 25(OH)D and PTH levels, both alone and in conjunction, to accurately predict stroke patients and healthy subjects were tested, as outlined by Model 1. According to this model, 25(OH)D levels had an accurate prediction rate of 48.3% for stroke patients and 70.9% for healthy subjects; it has an overall accurate prediction rate of 61.2% (wald = 12.215, $p = 0.000$). When the PTH level was used as the prediction marker, the accurate prediction rate was 35.6% for stroke patients, 81.8% for the controls, and 61.8% as an overall accurate prediction rate (wald = 8.129, $p = 0.004$). Models 1.1 and 1.2 are presented in Figures 1 and 2 graphically. When PTH and 25(OH)D were analyzed together (Model 1.3), the accurate prediction rate increased to 57.6% and the overall accurate prediction rate increased to 64%. The 20% increase in accurate prediction rate indicated that both factors were more effective for accurate prediction when used in conjunction (wald = 4.822, $p = 0.028$). Ten percent of 30 patients incorrectly diagnosed by 25(OH)D were accurately categorized by PTH (Model 1.4) in a statistically significant manner (wald = 3.911, $p = 0.048$).

25(OH)D and PTH, both alone and in conjunction, were also used as prediction tools in the presence of cardiac risk factors in Model 2 (Table 5). In this model, 25(OH)D was used in conjunction with risk factors such as CAD, MI, and AF (Model 2.1). The accurate prediction rate of the model was 53.3% ($p = 0.001$) for the stroke patients and 76.9% (p = NS) for the healthy subjects. When used in conjunction with cardiac risk factors, the accurate prediction rate of PTH was 45.8% ($p = 0.41$) (Model 2.2). This result was also statistically significant, although it was weaker than that of 25(OH)D. The accurate prediction rate for the healthy subjects was 90.8% (p = NS). When both 25(OH)D and PTH were used in conjunction with other cardiac risk factors (Model 2.3), the accurate prediction rate was the same as that obtained without taking cardiac risk factors into account (57.6%) ($p = 0.99$). Among all risk factors considered, CAD significantly contributed to accurate prediction. However, this effect was significantly weaker than those of 25(OH)D and PTH. Of 28 patients not accurately predicted by an analysis using cardiac risk factors and 25(OH)D together, only 7.1% were accurately predicted by PTH and IHD, and this difference did not reach statistical significance.

Model 3 used 25(OH)D and PTH in conjunction with other cardiovascular risk factors (HT, DM, and lipid levels) in addition to the cardiac risk factors studied in Model 2. Table 6 shows the effect and statistical significance of the predictors used in this analysis. The model in which 25(OH)D was used with all of these risk factors (Model 3.1) had an accurate prediction rate of 68.3% for the stroke patients. Compared to all other factors, 25(OH)D had the greatest effect on the accurate prediction rate ($p = 0.002$). The effect of HT was smaller, but statistically more significant than that of 25(OH)D ($p = 0.010$). Other risk factors had no effect on the accurate prediction rate. When PTH was used with all cardiovascular risk factors (Model 3.2) the accurate prediction rate was 59.3%. In this analysis, the effect of PTH on the result was statistically significant ($p = 0.019$), but less than that of HT. The most powerful effect in the analysis was that of HT ($p = 0.002$). Other risk factors had no effect on the result. When both 25(OH)D and PTH were used together with all other risk factors (Model 3.3), the accurate prediction rate was found to be 69.5%. In this analysis it was observed that both 25(OH)D and HT were two important indicators for predicting strokes, with the effect of 25(OH)D being more powerful than that of HT. Furthermore, effect of PTH was minimal and not statistically significant in this analysis. Model 3.4, in which PTH and other risk factors were used, was not successful for nineteen patients who were incorrectly diagnosed using 25(OH)D levels. In conclusion, a combined review of all analyses revealed that 25(OH)D was

Table 3: Comparison of 25(OH)D and PTH levels between the patients and controls.

	Group	N	Mean	Std. deviation	Test	Sig
25(OH)D	Patient	60	15.7117	4.27599	$Z = -3.147$	0.002
	Control	79	20.1608	8.05743		
PTH	Patient	59	82.8373	38.91614	$t = -2.998$	0.002
	Control	77	64.7416	28.80296		

Table 4: Rates of accurate prediction of patients and disease-free subjects by 25(OH)D and PTH level (Model 1).

	B	SE	Wald	df	Sig.	exp (B)	Accurate prediction rate of the patient group	Accurate prediction rate of the control group	Overall accurate prediction rate
Model 1.1									
25(OH)D	.113	.032	12.215	1	.000	1.119	48.3	70.9	61.2
Constant	−1.713	.577	8.814	1	.003	.180			
Model 1.2									
PTH	−.017	.006	8.129	1	.004	.983	35.6	81.8	61.8
Constant	1.477	.457	10.429	1	.001	4.379			
Model 1.3									
25(OH)D	.113	.034	10.903	1	.001	1.119	57.6	68.8	64.0
PTH	−.013	.006	4.822	1	.028	.987			
Constant	−.757	.790	.918	1	.338	.469			
Model 1.4 (the classification of 30 patients unclassified by Model 1.1)									
PTH	−.012	.006	3.911	1	.048	.988	10.0	98.7	73.8
Constant	1.810	.499	13.149	1	.000	6.111			

the most important factor for stroke prediction, followed by HT and PTH, in descending order. The effect of CAD was minimal and not statistically significant.

4. Discussion

In examining the association between serum PTH levels and cerebrovascular disease, as well as the contribution of 25(OH)D, this study made some important conclusions. First, serum PTH levels were significantly higher and 25(OH)D levels were significantly lower in the patient group compared to the control group. Numerous studies have stressed the association between serum 25(OH)D levels, cardiovascular disorders [9, 10], and mortality [11]. It has also been shown that 25(OH)D levels are lower in patients with stroke [12, 13]. Moreover, 25(OH)D deficiency increases risk of stroke and even affects stroke prognosis [14], causing more stroke-related fatal outcomes [15]. In contrast, the number of studies examining the association between increased PTH levels and cardiovascular diseases [6, 16], mortality [11, 17], and particularly, stroke [18, 19] is quite limited. Of these studies, one conducted by Sato et al. detected a decrease in serum 25(OH)D levels and BMD values and an increase in serum ionized calcium and PTH levels in female subjects with ischemic stroke. In the patient group the incidences of hypertension and coronary artery disease were higher than in the control group, as was the prevalence of lacunar infarcts; that group also entered menopause earlier [20]. A study examined the association between stroke and PTH in patients with hypercalcemia, and elevated PTH levels were associated with stroke, the incidence of which was 7.1% in patients with primary hyperparathyroidism [19].

The second finding of our study was that both PTH and 25(OH)D levels were two important markers that could be used to predict the future risk of stroke. Of these two predictors, 25(OH)D was a fairly powerful marker, while PTH was weaker, although still statistically. When both predictors were used together, the accurate prediction rate for stroke was further increased. This demonstrated that PTH is an important marker for defining stroke risk, but its predictive power is enhanced when used in conjunction with 25(OH)D. This suggests that combined use of these two markers would be a more powerful marker in risk assessment.

There exist some studies relating low 25(OH)D levels to increased risk for all cardiovascular disorders, including

Table 5: Rates of accurate prediction of patients and disease-free subjects by 25(OH)D and PTH used in conjunction with cardiac risk factors (Model 2).

	B	SE	Wald	df	Sig.	exp (*B*)	Accurate prediction rate of the patient group	Accurate prediction rate of the control group	Overall accurate prediction rate
Model 2.1									
25(OH)D	.130	.039	11.214	1	.001	1.139			
CAD(1)	1.169	.567	4.245	1	.039	3.217			
MI(1)	21.211	12641.233	.000	1	.999	1628236695.777	53.3	76.9	66.7
AF(1)	21.822	12669.616	.000	1	.999	3001279038.402			
Constant	−45.710	17897.485	.000	1	.998	.000			
Model 2.2									
PTH	−.013	.006	4.162	1	.041	.987			
IHD(1)	1.319	.561	5.527	1	.019	3.738			
MI(1)	20.396	13141.236	.000	1	.999	721159600.690	45.8	90.8	71.1
AF(1)	21.526	13937.969	.000	1	.999	2232101328.556			
Constant	−41.590	19156.176	.000	1	.998	.000			
Model 2.3									
25(OH)D	.132	.041	10.554	1	.001	1.141			
PTH	−.009	.007	1.773	1	.183	.991			
CAD(1)	1.134	.582	3.795	1	.051	3.108	57.6	81.6	71.1
MI(1)	20.780	12788.170	.000	1	.999	1058694576.339			
AF(1)	21.873	13324.999	.000	1	.999	3156046476.935			
Constant	−44.722	18468.707	.000	1	.998	.000			
Model 2.4 (the classification of 28 patients unclassified by Model 2.1)									
PTH	−.012	.007	2.694	1	.101	.988			
IHD(1)	.361	.741	.237	1	.626	1.435	7.1	98.7	74.3
Constant	1.513	.804	3.538	1	.060	4.540			

stroke [10, 21, 22], MI, and carotid atherosclerosis [14, 23]. However, studies have predominantly reported that increased PTH caused vascular abnormalities rather than stroke and mentioned it as a risk factor for cardiovascular disorders.

In two Swedish population studies of more than 1000 patients over 70 years of age, Hagström et al. reported that PTH was a powerful predictor for both clinical and subclinical atherosclerosis. In this study, plasma PTH levels above 50 pg/mL conferred a 20% risk for cardiovascular mortality [24]. In addition, several studies have supported the notion that increased PTH levels cause atherosclerosis or vessel wall dysfunction [16, 25, 26].

Anderson et al. found that PTH levels were higher in subjects with increased prevalence of cardiovascular risk factors (HT, DM). They also observed that PTH levels were elevated before other risk factors were apparent, which suggests that PTH may contribute to the development of such risk factors [2]. Another study found that increased serum PTH levels were correlated to the number of stenotic arteries, HT, and low ejection fraction.

In a population-based study by Wannamethee et al. examining the association between heart failure (HF) and PTH, increased PTH levels were correlated with HF risk, although such a risk was not related to mineral metabolism and 25(OH)D. This was explained by the hypothesis that, in the absence of chronic renal failure, PTH exerts its cardiac actions via PTH receptors found in myocardium [1]. In a study by Bansal et al. increased serum PTH levels were correlated with increased risk of HF and left ventricular mass, although such an association was absent for 25(OH)D [27].

Multiple studies have shown that PTH was predictive for vascular disease and death associated with disorders of mineral metabolism including primary and secondary hyperparathyroidism and CRF [18, 28, 29]. Hagström et al. explained the association between PTH and atherogenesis in the following way: vascular calcification and remodeling result from direct PTH receptor interaction on the vessel wall, indirect inflammation, and vascular dysfunction. In addition, increased PTH levels are associated with inflammation markers, which are now considered cardiovascular risk

Table 6: Rates of accurate prediction of patients and disease-free subjects by 25(OH)D and PTH used in conjunction with all cardiac risk factors (Model 3).

	B	SE	Wald	df	Sig.	exp (B)	Accurate prediction rate of the patient group	Accurate prediction rate of the control group	Overall accurate prediction rate
Model 3.1									
D_VIT_OH_25	.129	.042	9.325	1	.002	1.137			
CAD(1)	.472	.642	.539	1	.463	1.603			
MI(1)	21.250	12597.619	.000	1	.999	1692925884.235			
AF(1)	21.894	12648.666	.000	1	.999	3224649359.264			
HT(1)	1.251	.484	6.691	1	.010	3.495	68.3	80.8	75.4
DM(1)	−.255	.530	.232	1	.630	.775			
TRG	−.005	.004	2.421	1	.120	.995			
HDL	−.011	.025	.188	1	.665	.989			
LDL	.006	.007	.909	1	.340	1.006			
Constant	−44.958	17851.854	.000	1	.998	.000			
Model 3.2									
PTH	−.017	.007	5.467	1	.019	.983			
CAD(1)	.649	.623	1.083	1	.298	1.913			
MI(1)	20.437	12784.724	.000	1	.999	751248842.438			
AF(1)	21.428	13795.882	.000	1	.999	2023693999.998			
HT(1)	1.468	.470	9.770	1	.002	4.341	59.3	82.9	72.6
DM(1)	.294	.529	.309	1	.579	1.342			
TRG	−.003	.003	.752	1	.386	.997			
HDL	.017	.025	.453	1	.501	1.017			
LDL	.004	.007	.391	1	.532	1.004			
Constant	−42.241	18808.915	.000	1	.998	.000			
Model 3.3									
D_VIT_OH_25	.126	.044	8.225	1	.004	1.135			
PTH	−.012	.008	2.377	1	.123	.988			
CAD(1)	.467	.659	.503	1	.478	1.595			
MI(1)	20.884	12515.881	.000	1	.999	1174192607.473			
AF(1)	21.839	13255.218	.000	1	.999	3050522065.125			
HT(1)	1.403	.501	7.848	1	.005	4.067	69.5	80.3	75.6
DM(1)	.155	.561	.077	1	.782	1.168			
TRG	−.003	.004	.924	1	.337	.997			
HDL	.000	.027	.000	1	.995	1.000			
LDL	.006	.007	.696	1	.404	1.006			
Constant	−44.764	18230.411	.000	1	.998	.000			
Model 3.4 applied to 19 patients remained from Model 3.1									
PTH	−.011	.009	1.648	1	.199	.989			
CAD(1)	.625	.856	.533	1	.465	1.868			
HT(1)	.243	.556	.190	1	.663	1.275			
DM(1)	−.640	.762	.704	1	.401	.527	0.0	100.0	80.2
TRG	−.003	.004	.410	1	.522	.997			
HDL	−.006	.030	.038	1	.846	.994			
LDL	.001	.008	.006	1	.937	1.001			
Constant	2.574	1.971	1.706	1	.192	13.117			

factors [29]. The observation of a decreased incidence of CV disorders after the reduction of PTH levels by parathyroidectomy, renal transplantation, or calcimimetic agents supports the causal role of PTH in the development of CV disorders [29].

The third result of our study was that PTH, 25(OH)D, and HT were the most powerful markers for the prediction of stroke risk, even when all other cardiovascular risk factors were included in the analysis. When these three markers were compared with one another, 25(OH)D was the most powerful predictor, followed by HT and PTH in descending order. The weakest predictor was CAD, which has no significant predicting ability in the presence of the above three markers. Schierbeck et al. found that both PTH and vitamin D were independently associated with both cardiovascular and all-cause mortality [30]. These findings suggest that although both vitamin D and PTH appear to be separate risk factors for both stroke and all cardiovascular disorders combined, examining the two predictors in conjunction provides more accurate risk assessment for cardiovascular disorders. Our study limitation was the exclusion of an older patient group from the analysis to ensure equality in patient and control groups, which led to a relatively younger study population and, therefore, a potential bias.

In conclusion, PTH levels were increased, while 25(OH)D levels were decreased in patients with stroke. Both PTH and vitamin D appear to be separate risk factors for stroke. 25(OH)D was the most powerful marker for the predicting the stroke risk, followed by HT and PTH, in descending order. In addition to 25(OH)D, PTH serum levels should be considered, and both predictors should be assessed in conjunction for more accurate determination of stroke risk. Additional studies are needed to investigate the effect of PTH on stroke risk, the interaction of both predictors, and possible conditions that may develop as a result of dysregulated vitamin D and PTH synthesis.

Competing Interests

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

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Research Article

Insulin Resistance in Adipose Tissue but Not in Liver Is Associated with Aortic Valve Calcification

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Background. Insulin resistance is involved in the pathogenesis of cardiovascular disease, but its relationship with cardiovascular calcification has yielded conflicting results. The purpose of the present study was to investigate the role of hepatic and adipose tissue insulin resistance on the presence of coronary artery (CAC > 0) and aortic valve calcification (AVC > 0). *Methods*. In 1201 subjects (52% women, 53.6 ± 9.3 years old) without familiar and personal history of coronary heart disease, CAC and AVC were assessed by multidetector-computed tomography. Cardiovascular risk factors were documented and lipid profile, inflammation markers, glucose, insulin, and free fatty acids were measured. Hepatic insulin resistance (HOMA-IR) and adipose tissue insulin resistance (Adipo-IR) indices were calculated. *Results*. There was a significant relationship between HOMA-IR and Adipo-IR indices ($r = 0.758$, $p < 0.001$). Participants in the highest quartiles of HOMA-IR and Adipo-IR indices had a more adverse cardiovascular profile and higher prevalence of CAC > 0 and AVC > 0. After full adjustment, subjects in the highest quartile of Adipo-IR index had higher odds of AVC > 0 (OR: 2.40; 95% CI: 1.30–4.43), as compared to those in the lowest quartile. *Conclusions*. Adipo-IR was independently associated with AVC > 0. This suggests that abnormal adipose tissue function favors insulin resistance that may promote the development and progression of AVC.

1. Introduction

Aortic valve calcification (AVC) is defined as calcified and thickened aortic leaflets that do not impair the blood flow [1]. It is the most common heart valve disorder, increases with age, and may reflect a generalized process of atherosclerosis [2, 3]. Comparable to AVC, coronary artery calcification (CAC) is a specific atherosclerosis marker that correlates with plaque burden and has been a good predictor of future cardiovascular outcomes in the general population [3]. Some studies have shown that AVC and CAC share mechanistic similarities such as inflammatory processes, oxidative stress, dyslipidemia, and endothelial dysfunction [4, 5]. Most of these risk factors are systemic metabolic insults associated with the proatherogenic milieu of insulin resistance (IR) [6, 7]. IR is characterized by decreased insulin-mediated glucose disposal into peripheral tissues and has been commonly determined by the mathematical model described by Matthews et al. [8]. Using this model (HOMA-IR) some [9], but not all [10, 11], studies, have shown an association between IR and CAC. Similarly, although some recent reports have shown that IR, defined by high HOMA-IR, could play an important role in the mineralization of the aortic valve [1, 4, 6], other investigations showed that this association was not independent from cardiovascular risk factors [12].

Through the secretion of biological products such as free fatty acids, which impair glucose uptake by skeletal muscle, promote glucose production by the liver, and impair insulin release by pancreatic beta cells, adipose tissue has emerged as a key factor that contributes to the systemic IR development [13, 14]. Those adipocyte effects that can be measured as the product of fasting plasma free fatty acids by insulin concentration have been called adipose tissue insulin resistance (Adipo-IR) [15, 16]. Although Adipo-IR may contribute to the presence of cardiometabolic disorders [15–17], its role on the AVC has not been previously studied. Therefore, the aim of the present study was to investigate the association of HOMA-IR and Adipo-IR index with subclinical cardiovascular disease assessed as the presence of CAC or AVC.

2. Methods

The study population was recruited from controls participating in the Genetics of Atherosclerotic Disease (GEA) study. The GEA study is a cross-sectional and observational trial designed to examine the genomic bases of coronary heart disease (CHD) and to assess relationships between traditional and emerging risk factors with clinical and subclinical atherosclerotic vascular disease in an adult Mexican population [18]. Briefly, a convenience sample of 1200 CHD patients and 1500 control subjects aged 35 to 70 years was recruited from residents in Mexico City (July 2008 through November 2012). Patients with well-established premature CHD were selected from the outpatient clinic of the National Institute of Cardiology. Premature CHD was defined as history of myocardial infarction, angioplasty, revascularization surgery, or coronary stenosis >50% on angiography, diagnosed before the age of 55 in men and before 65 in women. Volunteer control participants with a negative family history of premature CHD and no personal history of cardiovascular disease were recruited from apparently healthy blood donors and through brochures posted in social service centers. Coronary patients and control subjects with personal history of renal, liver, thyroid, or malignant disease, as well as those on treatment with corticosteroids, were excluded. The GEA study was approved by the institution's ethics committee on research on humans of the National Institute of Cardiology and conducted according to the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from each participant included in the study.

2.1. Clinical Assessment. This study is a cross-sectional analysis of 1201 GEA control participants. We excluded 299 subjects with missing data for CAC, AVC ($n = 271$), or plasma free fatty acids ($n = 28$). All subjects were interviewed by a trained research staff and completed questionnaires to collect information pertaining to demographic characteristics, CHD history, medication, alcohol, and tobacco use. Positive history of tobacco was considered when individuals self-reported current smoking (≥1 cigarette per day) [19]. Physical activity index was calculated using the Baecke questionnaire [20], and total activity was obtained from the sum of work and leisure time activities. This questionnaire has been previously validated in adult population and provides reliable information. All participants had a complete clinical examination. Height was measured to the nearest 1 cm using a rigid stadiometer, and weight was measured to the nearest 0.1 kg with the use of a balance scale. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Systolic and diastolic blood pressure was measured after subjects rest for at least 10 minutes, and the average of the second and third of three consecutive measurements was used for the analysis. The presence of type 2 diabetes was considered according to the American Diabetes Association criteria [21] and when participants reported glucose-lowering treatment or a physician's previous diagnosis.

2.2. Biochemical Analysis. Venous blood samples were collected from subjects after 10-hour fasting. Plasma glucose, total and high density lipoprotein cholesterol (HDL-C), triglycerides, creatinine, and free fatty acids (FFA) were measured in fresh samples, using standardized enzymatic procedures in a Hitachi 902 analyzer (Hitachi LTD, Tokyo, Japan). Accuracy and precision of lipid measurements in our laboratory are under periodic surveillance by the Center for Disease Control and Prevention service (Atlanta, GA, USA). Low density lipoprotein cholesterol (LDL-C) was estimated by using the DeLong et al. method [22] and glomerular filtration rate (eGFR) was computed with the Chronic Kidney Disease Epidemiology Collaboration creatinine equation [23]. Total high-sensitivity C-reactive protein (hsCRP) levels were determined by immunonephelometry on a BN ProSpec nephelometer (Dade Behring, Marburg, Hesse, Germany), according to the manufacturer method. Interassay coefficients of variation for all these assays were less than 6%. Plasma insulin concentrations were determined by a radioimmunometric assay (Millipore, St. Charles, Missouri, USA) and serum total adiponectin was measured with a Quantikine ELISA kit (R&D Systems, Boston, Massachusetts, USA). IR was estimated with the use of the homeostasis model assessment (HOMA-IR = insulin [μIU/mL] × glucose [mmol]/22.5) [8] or the validated Adipo-IR index (Adipo-IR = FFA [mmol/l] × insulin concentration [μIU/L]) [15, 16]. Because percentile values for IR differed between sex, HOMA-IR, and Adipo-IR, quartiles were separately estimated for men or women.

2.3. Computed Tomography. Computed Tomography (CT) is a validated method for measuring visceral adipose tissue [24], CAC [25], and AVC [26]. In the present study, CT of the abdomen and chest were performed using a 64-channel multidetector helical system (Somatom Cardiac Sensation 64, Forchheim, Bavaria, Germany) and interpreted by experienced radiologists. Scans were read to assess and quantify total, subcutaneous, and visceral abdominal adipose tissue as described by Kvist et al. [27], as well as CAC and AVC using the Agatston score [25]. All foci with attenuation > 130 Hounsfield units were considered to obtain the total Agatston score, which was obtained by adding up the scores of individual lesions in coronary arteries or aortic valves. The

Table 1: Characteristics of the study population.

$n = 1{,}201$	
Age (years)	53.6 ± 9.3
Gender (men, %)	576 (48)
BMI (kg/m^2)	28.5 ± 4.5
Visceral AT (cm^2)	151 (112–194)
Systolic blood pressure (mmHg)	118 ± 18
Diastolic blood pressure (mmHg)	72 ± 10
LDL-C (mmol/L)	3.08 ± 0.83
HDL-C (mmol/L)	1.19 ± 0.34
Triglycerides (mmol/L)	1.68 (1.28–2.28)
Fasting glucose (mmol/L)	5.05 (4.7–5.5)
HOMA-IR	4.09 (2.7–5.9)
Adipo-IR	9.65 (6.24 – 14.49)
hsCRP (nmol/L)	15.3 (8.2–32.0)
Adiponectin (μg/mL)	7.9 (4.9–12.8)
Physical activity index	7.88 ± 1.22
Current smoking, *n* (%)	270 (22.5)
Statin use, *n* (%)	106 (8.8)
Type 2 diabetes, *n* (%)	161 (13.4)
Coronary artery calcification, *n* (%)	318 (26.5)
Aortic valve calcification, *n* (%)	226 (18.8)

Values of quantitative variables are expressed as mean ± standard deviation or median (interquartile range) and qualitative variables as number of subjects (percentage). BMI: body mass index; AT: adipose tissue; LDL-C: low density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol; HOMA-IR: homeostasis model assessment of insulin resistance; Adipo-IR: adipose tissue insulin resistance; hsCRP: high sensitive C-reactive protein.

presence of calcification was considered with an Agatston score > 0. Twenty different scans were randomly selected to evaluate consistency of interpretation; the intraobserved coefficient correlation was 0.99 ($p < 0.001$).

2.4. Statistical Analysis. Statistical analyses were performed in the pooled sample (men and women), after stratifying for HOMA-IR quartiles (Q1: <2.78, Q2: 2.78–4.12, Q3: 4.13–6.01, and Q4: >6.01 for men; Q1: <2.71, Q2: 2.71–3.97, Q3: 3.98–5.86, and Q4: >5.86 for women) or Adipo-IR quartiles (Q1: <5.57, Q2: 5.57–8.58, Q3: 8.59–12.48, and Q4: >12.48 for men; Q1: <6.98, Q2: 6.98–10.9, Q3: 10.9–16.23, and Q4: >16.23 for women). Variables were analyzed for normal distribution and expressed as mean ± standard deviation, median (interquartile range), or number of subjects (%). Comparisons of means, medians, and frequencies were made with ANOVA, Kruskal-Wallis, and chi squared tests, respectively. The association of CAC or AVC with IR was assessed by logistic regression analyses, using CAC > 0 or AVC > 0 as the dependent variable and HOMA-IR quartiles or Adipo-IR quartiles as independent variables. In each case, first quartile was considered as referent group. To confirm the association of Adipo-IR with AVC > 0, a forward stepwise logistic regression analysis was performed. All adjustments were done using variables that show significant association with both indices (Table 1) and those with known biological role on cardiovascular calcification such as LDL-C, smoking, statin use, and glomerular filtration rate. All analyses were carried out using the STATA 12 software (STATA CORP Texas, USA.); p values < 0.05 or 95% confidence intervals that excluded the unity were considered statistically significant.

3. Results

The studied population comprised 1201 subjects with a mean age of 53.6 ± 9.3 years (Table 1). The prevalence of diabetes was 13.4%, tobacco smoking 22.5%, statin use 8.8%, CAC > 0 26.5%, and AVC > 0 18.8%. Table 2 shows unadjusted clinical and biochemical characteristics of participants, in relation to HOMA-IR quartiles. Values of BMI, visceral adipose tissue, systolic and diastolic blood pressure, triglycerides, glucose, insulin, free fatty acids, Adipo-IR, and hsCRP, as well as diabetes prevalence, were directly associated with HOMA-IR. In contrast, HDL-C levels, adiponectin, and physical activity index decreased with increasing HOMA-IR quartiles ($p < 0.05$, for all). Table 3 shows similar associations of risk factors with Adipo-IR index and, as found for HOMA-IR, participants had a more adverse cardiovascular risk profile with increasing Adipo-IR index quartiles. Additionally, Adipo-IR index showed a direct and significant relationship with HOMA-IR ($r = 0.758$, $p < 0.001$).

In general, the proportions of subjects with CAC > 0 and AVC > 0 increased in parallel to insulin resistance levels. Figure 1(a) shows that the prevalence of both CAC > 0 (23.1%, 23.7%, 26.5%, and 33.0%) and AVC > 0 (12.7%, 18.0%, 18.1%, and 26.6%) was increasingly higher from the lowest to the highest HOMA-IR quartile (p trend < 0.05, for both), but the prevalence of CAC > 0 was significantly different only when HOMA-IR quartile 4 was compared with quartile 1, whereas a significant difference in AVC > 0 prevalence was observed when quartiles 3 and 4 were compared to the lowest quartile. Similarly, Figure 1(b) displays the prevalence of CAC > 0 (22.4%, 24.1%, 29.6%, and 30.1%) and AVC > 0 (11.7%, 18.7%, 18.2%, and 26.7%) in relation to Adipo-IR quartiles (p trend < 0.05, for both). It can be seen that the difference in prevalence of AVC > 0 is already significant when quartile 2 was compared to the lowest quartile, suggesting that insulin resistance in adipose tissue could be more closely associated with AVC > 0 than to CAC > 0 prevalence.

Multivariate logistic regression analyses were performed to investigate the independence of the association of CAC > 0 and AVC > 0 with hepatic or adipose tissue insulin resistance (Table 4). Although unadjusted analyses showed that the presence of CAC > 0 was associated with highest values of HOMA-IR and Adipo-IR index, addition of age, gender, and BMI to the adjustment (Model 1) attenuated these associations to no significant levels. On the other hand, AVC > 0 was related to higher values of HOMA-IR or Adipo-IR in Model 1. Despite the fact that inclusion of additional cardiovascular risk factors leads to nonsignificant association between HOMA-IR and AVC > 0 (Model 2), Adipo-IR remained significantly associated with ACV > 0 in model 2 and even after full adjustment (Model 3). In order to confirm this association, a stepwise logistic regression analysis was conducted using all variables in Model 3 plus HOMA-IR

Table 2: Characteristics of the study population by hepatic insulin resistance (HOMA-IR) quartiles.

	Quartile 1 $n = 300$	Quartile 2 $n = 300$	Quartile 3 $n = 299$	Quartile 4 $n = 301$	p trend
Age (years)	52.8 ± 9.7	54.0 ± 9.2	53.4 ± 8.9[a]	54.1 ± 9.3	0.365
Gender (men, %)	143 (47.7)	145 (48.3)	143 (47.8)	144 (47.8)	0.999
BMI (kg/m^2)	25.3 ± 3.2	27.8 ± 3.6[a]	29.7 ± 4.1[a,b]	31.2 ± 4.6[a,b,c]	<0.001
Visceral AT (cm^2)	109 (80–141)	140 (106–176)[a]	163 (131–201)[a,b]	188 (153–229)[a,b,c]	<0.001
Systolic BP (mmHg)	112 ± 16	117 ± 18	119 ± 17[a]	124 ± 19[a]	<0.001
Diastolic BP (mmHg)	69 ± 9	72 ± 9[a]	74 ± 9[a]	75 ± 10[a,b]	<0.001
LDL-C (mmol/L)	3.05 ± 0.78	3.10 ± 0.80	3.08 ± 0.85	3.08 ± 0.91	0.758
HDL-C (mmol/L)	1.33 ± 0.36	1.22 ± 0.34	1.15 ± 0.33[a,b]	1.08 ± 0.29[a,b,c]	<0.001
Triglycerides (mmol/L)	1.37 (1.02–1.83)	1.65 (1.20–2.25)[a]	1.80 (1.35–2.38)[a,b]	1.98 (1.49–2.77)[a,b,c]	<0.001
Glucose (mmol/L)	4.6 (4.4–4.9)	4.88 (4.6–5.2)[a]	5.21 (4.8–5.6)[a,b]	5.72 (5.2–7.3)[a,b,c]	<0.001
Insulin (μIU/L)	9.92 (7.9–11)	15.5 (14–17)[a]	21.1 (19–24)[a,b]	30.5 (26–37)[a,b,c]	<0.001
Free fatty acids (mmol/l)	0.54 (0.42–0.67)	0.56 (0.44–0.68)[a]	0.56 (0.43–0.71)[a]	0.60 (0.50–0.80)[a,b,c]	<0.001
HOMA-IR	2.13 (1.7–2.5)	3.35 (3.0–3.7)[a]	4.91 (4.4–5.4)[a,b]	7.96 (6.8–9.9)[a,b,c]	<0.001
Adipo-IR	5.1 (3.5–6.9)	8.6 (6.7–10.4)[a]	12.1 (8.8–15.1)[a,b]	18.2 (13.0–25.0)[a,b,c]	<0.001
hsCRP (mmol/L)	9.9 (6.0–20.0)	13.8 (10.0–35.0)[a]	18.7 (10.0–35.0)[a,b]	23.8 (11.0–40.0)[a,b,c]	<0.001
Adiponectin (μg/mL)	10.6 (6.6–16.9)	8.3 (5.5–13.6)[a]	7.3 (4.7–11.1)[a,b]	5.6 (3.5–9.3)[a,b,c]	<0.001
eGFR (mL/min/1.73 m^2)	99.9 ± 8.5	99.4 ± 8.2	100 ± 8.1	100 ± 12.1	0.818
Physical activity index	8.0 ± 1.1	7.9 ± 1.2[a]	7.9 ± 1.2	7.6 ± 1.2[a,b]	<0.001
Current smoking (%)	69 (23)	70 (23.3)	69 (23.1)	62 (20.6)	0.839
Statin use (%)	23 (7.7)	32 (10.7)	26 (8.7)	25 (8.3)	0.600
Type 2 diabetes (%)	9 (3)	14 (4.7)	39 (13.0)[b]	98 (32.6)[a,b,c]	<0.001

Values are expressed as mean ± standard deviation, median (interquartile range), or number of subjects (percentage). BMI: body mass index; AT: adipose tissue; BP: blood pressure; LDL-C: low density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol; HOMA-IR: homeostasis model assessment of insulin resistance; Adipo-IR: adipose tissue insulin resistance; hsCRP: high sensitive C-reactive protein; eGFR: estimated glomerular filtration rate. HOMA-IR range: Q1: <2.78; Q2: 2.78–4.11; Q3: 4.12–6.01; Q4: >6.01 for men and Q1: <2.71; Q2: 2.71–3.96; Q3: 3.97–5.85; Q4: >5.85 for women. [a]$p < 0.05$ versus Q1, [b]$p < 0.05$ versus Q2, and [c]$p < 0.05$ versus Q3.

(Table 5). The results showed that higher values of Adipo-IR, but not HOMA-IR, were independently associated with AVC > 0 (OR: 2.33; 95% C.I: 1.28–4.25).

4. Discussion

Although insulin resistance is involved in the pathogenesis of cardiovascular disease, the studies on the relation of this important metabolic abnormality with cardiovascular calcification have yielded conflicting results [1, 4, 6, 9–12]. These inconsistencies may be explained, at least in part, by the differential metabolic effects of insulin resistance on adipose tissue, liver, and skeletal muscle [9, 15, 16, 28]. Our aim was to investigate the role of insulin resistance on cardiovascular calcification, which has been associated with increased risk of cardiovascular disease. By the approach used we could compare the contribution of hepatic insulin resistance (HOMA-IR) with that of adipose tissue insulin resistance (Adipo-IR) to the coronary and aortic valve calcification. Our main findings were as follows: (1) HOMA-IR was significantly associated with CAC > 0, but this association was not independent of other cardiovascular risk factors; (2) HOMA-IR was also associated with AVC > 0, but the adjustment for some conventional risk factors attenuated the association, and the statistical significance was lost when physical activity, type 2 diabetes, and visceral adipose tissue were added to the model; (3) CAC > 0 was found to be associated with Adipo-IR but, similar to what was observed with HOMA-IR, the association was not independent from cardiovascular risk factors; and (4) AVC was associated with Adipo-IR and the association remained significant even in the full adjusted model (Model 3).

For decades AVC was thought to be a passive degenerative process related to aging [2, 3]. However, recent data suggest that constellation of systemic insulin resistance-related factors, such as visceral adiposity excess, inflammation, oxidative stress, dyslipidemia, and endothelial dysfunction, are involved in the calcification of heart valves [1, 5, 12]. HOMA-IR index is a mathematical model strongly correlated with the hyperinsulinemic-euglycemic clamp procedure and has been used to assess systemic insulin resistance in multiple epidemiological studies [8]. Results of investigations on the association of HOMA-IR with coronary heart disease are controversial. Recently, Ong et al. [9] reported a modest independent association of HOMA-IR with CAC (OR: 1.04; [95% CI: 1.01–1.08]). Using the same base cohort of the Multi-ethnic Study of Atherosclerosis, Bertoni et al. [10] showed that HOMA-IR was not independently associated with CAC > 0 in any of the four ethnic groups studied. In addition, the follow-up of the same population demonstrated that HOMA-IR was not an independent predictor of incidence or progression

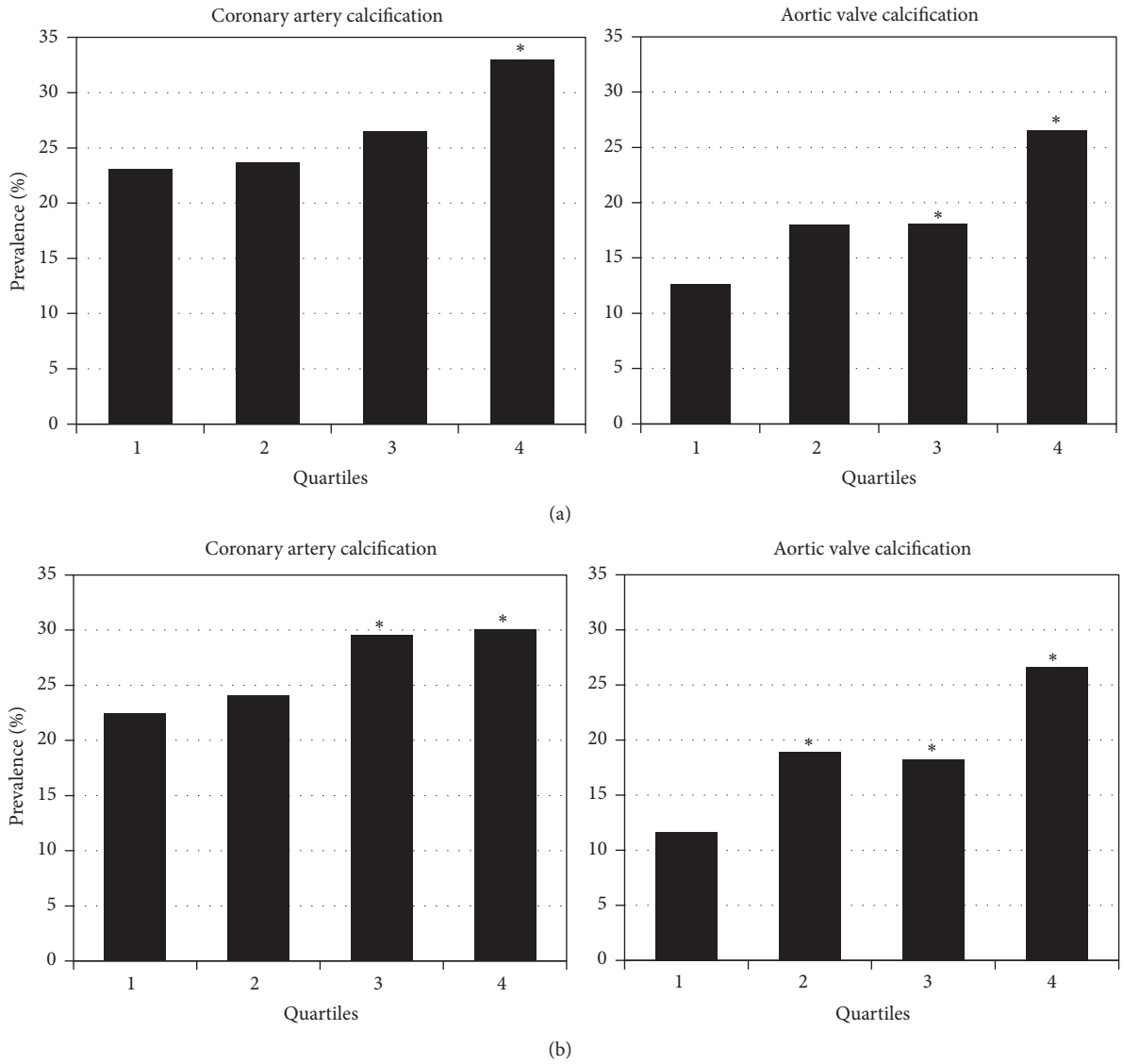

Figure 1: *Insulin resistance and cardiovascular calcification*. Prevalence of coronary artery calcification and aortic valve calcification according to quartiles of HOMA-IR (a) or quartiles of Adipo-IR (b). HOMA-IR: homeostasis model assessment of insulin resistance; Adipo-IR: adipose tissue insulin resistance. $^{*}p < 0.05$ versus Q1.

of CAC [11]. In agreement with those studies, our findings showed that HOMA-IR was not independently associated with CAC > 0. Similarly, Tison et al. [12] reported that association of HOMA-IR with AVC > 0 prevalence or incidence was not independent from traditional cardiovascular risk factors. Consistent with those results, our study showed that HOMA-IR was associated with AVC > 0, but significance was lost in the fully adjusted model. Of note, addition of type 2 diabetes to Model 2 only slightly attenuated the association (OR: 1.83; [95% CI: 1.04–3.32]). This observation suggests that physical activity and/or visceral adipose tissue could participate in the association of insulin resistance with AVC.

As mentioned above, insulin has different functions across organ systems. In the liver it reduces liver glucose production, in muscle it increases glucose uptake, and in adipose tissue it suppresses lipolysis [28]. Considering that, (1) hepatic glucose production is the primary determinant of the fasting plasma glucose concentration, (2) insulin levels are a primary regulator of hepatic glucose production, and (3) HOMA-IR index involves fasting insulin and glucose measurements; some researchers have reported that HOMA-IR reflects hepatic insulin resistance in a fasting state [29]. On the other hand, Adipo-IR index, which is derived from measurements of fasting insulin concentration and of fasting free fatty acids (principally released by adipose tissue during fasting state), could be a method mainly reflecting adipose tissue insulin resistance [15, 16]. Although no previous studies have analyzed whether Adipo-IR index is related to heart calcification, some evidence indicates relationships between this index and cardiovascular risk factors such as nonalcoholic fatty liver disease [16], metabolic syndrome [30], adipocytokines [30], and type 2 diabetes [31].

Table 3: Characteristics of the study population by adipose tissue insulin resistance quartiles.

	Quartile 1 $n = 300$	Quartile 2 $n = 299$	Quartile 3 $n = 302$	Quartile 4 $n = 300$	p trend
Age (years)	52.5 ± 9.4	54.1 ± 9.0	54.5 ± 8.9	53.4 ± 9.8	0.660
Gender (men, %)	144 (48)	144 (48.2)	144 (47.7)	144 (48)	0.990
BMI (kg/m^2)	25.7 ± 3.5	28.0 ± 3.9[a]	28.9 ± 3.7[a]	31.4 ± 4.5[a,b,c]	<0.001
Visceral AT (cm^2)	109 (82–150)	144 (109–180)[a]	162 (126–205)[a,b]	181 (150–230)[a,b,c]	<0.001
Systolic BP (mmHg)	112 ± 16	117 ± 17	120 ± 18[a]	123 ± 19[a,b]	<0.001
Diastolic BP (mmHg)	69 ± 9	72 ± 9	74 ± 10	75 ± 10[a,b]	<0.001
LDL-C (mmol/L)	3.05 ± 0.27	3.08 ± 0.82	3.18 ± 0.88	3.03 ± 0.85	0.784
HDL-C (mmol/L)	1.28 ± 0.37	1.21 ± 0.32	1.19 ± 0.33	1.10 ± 0.33[a,b,c]	<0.001
Triglycerides (mmol/L)	1.42 (1.04–1.89)	1.67 (1.22–2.18)[a]	1.81 (1.33–2.46)[a,b]	1.94 (1.46–2.72)[a,b,c]	<0.001
Glucose (mmol/L)	4.77 (4.50–5.11)	4.99 (4.70–5.30)[a]	5.10 (4.80–5.60)[a]	5.40 (4.90–6.10)[a,b,c]	<0.001
Insulin (μIU/L)	10.7 (7.9–13)	14.9 (12–18)[a]	20.0 (17–24)[a,b]	29.3 (23–36)[a,b,c]	<0.001
Free fatty acids (mmol/l)	0.42 (0.32–0.53)	0.54 (0.44–0.65)[a]	0.60 (0.48–0.72)[a,b]	0.70 (0.60–0.80)[a,b,c]	<0.001
HOMA-IR	2.28 (1.70–2.90)	3.36 (2.70–4.50)[a]	4.70 (3.70–5.70)[a,b]	7.22 (5.40–9.60)[a,b,c]	<0.001
Adipo-IR	4.46 (3.30–5.40)	7.98 (7.10–8.90)[a]	11.8 (10.30–13.40)[a,b]	19.9 (17.0–25.0)[a,b,c]	<0.001
hsCRP (mmol/L)	10.4 (6.0–19.0)	15.4 (8.0–31.0)[a]	17.6 (10.0–33.0)[a]	21.9 (11.0–39.0)[a,b]	<0.001
Adiponectin (μg/mL)	9.2 (6.2–15.3)	8.6 (5.2–13.7)[a]	7.1 (4.6–11.6)[a,b]	6.4 (3.8–9.9)[a,b,c]	<0.001
eGFR (mL/min/1.73 m^2)	100 ± 7.9	99.8 ± 8.4	102 ± 8.3	100 ± 12	0.398
Physical activity index	8.15 ± 1.2	7.79 ± 1.3	7.90 ± 1.2	7.71 ± 1.2[a]	0.029
Current smoking (%)	76 (25.3)	61 (20.4)	71 (23.5)	62 (20.7)	0.408
Statin use (%)	23 (7.67)	33 (11)	29 (9.6)	21 (7)	0.285
Type 2 diabetes (%)	24 (8)	37 (12.4)[a]	42 (13.9)[a]	58 (19.33)[a,b]	0.001

Values are expressed as mean ± standard deviation, median (interquartile range), or number of subjects (percentage). BMI: body mass index; AT: adipose tissue; BP: blood pressure; LDL-C: low density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol; HOMA-IR: homeostasis model assessment of insulin resistance; Adipo-IR: adipose tissue insulin resistance; hsCRP: high sensitive C-reactive protein; eGFR: estimated glomerular filtration rate. Adipo-IR range: Q1: <5.57; Q2: 5.57–8.58; Q3: 8.59–12.48; Q4: >12.48 for men and Q1: <6.98; Q2: 6.98–10.89; Q3: 10.60–16.22; Q4: >16.23 for women. [a]$p < 0.05$ versus Q1, [b]$p < 0.05$ versus Q2, and [c]$p < 0.05$ versus Q3.

Adipo-IR was not associated with CAC > 0 in the present study. These results, as well as those from other studies [10, 11], suggest that insulin resistance is not more important than conventional cardiovascular risk factors for coronary calcium accumulation. Conversely, our data highlight the idea that Adipo-IR was strongly and independently associated with AVC > 0. In addition, we found that Adipo-IR/AVC > 0 association was not importantly modified by physical activity and visceral adipose tissue mass (Model 3, Table 4). This finding suggests that adipose tissue function could be more important than the amount of adipose tissue for the association of insulin resistance with AVC. This hypothesis is supported by several recent studies showing a greater effect of dysfunctional adipose tissue on insulin resistance, lipid abnormalities, inflammation, endothelial dysfunction, adipokine imbalance, and inflammasome and/or oxidative stress activation than that of adipose tissue mass [32, 33]. From a clinical point of view, all these results suggest that Adipo-IR may offer a therapeutic advantage (i.e., physical activity or weight loss) to prevent the development of AVC in metabolically unhealthy subjects.

Given that CAC and AVC share common risk factors and display similarities in their pathophysiology [3, 12, 34], the differences we found in the associations of these two conditions with insulin resistance suggest a different calcification process in each of these regions. Support for this hypothesis is given by results of recent studies showing that calcification in the valve appears largely unrelated to calcifying activity in coronary atherosclerosis [34], and cardiovascular risk factors such as receptor for advanced glycation end products or oxidized low density lipoproteins are implicated in the mechanistic production of reactive oxygen species and bone morphogenetic protein, which promotes valvular interstitial cells activation and leads to osteogenic activity, inflammation, matrix remodeling, fibrosis, and calcification [35]. Additionally, another study reported important differences in the mechanisms promoting oxidative stress, which is believed to be a key trigger of the procalcific processes both in the aortic valve and in the coronary arteries [36]. Furthermore, randomized trials with statin therapy have failed to impact valve disease progression [37, 38].

Strengths of the present work included (1) the extensive clinical and biochemical characterization of population, which allowed adjustment for multiple cardiovascular risk factors; (2) the detection of CAC and AVC simultaneously by CT; and (3) the large sample size studied. There are also some limitations. First, causality cannot be determined due to the

Table 4: Unadjusted and multivariate adjusted associations of HOMA-IR and Adipo-IR indices with CAC > 0 and AVC > 0.

		Unadjusted	Model 1	Model 2	Model 3
			HOMA-IR		
CAC > 0	Q1	1 (reference)	1 (reference)	1 (reference)	1 (reference)
	Q2	1.03 (0.71–1.51)	0.83 (0.53–1.29)	0.88 (0.53–1.43)	0.79 (0.47–1.32)
	Q3	1.20 (0.83–1.75)	1.031 (0.66–1.62)	1.17 (0.70–1.96)	0.78 (0.45–1.33)
	Q4	**1.64 (1.14–2.36)**	1.34 (0.85–2.12)	0.79 (0.49–1.28)	0.92 (0.51–1.67)
AVC > 0	Q1	1 (reference)	1 (reference)	1 (reference)	1 (reference)
	Q2	1.51 (0.96–2.37)	1.29 (0.79–2.13)	1.09 (0.63–1.89)	1.01 (0.57–1.80)
	Q3	1.52 (0.96–2.38)	1.34 (0.80–2.22)	1.17 (0.67–2.07)	0.97 (0.53–1.78)
	Q4	**2.50 (1.63–3.81)**	**2.04 (1.22–3.39)**	1.64 (0.93–2.92)	1.38 (0.72–2.52)
			Adipo-IR		
CAC > 0	Q1	1 (reference)	1 (reference)	1 (reference)	1 (reference)
	Q2	1.09 (0.75–1.61)	0.90 (0.58–1.39)	0.82 (0.51–1.32)	0.64 (0.39–1.07)
	Q3	**1.45 (1.01–2.10)**	1.21 (0.78–1.86)	0.96 (0.60–1.55)	0.92 (0.55–1.53)
	Q4	**1.49 (1.03–2.15)**	1.22 (0.77–1.94)	1.01 (0.61–1.68)	0.94 (0.54–1.61)
AVC > 0	Q1	1 (reference)	1 (reference)	1 (reference)	1 (reference)
	Q2	1.74 (1.105–2.75)	1.52 (0.92–2.50)	1.60 (0.93–2.80)	1.55 (0.87–2.76)
	Q3	1.69 (1.06–2.66)	1.36 (0.82–2.26)	1.24 (0.70–2.19)	1.20 (0.65–2.19)
	Q4	**2.75 (1.78–4.26)**	**2.38 (1.42–3.98)**	**2.19 (1.22–3.93)**	**2.18 (1.18–4.09)**

Model 1: Adjusted for age, gender, and BMI.
Model 2: Adjusted for age, gender, BMI, current smoking, physical activity index, statin use, SBP, DBP, LDL-C, HDL-C, triglycerides, and eGFR.
Model 3: Adjusted for age, gender, BMI, current smoking, physical activity index, statin use, SBP, DBP, LDL-C, HDL-C, triglycerides, eGFR, hs C-reactive protein, adiponectin, type 2 diabetes, and visceral adipose tissue.
Odds ratios (95% CI) for CAC > 0 or AVC > 0 in participants stratified by HOMA-IR or Adipo-IR quartiles (Q). Bold numbers: $p < 0.05$.
HOMA-IR: homeostasis model assessment of insulin resistance; Adipo-IR: adipose tissue insulin resistance; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; LDL-C: low density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol; eGFR: estimated glomerular filtration rate.

Table 5: Association of cardiovascular risk factors with aortic valve calcification presence (AVC > 0) in forward stepwise logistic regression analysis.

	Odds ratio (95% C.I.)	Probability
Age	1.14 (1.11–1.18)	<0.001
Gender	2.71 (1.85–3.98)	<0.001
Body mass index	1.04 (0.99–1.09)	0.069
Triglycerides	1.00 (0.99–1.00)	0.118
Current smoking	0.47 (0.28–0.78)	0.004
Use of statins	1.58 (0.90–2.76)	0.106
Estimated glomerular filtration rate	1.02 (0.99–1.05)	0.052
Low density lipoprotein cholesterol	1.01 (1.01–1.017)	<0.001
Type 2 diabetes	1.58 (1.01–2.47)	0.045
Adipo-IR quartile 1	1 (reference)	
Adipo-IR quartile 2	1.53 (0.86–2.71)	0.142
Adipo-IR quartile 3	1.22 (0.68–2.21)	0.490
Adipo-IR quartile 4	2.33 (1.28–4.25)	0.006

Adipo-IR: adipose tissue insulin resistance.
Variables that drop out of the model: physical activity index, systolic blood pressure, diastolic blood pressure, high density lipoprotein cholesterol, homeostasis model assessment of insulin resistance (HOMA-IR), hs C-reactive protein, adiponectin, and visceral adipose tissue.

cross-sectional nature of the study design. Second, HOMA-IR and Adipo-IR indices are inferior in assessing insulin resistance than dynamic test such as hyperinsulinemic-euglycemic clamp or adipose tissue microdialysis [39], respectively; however, this limitation is offset by its practical application in the study of a large number of subjects. Finally, it is not possible to discard residual confounding by some unmeasured factors like inflammatory mediators (i.e., TNF-α and ferritin) as well as procalcifying molecules (e.g., sclerostin and osteoprotegerin).

5. Conclusion

Our results show that traditional cardiovascular risk factors largely explain the association of HOMA-IR with CAC and AVC. The novel finding of our study is that Adipo-IR, but not HOMA-IR, is independently associated with calcification of the aortic valve. This could suggest that abnormal adipose tissue function has a role in the occurrence of insulin resistance that may favor the development and progression of abnormal cardiovascular conditions such as AVC. The independent association of Adipo-IR with this valve condition suggests that oxidative stress or other adipose tissue related abnormalities could participate in the aortic valve damage. Further studies are needed to corroborate our findings and to better elucidate the underlying mechanisms responsible for this association. From a clinical point of view, the present results may be useful to identify an abnormal metabolically condition, which precedes chronic complications, and to improve the therapeutic approach in subjects with early insulin resistance.

Competing Interests

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

Authors' Contributions

Esteban Jorge-Galarza, Carlos Posadas-Romero, and Juan G. Juárez-Rojas participated in conception, design, analysis, interpretation of data, and final approval of the manuscript submitted. Margarita Torres-Tamayo, Aida X. Medina-Urrutia, Marco A. Rodas-Díaz, Rosalinda Posadas-Sánchez, Gilberto Vargas-Alarcón, María del Carmen González-Salazar, and Guillermo C. Cardoso-Saldaña contributed in data collection, drafting of the manuscript, and revising it critically for important intellectual content.

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Research Article

Handheld Capillary Blood Lactate Analyzer as an Accessible and Cost-Effective Prognostic Tool for the Assessment of Death and Heart Failure Occurrence during Long-Term Follow-Up

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Impact of tissue lactate accumulation on prognosis after acute myocardial infarction (AMI) is biased. The study aimed to assess the prognostic role of lactate concentration (LC) in patients with AMI during one year of follow-up. 145 consecutive patients admitted due to AMI were enrolled. The data on the frequency of endpoint occurrence (defined as I, death; II, heart failure (HF); and III, recurrent myocardial infarction (re-MI)) were collected. The patients were divided into group A (LC below the cut-off value) and group B (LC above the cut-off value) for the endpoints according to receiver operating characteristic (ROC) analysis. The cumulative survival rate was 99% in group I-A and 85% in group I-B ($p = 0.0004$, log-rank test). The HF-free survival rate was 95% in group II-A and 82% in group II-B ($p = 0.0095$, log-rank test). The re-MI-free survival rate did not differ between groups. A multivariate Cox analysis showed a statistically significant influence of LC on death [Hazard Ratio (HR): 1.41, 95% Confidence Interval (CI) (1.13–1.76), and $p = 0.002$] and HF [HR: 1.21, 95% CI (1.05–1.4), and $p = 0.007$] with no impact on re-MI occurrence. LC in capillary blood may be considered a useful prognostic marker of late-onset heart failure and death after AMI.

1. Introduction

Cardiovascular diseases are the most common cause of death in Poland, with an annual death rate of 452 per 100,000. Cardiovascular diseases thus represent a serious health problem for the country [1]. The morbidity rate of acute myocardial infarction (AMI), as shown by Yeh et al. [2], showed a tendency to decrease systematically. Irrespective of a broad spectrum of activities, a further decrease in the mortality rate primarily depends on innovation in pharmacotherapy and primary percutaneous coronary intervention (PCI) delay shortening. The abovementioned actions may positively impact the AMI in-hospital mortality rates being reported in Europe as 7% in the STEMI and 3–5% in the NSTEMI group of patients [3–6]. Patient-dependent delay plays a crucial role in the potential improvement of AMI treatment results; there is thus a need for the implementation of an easy-to-use and cost-effective diagnostic and prognostic tool. AMI is associated with an anaerobic switch phenomenon, which is primarily due to coronary artery occlusion, leading to the acute impairment of myocardium oxygen supply. A deterioration in left ventricle contractility leading to a reduction in cardiac output may result in peripheral tissue hypoxemia and inhibit glycolysis, which represents the primary source of adenosine triphosphate (ATP) supply, providing thirty-eight moles of ATP from one molecule of glucose. Pyruvate

Table 1: Patient characteristics according to the type of myocardial infarction.

	STEMI ($n = 60$)	NSTEMI ($n = 85$)	p
Age	59.85	62.86	ns
Women	21 (35%)	25 (30%)	ns
Hypertension	50 (83%)	76 (89%)	ns
Diabetes	20 (33%)	20 (24%)	ns
Smoking	36 (60%)	37 (44%)	ns
Atrial fibrillation	5 (8%)	6 (7%)	ns
Systolic BP (mmHg)	136	140	ns
Diastolic BP (mmHg)	81	83	ns
Shock	7 (12%)	1 (1%)	ns
IABP	2 (3%)	0 (0%)	ns
eGFR < 60 (ml/min/1.73^2)	5 (8%)	11 (13%)	ns
Previous CABG	1 (2%)	3 (4%)	ns
Previous cardiac arrest	3 (5%)	2 (2%)	ns
GPIIb/IIIa inhibitors	29 (48%)	8 (9%)	$p < 0.001$
EDV (ml)	97	90	ns
LVEF (%)	42	49	$p < 0.005$
Hospitalization time (days)	8.8	8.0	ns
Heart rate (1/min)	78	82	ns
BMI	27.7	28.6	ns
Time from symptom onset to FMC (hours)	7.77 ± 7.19; 6.00 (3.25, 9.00)	22.04 ± 21.68; 12.00 (8.00, 24.00)	$p < 0.001$

Abbreviations: BMI: body mass index. BP: blood pressure. CABG: coronary artery bypass grafts. EDV: end-diastolic volume. eGFR: estimated glomerular filtration rate. GPIIb/IIIa: glycoprotein IIb/IIIa inhibitors. IABP: intra-aortic balloon pump. LVEF: left ventricular ejection fraction. NSTEMI: non-ST segment elevation myocardial infarction. STEMI: ST segment elevation myocardial infarction.

reduction into lactate restores only two moles of ATP in anaerobic conditions and is thus largely ineffective. Lactate was discovered by Scheele [7] in 1780 and was implemented into a clinical setting more than sixty years later by Johann Scherer [8] for various methods of assessment including a capillary blood handheld analyzer. The accumulation of lactate increases tissue acidosis with subsequent acid-base homeostasis disturbance, resulting in extreme conditions and symptoms of shock (tachycardia, tachypnea, cyanosis, pallor, third heart sound, and cold extremities) [9]. Whether the type of shock (hypovolemic, septic, or cardiogenic) differentially impacts the lactate concentration (LC) remains to be addressed with appropriate randomized multicenter studies.

2. Aim of the Study

Aim of the study was to assess LC in AMI patients; to evaluate the potential relationships between LC and other clinical and biochemical factors; to assess the possible prognostic impact of LC on death, heart failure (HF), and recurrent myocardial infarction (Re-MI) occurrence in one year of follow-up.

3. Methodology and Patients

We enrolled 145 consecutive patients into a prospective cohort study. They were admitted to our center (a tertiary university hospital) due to AMI between August and December 2012 and in them we performed LC measurement using the handheld The Edge® device (provided by the APEXBIO Company, Taiwan). The total cost of the equipment, including the electronic analyzer device and the measurement strips, was 410 USD, which equals 2.83 USD per patient. The study complies with the Declaration of Helsinki and was approved by the local ethics committee (approval number KNW/0022/KB1/99/12), and all patients gave informed consent prior to enrollment. The inclusion criteria included AMI according to the current European Society of Cardiology (ESC) definition [4] and age between 18 and 80 years. All enrolled patients were submitted for invasive coronary angiography. The exclusion criteria were a lack of informed consent (unconscious patients) or known malignant disease. Patients received typical treatment according to the current ESC guidelines, which were independent of the results of the LC measurement and were submitted for revascularization via percutaneous coronary intervention (PCI) coronary artery bypass grafts (CABG) or optimal medical therapy (OMT) either ad hoc or after a heart team decision process. The enrolled patients were subsequently analyzed according to the defined endpoints, which included death, HF, and re-MI occurrence in one year of follow-up performed in the majority of cases during clinical examination (90%) or telephone survey (10%). Nevertheless each endpoint which occurred during follow-up had to be confirmed by the data received from the national health care system digital database. The implemented system of blinded control during follow-up tended to improve the objectivity of the acquired data. The patients characteristics divided into STEMI and NSTEMI group were presented in the Table 1.

Table 2: Biochemical parameters in STEMI versus NSTEMI patients.

Parameter	STEMI ($n = 60$)	NSTEMI ($n = 85$)	p
Lactate concentration (mmol/l)	4.0	4.1	ns
Glycemia (mmol/l)	8.4	7.3	$p < 0.001$
Total cholesterol (mg/dl)	220	215	ns
Triglycerides (mg/dl)	126	124	ns
HDL (mg/dl)	60	55	ns
LDL (mg/dl)	135	136	ns
cTn (ng/l)	847	357	ns
CK-MB (IU/l)	64	49	ns
Creatinine (umol/l)	78	81	ns
RBC (10^6/ul)	4.8	4.8	ns
WBC (10^3/ul)	12	10	$p < 0.025$
PLT (10^3/ul)	259	239	ns
HGB (g/dl)	14	14	ns

CK-MB: creatine kinase MB. cTn: cardiac troponin. HDL: high-density lipoproteins. HGB: hemoglobin. LDL: low-density lipoproteins. NSTEMI: non-ST elevation myocardial infarction. PLT: platelet count. RBC: red blood cell count. STEMI: ST elevation myocardial infarction.

4. Statistical Analysis

The distributions of the examined parameters were analyzed using the Shapiro-Wilk test. Values were presented as the means and standard deviation (SD) or as the median in the 25th and 75th percentiles. Nominal and categorical values were expressed in percentages or proportional rates. Linear variables with a normal distribution were compared using Student's t-test. Variables with an abnormal distribution were compared using the Kolmogorov-Smirnov and Mann–Whitney U tests. Categorical variables of abnormal distribution were compared using a Chi-square test with Yates correction. A Kaplan-Meier analysis (log-rank test) was used to demonstrate the frequency of endpoint occurrence during the follow-up period for the patients, which were divided into two groups with low (A) and high (B) LC according to the calculated cut-off value (acquired from receiver operating characteristic (ROC) analysis). Cut-off values for each endpoint were calculated using ROC with subsequent sensitivity and specificity, area under curve (AUC), and Confidence Interval estimation. Cox proportional hazard regression was used to evaluate the risk of endpoint occurrence. The independent variables included in the multivariate model were variables that reached statistical significance (set at 0.1) in the univariate analysis of all parameters. Independent predictors of endpoint occurrence were presented as the Hazard Ratio (HR) with a Confidence Interval (CI). Differences between the values were considered statistically significant if $p < 0.05$. Analyses were performed using Statistica 10 with the medical package (StatSoft Inc.).

5. Results

STEMI patients are characterized by the more frequent use of GPIIb/IIIa receptor blockers (48 versus 9%, $p < 0.001$), lower baseline left ventricular ejection fraction (LVEF) (42 versus 49%, $p < 0.005$), and shorter time from the onset of symptoms to first medical contact (FMC) (8 versus 22 hours, $p < 0.001$). Patient characteristics according to AMI type are presented in Table 1. STEMI patients had a higher serum blood concentration of glucose (8.4 versus 7.3 mmol/l, $p < 0.001$) and white blood cell count (12 versus $10 * 10^3$/ul, $p < 0.025$). Data are presented in Table 2.

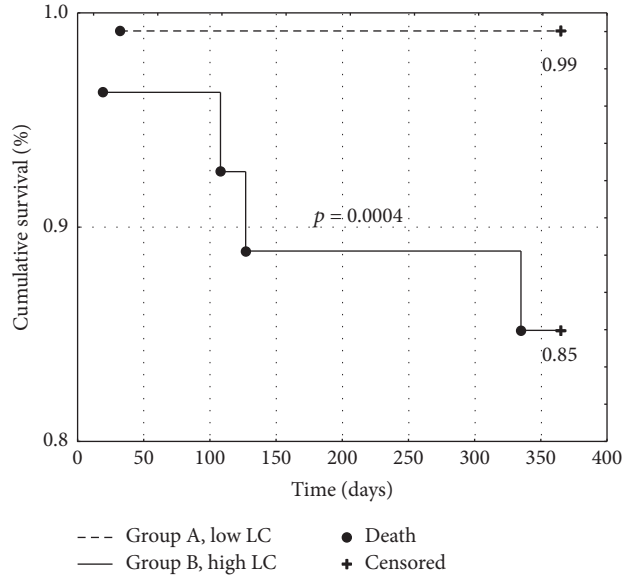

Figure 1: Kaplan-Meier survival curves for all-cause mortality of patients with low (group A) versus high (group B) lactate concentration. LC: lactate concentration.

The highest AUC of 0.9 (with a specificity and sensitivity of 80% at the LC cut-off value of 5.35 mmol/l) was observed for the death endpoint; this was marked as I. All data are presented in Table 3

Kaplan-Meier curves were used to assess the cumulative event-free survival for each endpoint. Patients with an LC below the cut-off value had a significantly higher cumulative survival rate (0.99 versus 0.85, $p < 0.001$, Figure 1) and

Table 3: ROC-derived cut-off values of LC for each endpoint.

Endpoint during follow-up	LC (mmol/l)	Sensitivity and specificity (%)	AUC ± SD	95% CI
Death (I)	5.35	80	0.9 ± 0.05	(0.80–0.99)
Heart failure (II)	4.14	69	0.74 ± 0.07	(0.61–0.88)
Re-MI (III)	3.28	49	0.56 ± 0.07	(0.43–0.69)

AUC: area under the curve. CI: confidence interval. LC: lactate concentration. Re-MI: recurrent myocardial infarction. ROC: receiver operating characteristic. SD: standard deviation.

Table 4: Cox regression model for death occurrence in one year of follow-up.

Endpoint I	Univariate Cox regression analysis			Multivariate Cox regression analysis		
	HR	95% CI	p	HR	95% CI	p
HGB	0.68	(0.47–0.97)	0.030	—	—	—
LC	1.29	(1.11–1.51)	0.001	1.41	(1.13–1.76)	0.002
LDL	0.98	(0.97–0.99)	0.004	0.97	(0.95–0.99)	0.008
LM > 50%	7.16	(1.15–44.47)	0.031	11.06	(1.34–91.04)	0.023

HGB: hemoglobin. LC: lactate concentration. LDL: low-density lipoproteins. LM: left main coronary artery.

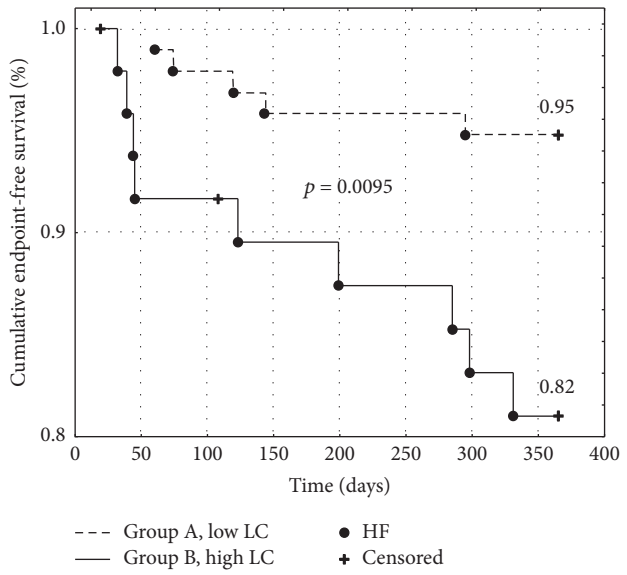

Figure 2: Kaplan-Meier event-free survival curves for heart failure occurrence in patients with low (group A) versus high (group B) lactate concentration. HF: heart failure. LC: lactate concentration.

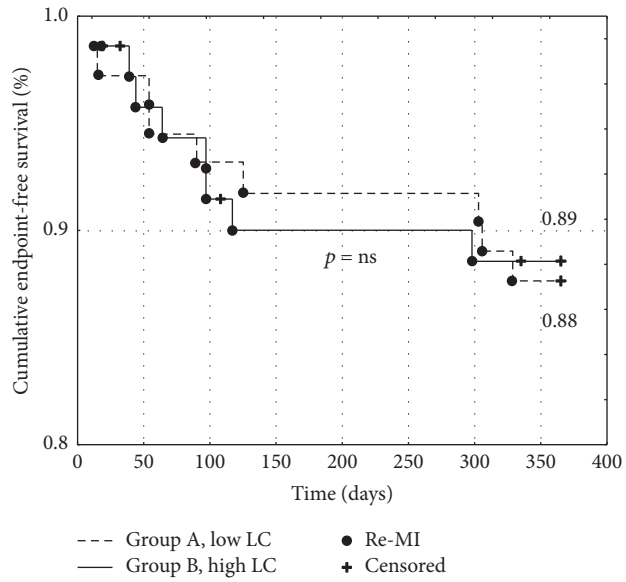

Figure 3: Kaplan-Meier event-free survival curves for recurrent myocardial infarction occurrence in patients with low (group A) versus high (group B) lactate concentration. LC: lactate concentration. Re-MI: recurrent myocardial infarction.

HF-free episode survival rate (0.95 versus 0.82, $p < 0.01$, Figure 2) with no differences in the re-MI episode survival rate (0.89 versus 0.88, p = ns, Figure 3).

We performed a univariate Cox regression analysis; we then constructed multivariate Cox regression analysis models using the backward stepwise method to predict the occurrence of each endpoint (I, death; II, HF; III, re-MI). A statistically significant influence of LC on death [Hazard Ratio (HR): 1.41, 95% Confidence Interval (CI) (1.13–1.76), and $p = 0.002$] and HF occurrence [HR: 1.21, 95% CI (1.05–1.4), and $p = 0.007$] was reported. The relationship between LC and re-MI during follow-up was not observed. The Cox regression analysis models are presented in Tables 4–6.

6. Discussion

The blood acquisition site substantially impacts LC. What should be considered a significant elevation of LC is in this condition questioned. In our study, a significant elevation was described by the production of more than 2.5 mmol/l; however, LC elevation and its role in the diagnosis of certain clinical states (in addition to prognosis and survival) have not been thoroughly elucidated. Boldt et al. [10] reported the inability to discriminate serious significant differences between LC as assessed using the handheld Accusport diagnostic tool by Roche Diagnostics in addition to the

Table 5: Cox regression model for heart failure occurrence in one year of follow-up.

Endpoint II	Univariate Cox regression analysis			Multivariate Cox regression analysis		
	HR	95% CI	p	HR	95% CI	p
AF	3.53	(0.96–12.98)	0.05	—	—	—
BMI	1.12	(1.01–1.24)	0.03	1.20	(1.07–1.35)	0.002
CREAT	1.03	(1.01–1.04)	0.001	1.04	(1.02–1.06)	0.0004
GPIIb/IIIa	3.01	(1.03–8.78)	0.04	5.00	(1.52–16.42)	0.007
IABP	8.23	(1.03–66.06)	0.04	—	—	—
LC	1.17	(1.04–1.31)	0.01	1.21	(1.05–1.40)	0.007
LM > 50%	4.53	(1.39–14.82)	0.01	—	—	—
Diabetes	2.73	(0.94–7.96)	0.06	—	—	—
Shock	5.39	(1.46–19.88)	0.01	—	—	—
STEMI	2.59	(0.85–7.91)	0.09	—	—	—
WBC	1.12	(0.99–1.25)	0.06	—	—	—

AF: atrial fibrillation. BMI: body mass index. CREAT: creatinine. GPIIb/IIIa: glycoprotein IIb/IIIa inhibitors. IABP: intra-aortic balloon pump. LC: lactate concentration. LM: left main coronary artery. STEMI: ST elevation myocardial infarction. WBC: white blood cells.

Table 6: Cox regression model for recurrent myocardial infarction in one year of follow-up.

Endpoint III	Univariate Cox regression analysis			Multivariate Cox regression analysis		
	HR	95% CI	p	HR	95% CI	p
CREAT	1.02	(1.01–1.04)	0.00	1.02	(1.01–1.04)	0.001
DES	2.54	(0.88–7.37)	0.08	—	—	—
Glycemia	1.13	(1.03–1.25)	0.01	—	—	—
HDL	1.01	(1.00–1.02)	0.01	—	—	—
LAD > 50%	4.52	(1.00–20.37)	0.05	7.08	(1.11–44.97)	0.034
LVEF	0.96	(0.92–1.00)	0.03	—	—	—
Number of stents	1.79	(1.04–3.08)	0.03	1.69	(1.02–2.81)	0.037
Previous SCA	4.27	(0.95–19.26)	0.05	23.55	(3.25–170.86)	0.001
Smoking	0.20	(0.06–0.71)	0.01	0.14	(0.04–0.56)	0.005
Stent length	1.02	(1.00–1.04)	0.08	—	—	—

CREAT: creatinine. DES: drug-eluting stent. HDL: high-density lipoproteins. LAD: left anterior descending coronary artery. LVEF: left ventricular ejection fraction. SCA: sudden cardiac arrest.

Chiron Diagnostics 865 and Lactate PAP tools by Analyticon; however, the initial incoherence between the arterial and capillary blood LC assessments were potentially due to technical (different volumes submitted to examination and method of acquisition) and clinical reasons including reduced intravascular osmotic pressure and capillary edema caused by intravenous liquid administration. The delay in LC assessments using the reference method varied between 45 and 168 minutes (mean of 85 minutes) and was twice as expensive as the electronic analyzer with a total cost of five hundred United States dollars (USD). A tight correlation between fingertip and whole blood point-of-care (POC) LC assessment in emergency department patients compared with a standard laboratory analyzer with intraclass correlation coefficients of 0.90 and 0.92, respectively, was reported by Gaieski et al. [11]. Notably, the time of LC assessment using an electronic analyzer compared with a reference method was 65 minutes shorter in this setting. The impact of LC on infection frequency was previously reported by Claridge et al. [12] and his group in patients hospitalized in the intensive care unit (ICU) after trauma or surgical intervention. In the study, the normalization of LC in time corresponded to the infection occurrence rate (a longer period of time was needed to normalize the higher incidence of infection). The mortality rate was higher in patients with the infection (7.9 versus 1.9%, $p < 0.05$) and was indirect proof of LC utility in the early risk assessment and determination of prognosis. Notably, the group of patients with an LC above 2.4 mmol/L was characterized by a higher rate of death, longer hospital stay, and increased cost of hospitalization. These findings are in accordance with our results, although we examined different group of patients with AMI submitted for invasive coronary angiography and followed up for one year. We believe that this novel approach might be useful in early risk stratification, especially considering low cost of the single LC assessment and its accessibility, although further studies in larger groups of patients and different clinical scenarios would improve the knowledge in the field. Meregalli et al.

[13] reported the role of LC in the prognosis of patients after surgical treatment followed by admittance to the ICU. They demonstrated its effectiveness in assessing the risk of death and performed serious complications risk stratification with the area under ROC curves only slightly lower than those for the widely used SAPS II (New Simplified Acute Physiology Scale) [0.583 versus 0.705 (for death); 0.646 versus 0.678 (for serious complications)]. LC was thus postulated to be more reliable than other indices of hemodynamic state deterioration, such as heart rate, diuresis and the mean arterial pressure, or metabolic acidosis. The elimination of the clinical signs of shock does not exclude the possibility of hypoperfusion as an important risk factor of serious complications. It is worth mentioning that, after excluding patients with the clinical signs of shock, LC was significantly higher in those who reached endpoints I (8.7 versus 3.8, $p = 0.008$) and II (5.0 versus 3.8, $p = 0.028$). This relationship did not occur for endpoint III (3.7 versus 4.0, $p =$ ns). Howell et al. [14] reported that increased LC (≥4.0 mmol/l) had serious implications for the mortality rate even in normotensive patients. The mortality rate in this group of patients was 15%; however, the patients presenting with septic shock or an LC above 4.0 mmol/l had a mortality rate of 28.3%, which was significantly higher than that in patients who had neither (2.5%, $p < 0.0001$). In a model with good discrimination (AUC = 0.87) consisting of age, blood pressure, malignancy, platelet count, and blood urea nitrogen, LC was considered to be associated with mortality. These findings were also consistent with our results despite the fact that we focused on the potential relationship between LC evaluated during AMI and symptoms of late-onset. The construction of a Cox proportional hazard regression models required the implementation of advanced statistical methods. The ROC-derived cut-off values for each of the three analyzed endpoints varied between 3.3 and 5.4 mmol/l. Jansen et al. [15] analyzed over one hundred patients and reported an increased mortality rate in those with an LC above 3.5 mmol/l (41 versus 12%, $p < 0.001$, area under ROC curve 0.69). These findings are consistent with our results; however, the cardiovascular-related etiology of admission was observed in only 30% of Jansen's patients (compared with 100% of our patients). Lazzeri et al. [16] reported a significant impact of LC on prognosis only in patients in the worse hemodynamic state that were assessed on the basis of the Killip-Kimball classification and LVEF assessment; their results were also consistent with our findings. It is worth emphasizing that in contrary to the mentioned study our group consisted in nearly 60% of NSTEMI patients all of whom had invasive coronary angiography. The LC was significantly elevated (4.7 versus 3.7 mmol/l, $p < 0.025$) in patients with deteriorated systolic function of the left ventricle that we arbitrarily defined as an LVEF below 45% in the acute echocardiographic assessment prior to admission to cath lab. Vermeulen et al. [17] reported that an acute hemodynamic state in the patient (expressed by symptoms like increased heart rate, decreased blood pressure, and the presence of diabetes and distal embolization of coronary arteries) significantly impacted LC elevation and the 30-day mortality rate. These findings were also consistent with our results.

7. Conclusions

The point-of-care LC assessment of capillary blood using a handheld analyzer is a safe, easy, and cost-effective method to stratify the risk of late-onset heart failure and death after AMI. Its prognostic potential was conserved irrespective of clinical signs of shock.

8. Limitations of the Study

This is a pilot study; thus, the number of patients is relatively small. It was also a single-center study. However, the authors collecting the data did not interfere with the management process. Due to the design of the study and the national law regulations, all patients had to give informed and written consent; hence, the patients who were unconscious were excluded from the study. This could have impacted the potential selection bias and may have resulted in the absence of in-hospital deaths.

Competing Interests

The authors report no financial relationships or conflict of interests regarding the content in the paper.

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Research Article

Relationship between Serum Levels of Metalloproteinase-8 and Tissue Inhibitor of Metalloproteinases-1 and Exercise Test Results in Postmenopausal Women

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Physical activity as a part of the lifestyle is a significant factor influencing health condition. Exercises that require stamina are of particular importance. Oxygen metabolism, which is a significant part of all longer training processes, has an influence on cardiovascular and respiratory system functioning as well as all the processes taking part in maintenance of efficient homeostasis. Presentation of the correlation between exercise test results and MMP-8 (metalloproteinase-8) and TIMP-1 (tissue inhibitor of metalloproteinases-1) levels was attempted in this work. MMP-8 is a proteolytic enzyme taking part in progression of diseases related to process of ageing. 62 healthy women in postmenopausal period were qualified for the study (mean age: 54 ± 3.6). There was exercise test on the treadmill according to Bruce's protocol performed. MMP-8 and TIMP-1 serum levels were measured. There was statistically important correlation between increased level of MMP-8 and increased level of TIMP-1 with lower results of exercise test observed. The conducted study provides further biochemical arguments for prophylactic role of physical activity, which lowers the risk of noninfectious diseases, typical for middle adulthood, by influencing physical capacity.

1. Introduction

Systematic physical activity improves expression of genes which influence health condition [1]. Knowledge of these mechanisms enables considering moderate physical activity as one of the main means of chronic diseases prevention. An adequately high level of physical capacity in various age groups is one of health state indicators [2]. The assessment of body's morphological adaptation to physical exertion is a basis of reliable health-related training planning and physical rehabilitation. Research on connections between physical condition and level of chosen biochemical parameters (e.g., enzymes) may provide more arguments for thinking of physical activity as a factor influencing functioning of the body in various stages of life.

The postmenopausal period is a special time in women's life. Aside from frequent occurrence of typical ailments [3, 4], risk of cardiovascular events, which is significantly lower in premenopausal period in comparison with men, significantly increases [5–9]. Insufficient level of estrogens, which occurs in women in postmenopausal period, did not turn out to be influential enough in cardiovascular diseases pathogenesis to fulfill hopes related to hormone replacement therapy [10]. In women in this period of life cardiovascular diseases risk factors, such as hypertension [11–13], psychological disorders [14–17], overweight and obesity, dyslipidemia, and low physical activity [18–21], are commonly observed. The consequence of low physical activity is decreased physical capacity, which leads to premature involution processes and occurrence of risk factors, which frequently cause diseases of affluence. There is a connection between maximal oxygen consumption, VO_2max, and chronic diseases [2]. Research on connections between chosen physiological, structural, and biochemical parameters and active lifestyle allows the understanding of this relation.

2. Aim of the Study

The aim of the study is to assess the connection between physical capacity measured with exercise test and indicator of early vascular changes, increased matrix metalloproteinase (MMP), and its inhibitor activity, in clinically healthy women in postmenopausal period. Matrix metalloproteinases are the main proteolytic enzymes responsible for apoptosis and angiogenesis. They are involved in pathogenesis of diseases related to the process of ageing, such as arthritis, atherosclerosis, and neoplasms [22]. Assessment of the connection between physical capacity and level of metalloproteinase activity could show protective influence of physical activity on lowered risk of these diseases.

3. Material and Methods

The examined group consisted of 62 Caucasian women in the postmenopausal period at the age of 54.6 ± 3.6. Patients with medical history or clinical or laboratory evidence of serious or unstable disorders, such as coronary artery disease, stroke, or other cerebrovascular events, peripheral vessels diseases diagnosed earlier, heart failure, cardiomyopathy, mitral valve leaflet prolapse syndrome confirmed with echocardiography, preexcitation syndrome present in electrocardiography (ECG) or in medical history, left bundle branch block, myocardial bridge in medical history, musculoskeletal diseases not allowing the performance of the exercise test, diabetes or other severe systemic or organs diseases, clinical signs of hyperandrogenism, and thyroid disease, were excluded from the study. Women treated with hormone replacement therapy were also excluded from the study.

Methods used in the study included history taking, physical examination, exercise test, and assessing activity of metalloproteinase-8 (MMP-8) and tissue inhibitor of metalloproteinases-1 (TIMP-1) in serum. Women were defined as postmenopausal according to following criteria: (1) duration of amenorrhea > 12 months and (2) blood concentration of follicle stimulating hormone (FSH) > 30 IU/mL [23, 24]. Examination was performed in the Department of Internal Diseases, Medical University of Lublin, Poland.

Medical history data and physical examination results were registered in a questionnaire. Waist circumference was measured; body mass index (BMI) and waist-hip ratio (WHR) were calculated. Waist circumference was taken as the minimum circumference between the umbilicus and the xiphoid process and measured to the nearest 0.5 cm. BMI was calculated as weight in kilograms divided by the square of height in meters.

ECG exercise stress tests were performed with the treadmill stress test (GE Medical Systems, Freiburg, Germany) according to Bruce's protocol [25]. Test was discontinued after the pulse limit was reached or in case of chest pain, vertigo, dyspnoea, changes in ECG which indicated ischemia, dysrhythmia, ventricular or supraventricular tachycardia, gradual pressure decrease, increase of systolic pressure (SBP) above 260 mm Hg, increase of diastolic pressure (DBP) above 115 mm Hg, and bradycardia. In the exercise test assessment initial pressure, maximum systolic and diastolic pressure measured in exertion, increase of systolic and diastolic pressure, maximum heart rate, pulse increase, metabolic equivalent of task (MET), and duration of exertion were taken into consideration. Test was discontinued according to common rules; observation also included recovery period.

Table 1: General characteristics of the examined group ($n = 62$) (mean ± standard deviation).

Age (years)	54.6 ± 3.6
Height (cm)	160.7 ± 4.8
Weight (kg)	71.5 ± 13.0
BMI (kg/m^2)	27.7 ± 4.6
Waist circumference (cm)	89.4 ± 13.1
Waist-hip ratio	0.831 ± 0.060

Metalloproteinase-8 (MMP-8) and tissue inhibitor of metalloproteinases-1 (TIMP-1) levels in blood serum were assessed with immunosorbent tests using ELISA method with R&D Systems kits (Minneapolis, MN 55413, USA). Blood samples at rest, before exertion, were taken between 10 a.m. and 1 p.m. Blood level of metalloproteinase-8 (MMP-8) was assessed with kit whose catalogue number was DMP800; tissue inhibitor of metalloproteinase (TIMP-1) level was assessed with kit with catalogue number DTM100. Producer's instructions were followed. In every case calibration curve was made. Examination results were assessed with III universal microplatelets reader Bio-Tek ELX 800. Blood levels of MMP-8 and TIMP-1 were measured in ng/mL.

Data were processed using Statistica 10 (StafSoft) computer programme. Data are shown as means ± standard deviation (SD). Correlations between selected parameters were calculated using nonparametric test (R-Spearman's rank correlation coefficient).

The procedures of investigation were in accordance with the ethical standards of the responsible committee on human experimentation (Committee on Bioethics, Medical University of Lublin, KE-0254/185/2006) and with the Helsinki Declaration of 1975, as revised in 1983.

4. Results and Discussion

4.1. Results. The group of 62 women in the postmenopausal life period, confirmed by FSH level higher than 30 IU/mL, was examined. Mean menopause duration was 4.7 ± 4.5 years. The anthropometric data of studied group are presented in Table 1. None of examined women showed systematic physical activity. Their BMI values (Table 1) are indirect evidence of rather low physical activity [26]. In exercise test performed on treadmill mean exertion load was 9 MET and mean exertion duration was 7.9 min (Table 2). It allowed reaching average 83.2% of maximal pulse; average pulse increase from the initial pulse at rest was 68/min.

Mean metalloproteinase-8 level in blood serum in the examined group was 12.5 ± 7.5 ng/mL and mean tissue inhibitor of metalloproteinases-1 level was 221.7 ± 57.7 ng/mL. Concentration of MMP-8 in blood serum was statistically significantly negatively correlated with increase of heart rate during exertion test ($R = -0.523$; $p = 0.004$) (Figure 1). Positive correlation between MMP-8 level and diastolic pressure

Table 2: Exercise test results in examined group of women in postmenopausal period ($n = 62$) (mean ± standard deviation).

Exercise test duration (min)	7.9 ± 1.8
MET	9.0 ± 2.1
HR at rest (beats/min)	76.6 ± 11.8
SBP at rest (mm Hg)	127.4 ± 14.1
DBP at rest (mm Hg)	81.8 ± 10.5
HR reached during exercise (beats/min)	143.7 ± 17.4
HR increase during exercise (beats/min)	69.3 ± 23.0
HR max (beats/min)	172.8 ± 21.4
HR during exercise/HR max (%)	83.6 ± 12.3
SBP reached during exercise (mm Hg)	165.4 ± 17.1
DBP reached during exercise (mm Hg)	89.9 ± 9.0
SBP increase during exercise (mm Hg)	38.6 ± 18.0
DBP increase during exercise (mm Hg)	10.6 ± 8.9

SBP: systolic blood pressure; DBP: diastolic blood pressure; HR: heart rate; MET: metabolic equivalent of task.

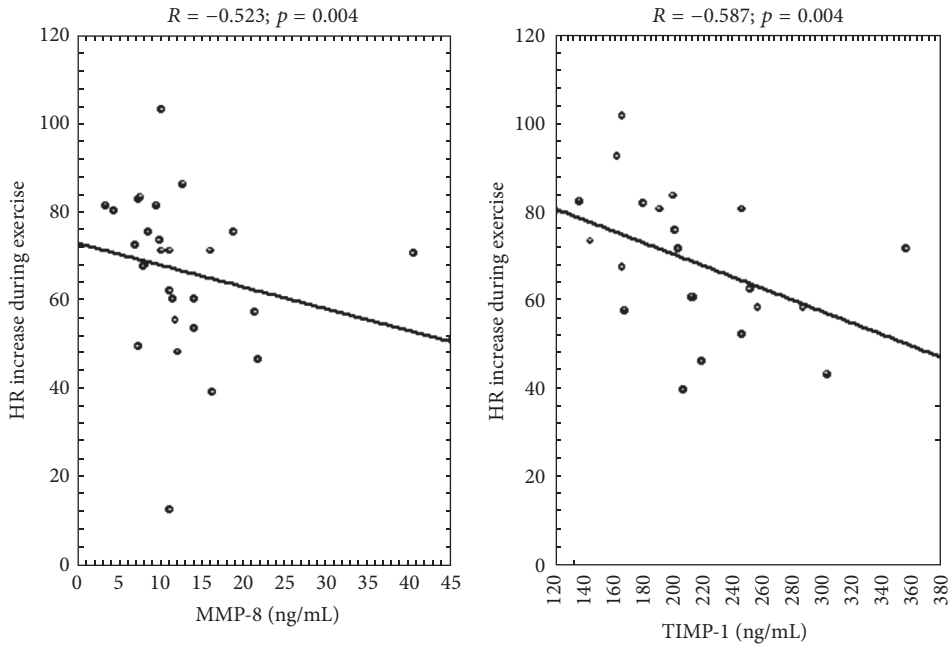

Figure 1: MMP-8 and TIMP-1 in relation to exertional heart rate increase. MMP-8: matrix metalloproteinase-8; TIMP-1: tissue inhibitor of metalloproteinases; HR: heart rate.

at rest was on the border of statistical significance ($R = 0.343$; $p = 0.074$). There were no statistical correlations between MMP-8 in serum and other exertion test parameters, MET, duration, and blood pressure (BP) in exertion and at rest (Table 3). TIMP-1 concentration in serum was significantly negatively correlated with heart rate increase ($R = -0.587$; $p = 0.004$) as well as maximal heart rate in exertion test ($R = -0.432$; $p = 0.045$). Other parameters of exertion test were not significantly correlated with TIMP-1 level in serum.

4.2. Discussion. Research on connection between active lifestyle and chosen physiological, structural, and biochemical parameters contributes to recognizing mechanisms of chronic diseases, such as cardiovascular diseases, which are the main cause of death and disability in group over 50 years old. Physical training improves the endothelium function [27] and is one of the most important cardioprotective means [28].

Clinical course of ischemic heart disease in women in postmenopausal period is specific. More often there are microcirculatory abnormalities, whose sign is positive electrocardiographic exercise test with no significant hemodynamic changes in the epicardial arteries. What is more, false positive and false negative results of the exercise test are more frequent, what limits usefulness of this examination in diagnostics of ischemic heart disease among women in postmenopausal period [29]. Exertion test may be a useful tool in planning health-related training; thus, it may be used in cardiovascular diseases primary prevention, particularly in groups with increased risk. One of these groups is women

Table 3: Exercise test parameters correlation with MMP-8 and TIMP-1 concentration in examined group of women in postmenopausal period ($n = 62$).

	MMP-8 (ng/mL)		TIMP-1 (ng/mL)	
	R	p	R	p
Exercise test duration (min)	−0.237	0.226	−0.138	0.551
MET	−0.103	0.601	−0.326	0.138
HR at rest (beats/min)	0.187	0.340	0.120	0.594
HR reached during exercise (beats/min)	−0.047	0.814	−0.432*	0.045
HR increase during exercise (beats/min)	−0.523*	0.004	−0.587*	0.004
SBP at rest (mm Hg)	0.224	0.252	−0.074	0.744
DBP at rest (mm Hg)	0.343	0.074	−0.262	0.239
SBP reached during exercise (mm Hg)	−0.090	0.650	−0.076	0.738
DBP reached during exercise (mm Hg)	0.306	0.113	0.074	0.744
SBP increase during exercise (mm Hg)	−0.225	0.270	0.203	0.379
DBP increase during exercise (mm Hg)	0.074	0.718	0.153	0.508

SBP: systolic blood pressure; DBP: diastolic blood pressure; HR: heart rate; MET: metabolic equivalent of task.
*Statistically significant.

in postmenopausal period, independently from discussions about hormonal changes influence on the circulatory system. Physical capacity can be determined in the exertion tests using several measured parameters: heart rate at rest, its increase during exercise, blood pressure at rest and during exercise, and, finally, calculated metabolic equivalent of task.

One of the cardiovascular risk factors is low physical activity, which may modify exercise test results by influencing physical capacity of women after menopause. That is why the question regarding whether there is a correlation between exercise test results and early biochemical indicators of pathological process within the arteries remains to be answered. These indicators include metalloproteinases, enzymes which take part in extracellular matrix remodeling. They are secreted as proenzymes and activated by proteinases, plasmin, trypsin, chymotrypsin, kallikrein, cathepsin, and some MMPs. The main sources of all metalloproteinases are inflammatory cells [30, 31]. Metalloproteinases include cell membrane metalloproteinases which are related to cell membrane (MMP-14, MMP-15, MMP-16, and MMP-17), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-7, MMP-10, MMP-11, and MMP-12), and collagenases (MMP-1, MMP-8, and MMP-13). Metalloproteinases take part in many physiological and pathological processes and they are responsible for remodeling and degrading the connective tissue [32]. MMP-8 is elevated in periodontal diseases in humans [33]. It is not specific and can be considered as general inflammation marker. However, there is a growing evidence suggesting a link between periodontal state and coronary heart disease [34]. Thus, a potential role of MMP-8 as a marker of increased risk of myocardial ischemia can be hypothesized.

Metalloproteinases activity may be moderated by tissue inhibitors, which include TIMP-1. Metalloproteinases inactivation is based on forming complexes which consist of active forms of MMP or some proenzymes and tissue inhibitor, complexes MMP-TIMP [35, 36]. TIMP-1 inhibits activity of majority of MMPs by forming irreversible complexes. Complexes MMP-TIMP take part in inflammatory processes, cardiomyocytes damage, forming aneurysms of the aorta, blood pressure regulation by influencing tension of arteries walls, vessels remodeling, coagulation, and angiogenesis processes [35–37].

In the atherosclerotic lesions MMP-8 expression was found. MMP-8 inactivation in experiments conducted on mice causes significant decrease of atherosclerotic lesions formation. Genetic knockout of MMP-8 in mice also results in lower angiotensin II concentration, lower blood pressure, and decrease of adhesive molecules number in atherosclerotic plaque, which lowers leucocytes adhesion to the endothelium. These data indicate an important role of MMP-8 in atherosclerosis process [37, 38]. That is why increased expression of MMP-8 along with decreased expression of its tissue inhibitor is disadvantageous and may be a sign of forming vessels changes of atherosclerotic nature, which is not necessarily clinically noticeable. In the studied group of women in postmenopausal period there was statistically significant correlation of MMP-8 increase in serum and small heart rate increase in exertion found. What is more, the disadvantageous changes in TIMP-1 were observed in the same group of patients. It is possible that higher heart rate at rest naturally resulting in lower difference between heart rates at rest and in exertion, which is characteristic for persons with lower physical activity and is one of atherosclerosis risk factors, causes tendency towards MMP-8 and its tissue inhibitor level increase in serum. What is more, disadvantageous changes in MMP-8 level seem to occur in patients with higher diastolic pressure at rest, though this correlation is on border of statistical significance, $p = 0.074$. Hence, MMP-8 level seems to be a potential candidate to a novel marker of cardiovascular risk among postmenopausal women.

To our best knowledge, there have not been any researches indicating correlations between exercise test results and metalloproteinases and their inhibitors blood levels in clinically healthy women in postmenopausal period yet.

Confirmation of results of this study and their practical usefulness demand further research on bigger group of women.

5. Conclusions

(1) Increased level of MMP-8 in serum is correlated with lower heart rate increase in exercise test in women in postmenopausal period.

(2) Tissue inhibitor of metalloproteinases activity is higher among women in postmenopausal period who present lower maximal heart rate and lower heart rate increase in exertion.

(3) These changes may constitute evidence of pathological process within the arteries in women with lower than common for this group physical capacity.

Competing Interests

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

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Research Article

Is Urinary NGAL Determination Useful for Monitoring Kidney Function and Assessment of Cardiovascular Disease? A 12-Month Observation of Patients with Type 2 Diabetes

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Background. Diabetic kidney disease (DKD) may start as glomerular or tubular damage. We assessed kidney function during one-year-long observation of patients with type 2 diabetes mellitus (T2DM) after initiation of nephroprotective treatment, with emphasis on the changes in urinary neutrophil gelatinase-associated lipocalin (uNGAL), and evaluated the association between tubular damage and cardiovascular complications of T2DM. *Materials and Methods*. Adult T2DM patients (55) were assessed initially and 30 patients after 1 year. Albumin and uNGAL and creatinine were measured in first morning urine. Albumin/creatinine (uACR) and uNGAL/creatinine (uNCR) ratios were calculated. *Results*. In logistic regression, both uACR above 30 mg/g and uNCR the median (21.3 μg/g) were associated with cardiovascular complications, independently of classical risk factors and diabetes duration. One year after initiation of treatment, a significant reduction in HbA_{1c} was observed. BMI and lipid profiles did not change. Increase in serum creatinine and reduction in eGFR occurred, along with decrease in uNGAL and uNCR. Increasing uNCR and uACR were associated with higher control HbA_{1c}. The increase in uNCR was more frequent in patients with hypertension. *Conclusions*. Better glycemic control in T2DM patients results in improved tubular function, as reflected by reduced uNCR and uNGAL. First morning urine uNGAL and uNCR may be useful to assess renal function and cardiovascular risk, along with albuminuria and eGFR.

1. Introduction

The prevalence of diabetes worldwide is over 9 percent, and it is gradually increasing [1]. The World Health Organization (WHO) estimates that, in highly developed countries, 85 percent of population will suffer from type 2 diabetes mellitus (T2DM) [2]. The mortality due to diabetes complications, resulting from diabetic macroangiopathy, microangiopathy, or neuropathy, is an important social and clinical issue. In accordance with the latest 2016 European Society of Cardiology (ESC) guidelines, diabetic patients are considered to be at a very high or high risk of developing cardiovascular disease (CVD) [3]. It is assumed that 30–35 percent of genetically predestined individuals, receiving no or inadequate treatment, may develop angiopathy in renal microcirculation and diabetic kidney disease [4]. Glomerular filtration rate (GFR) lower than 60 mL/min/1.73 m^2 further increases the risk of developing CVD [3]. Available studies clearly point

to increased mortality rate of patients suffering from T2DM associated with nephropathy. However, early recognition of T2DM and adequate treatment inhibit progression of changes in blood vessels and contribute to favorable prognosis [3].

Kidney diseases are diagnosed on the basis of serum creatinine concentration and estimated GFR (eGFR), albuminuria, renal imaging, and histology following renal biopsy. However, low accuracy or invasiveness of these tests causes the fact that they frequently do not meet expectations of clinicians. In accordance with American Diabetes Association (ADA) 2016 standards, in order to detect or assess DKD, urinary albumin excretion and eGFR have to be measured annually [4]. Taking into account a limited diagnostic value of eGFR in detection of early renal dysfunction [5], as well as the fact that not all people with DKD have increased albuminuria, a search for new markers for kidney damage seems necessary. The new markers should be characterized by higher diagnostic sensitivity and specificity and should allow detecting the nonglomerular kidney damage [6–8].

Neutrophil gelatinase-associated lipokalin (NGAL), a member of the lipocalin protein family, has been recognized as one of the most promising biomarkers of early stages of kidney damage. It is a secreted protein with a molecular weight of 25 kDa, found in the neutrophil granules [9]. Recent studies focus mainly on the role of NGAL as a biomarker of acute kidney injury (AKI) [10, 11]. However, NGAL may also serve as a marker of chronic kidney disease (CKD), including DKD [12, 13]. Due to high biological variability of urinary NGAL (uNGAL), in CKD patients, uNGAL measurements should be accompanied by the assessment of urine creatinine concentration and calculation of uNGAL/creatinine ratio (uNCR) [14]. Prospective studies in DKD patients indicated the association between increasing uNGAL and the progression of kidney disease [15, 16] and negative correlation between uNCR and eGFR [16, 17], irrespective of albuminuria, although a positive correlation with albuminuria was also observed in some studies [16]. These observations in T2DM patients encourage the hypothesis that NGAL may be an earlier biomarker of DKD than albuminuria. The measurements of initial values of uNGAL and uNCR, and subsequent regular monitoring of their changes, seem to be useful in the assessment of kidney function in T2DM patients. Promising preliminary results were also obtained in our studies: namely, we found that uNCR exceeding 21.3 μg/g may be useful for early prediction of renal tubular damage in the course of DKD [18].

The aim of the study was to assess the changes in renal function of T2DM patients during a 12-month observation, following the introduction of nephroprotective treatment according to ADA 2016 standards. Special attention was paid to the changes in uNGAL concentrations and uNCR values after treatment. We evaluated the correlations between the changes in markers of kidney function after a year-long treatment and the diabetes duration, blood glucose concentrations, and the use of medications affecting the renin-angiotensin-aldosterone system (RAAS) and the lipid metabolism. Additionally, the relationship between uACR and uNCR values and cardiovascular complications were assessed at the beginning of the study.

2. Materials and Methods

The study group consisted of women and men diagnosed with T2DM and undergoing treatment at the Department of Nephrology at St. Queen Jadwiga Clinical District Hospital No. 2 in Rzeszow, Poland. The inclusion criteria were age above 18 years, T2DM, and eGFR $\geq$60 mL/min/1.73 m^2. The exclusion criteria were anemia, overt proteinuria or urinary albumin/creatinine ratio (uACR) > 300 mg/g, hematologic malignancies, systemic connective tissue diseases, allergies, infections, uncontrolled hypertension, and treatment with potentially nephrotoxic medications. Patients gave written informed consent for the study. The protocol received permission from the Bioethics Committee of the Regional Medical Chamber in Rzeszow, Poland (approval number 70/2014/B issued on 19 September 2014).

A cross-sectional analysis was conducted using data obtained at the first visit of patients. A prospective, observational study was conducted in patients with available follow-up data. At the beginning of the study patients underwent a careful clinical examination, including the assessment of body mass index (BMI) and blood pressure, as well as laboratory tests. Cardiovascular complications of T2DM were diagnosed in patients with ischemic heart disease, heart failure, systemic atherosclerosis, or ischemic stroke that occurred after the diagnosis of T2DM. In accordance with current clinical regulations [19], nephroprotective treatment was initiated, hypertension treatment was modified taking into account documented, outpatient blood pressure readings, diabetes treatment was adjusted taking into account outpatient measurements of glycemic status and the measurements of glycated hemoglobin (HbA_{1c}), fluid and electrolyte balance was regulated, and hypolipemizing treatment was initiated or modified in order to control the lipid profile and the liver function parameters. Twelve months after the initiation of the study a subsequent clinical assessment of patients and laboratory tests were conducted.

Laboratory tests included fasting serum glucose, HbA_{1c}, complete blood count, triglycerides, total cholesterol, low-density lipoprotein- (LDL-) and high-density lipoprotein-(HDL-) cholesterol, and serum creatinine. eGFR was calculated on the basis of CKD-EPI equation [20]. The urine tests included examination of the sediment, concentrations of uNGAL, albumin, and creatinine in first morning urine sample. uNGAL was measured using CMIA (chemiluminescent microparticle immunoassay), on the immunochemistry platform ARCHITECT® (ARCHITECT Analyzer, Abbott Diagnostics, Abbott Park, USA). uNCR and uACR were calculated. The laboratory tests were performed at the Department of Laboratory Diagnostics at St. Queen Jadwiga Clinical District Hospital No. 2 in Rzeszow (Poland) at the day of blood collection.

2.1. Statistical Analysis. Numbers of patients (percentages of the study group) were reported for categories. Mean ± standard deviation or median (upper-lower quartile) was reported for quantitative variables (depending on distribution as evaluated with Shapiro-Wilk's test). Chi-squared test was used to analyze contingency tables. The parametric tests

Table 1: Baseline characteristics of the studied group of 55 T2DM patients with respect to cardiovascular complications of diabetes.

	Patients with cardiovascular complications ($N = 19$)	Patients without cardiovascular complications ($N = 36$)	p
Age, years	70 ± 11	59 ± 15	0.022
Female sex	10 (53)	19 (53)	1.0 [NS]
Ischemic heart disease, N (%)	17 (89)	—	—
Heart failure, N (%)	6 (32)	—	—
Systemic atherosclerosis, N (%)	5 (26)	—	—
T2DM duration, years	7 (4–10)	5 (1–10)	0.1 [NS]
Hypertension, N (%)	16 (84)	26 (72)	0.3 [NS]
Dyslipidemia, N (%)	18 (95)	33 (92)	0.7 [NS]
BMI, kg/m^2	32.6 ± 7.6	30.8 ± 5.2	0.5 [NS]
HbA_{1c}, %	6.2 (6.1–6.6)	7.5 (6.2–9.4)	0.2 [NS]
WBC, $10^3/\mu$L	8.0 (5.3–9.7)	7.1 (5.9–8.4)	0.8 [NS]
Serum creatinine, μmol/L	64.5 (60.1–82.2)	65.8 (58.8–76.9)	0.6 [NS]
eGFR, mL/min/1.73 m^2	87 (71–94)	95 (81–99)	0.07 [NS]
Albuminuria, mg/L	12.3 (7.2–41.2)	9.5 (6.3–13.4)	0.048
uACR, mg/g	16.0 (7.5–53.6)	7.8 (3.4–13.2)	0.005
uNGAL, μg/L	22.3 (10.4–56.9)	18.8 (8.8–42.6)	0.7 [NS]
uNCR, μg/g	29.1 (13.4–58.8)	16.2 (9.5–38.8)	0.038

T2DM, type 2 diabetes mellitus; N, number of patients; BMI, body mass index; HbA_{1c}, hemoglobin A_{1c}; WBC, white blood cells; eGFR, estimated glomerular filtration rate; uNGAL, urine neutrophil gelatinase-associated lipocalin; uNCR, urinary NGAL/creatinine ratio; uACR, urinary albumin/creatinine ratio; NS, nonsignificant result.

were used for normally distributed data, and nonparametric tests were used for nonnormally distributed data. In detail, the results obtained at the beginning of the study and after 12 months were compared using paired t-test or Wilcoxon test. The differences between groups were tested with unpaired t-test or Mann–Whitney test. Correlations were assessed using Pearson's or Spearman's correlation coefficients. In particular, the correlations between changes in the markers of kidney function were evaluated; the change in the value of a given marker was defined as the difference between the control value (value after 12 months of treatment) and the initial value (at the beginning of the study). Multiple logistic regression was calculated to study the association between cardiovascular complications and uACR and uNCR values, with adjustment for classical cardiovascular risk factors. Results were considered statistically significant at $p < 0.05$. Statistica 12 (StatSoft, Tulsa, USA) software was used for computations.

3. Results

Initially, 55 patients were qualified for the study. Nineteen of them were diagnosed with cardiovascular complications of T2DM. Patients with cardiovascular complications were characterized with older age, higher albuminuria and uACR values, and higher uNCR values (Table 1). Median uNCR value in the group of 55 T2DM patients was 21.3 μg/g. In multiple logistic regression, both uACR above 30 mg/g and uNCR the median were associated with cardiovascular complications independently of classical cardiovascular risk factors and diabetes duration (Table 2).

The follow-up data after 12 months from initiation of nephroprotective treatment were available for 30 patients. The prospective study group included 17 women (56%) and 13 men (44%), aged 64 ± 13 years. The median duration of T2DM at the beginning of the study was 9 (2–11) years. At the beginning of the study most patients were diagnosed with comorbidities: hypertension in 23 (77%), ischemic heart disease in 17 patients (31%), including one with the history of non-ST elevation myocardial infarction, systemic atherosclerosis in 5 (9%), and heart failure in 6 (11%) patients. Additionally, one patient had history of transient ischemic attack. No new cardiovascular complications were diagnosed during the follow-up. Most patients with hypertension received medications affecting the RAAS (angiotensin converting enzyme inhibitors or angiotensin receptor blockers). These medications were used by 21 patients (70%) at the beginning of the study. Only in 2 patients with hypertension, because intolerance was not treated by the RAAS inhibitors, one of these patients was repeatedly prone to develop hyperkalemia, whereas the other developed hypotension when RAAS inhibitors were added to alpha-blocker used because of urological disorder. During the study, 28 patients (93%) were treated with RAAS inhibitors. Twelve patients (40%) were treated with statins.

The characteristics of the study group at the beginning of the study and after 12 months are presented in Table 3. At the beginning of the study 26 patients (87%) had a BMI ≥25 kg/m^2, and the percentage remained the same after 12 months. During the study, BMI decreased in 16 patients (53%), increased in 9 (30%), and did not change in the remaining 5 patients (17%). Mean HbA_{1c} decreased after

Table 2: Multiple logistic regression model showing the association between selected variables and cardiovascular complications among 55 T2DM patients evaluated at the beginning of the study.

Independent variables	Odds ratio (95% confidence interval)	p
Age, years	1.11 (0.99–1.25)	0.07 NS
Female sex	0.44 (0.05–3.80)	0.4 NS
T2DM duration, years	1.03 (0.84–1.26)	0.8 NS
BMI, kg/m^2	1.22 (1.00–1.48)	0.041
Hypertension	5.21 (0.15–185)	0.3 NS
Dyslipidemia	0.16 (0.02–1.68)	0.1 NS
uACR > 30 mg/g	25.20 (1.01–639)	0.042
uNCR > 21.3 μg/g	14.99 (1.01–247)	0.048
Whole model	chi^2 = 19.8; p = 0.011	

For abbreviations, see Table 1.

Table 3: Characteristics of 30 T2DM patients with available follow-up data at the beginning of the study (baseline results) and after 12-month follow-up (control results).

	Baseline results	Control results	p
BMI, kg/m^2	30.9 ± 5.5	31.4 ± 5.7	0.6 NS
HbA_{1c}, %	7.98 ± 1.99	6.31 ± 0.93	0.037
Hemoglobin, g/dL	14.2 ± 1.4	13.9 ± 1.4	0.019
WBC, $10^3/\mu$L	7.59 ± 2.39	7.34 ± 2.67	0.5 NS
Total cholesterol, mmol/L	5.38 (4.11–5.95)	4.78 (4.01–5.86)	0.3 NS
LDL-cholesterol, mmol/L	3.10 (2.07–3.75)	2.62 (1.98–3.94)	0.3 NS
HDL-cholesterol, mmol/L	1.32 (1.01–1.53)	1.36 (1.03–1.49)	0.1 NS
Triglycerides, mmol/L	1.57 (1.13–1.89)	1.53 (1.16–2.03)	0.5 NS
Serum creatinine, μmol/L	68.1 (60.1–76.9)	69.0 (61.9–77.8)	0.035
eGFR, mL/min/1.73 m^2	94.4 (79.7–98.3)	87.0 (74.6–99.0)	0.023
Albuminuria, mg/L	8.53 (6.59–13.53)	5.55 (2.14–19.75)	0.2 NS
uACR, mg/g	7.49 (3.39–13.38)	4.69 (2.86–43.41)	0.1 NS
uNGAL, μg/L	18.00 (9.00–32.20)	9.35 (2.50–19.30)	0.018
uNCR, μg/g	16.18 (10.00–33.72)	8.82 (3.09–26.83)	0.037
Leukocyturia, n/(%)	3 (10)	5 (17)	0.4 NS

LDL, low-density lipoprotein; HDL, high-density lipoprotein; see Table 1.

12 months of treatment (Table 1), and in 19 patients (63%) good glycemic control was achieved (HbA_{1c} < 6.5%). The treatment had no influence on the concentrations of total cholesterol, HDL- and LDL-cholesterol, or triglycerides (Table 3).

After 12 months, serum creatinine increased in 17 patients (57%) along with a decrease in eGFR (by 7 mL/min/1.73 m^2 on average and 16 maximum). In 4 patients (13%) eGFR did not change, whereas in 9 (30%) it increased (by 4 mL/min/1.73 m^2 on average and 10 maximum). Overall, average creatinine concentrations slightly increased, and average eGFR values in the whole study group decreased (Table 3). Simultaneously, average concentrations of uNGAL and urinary albumin decreased; however, in the case of albuminuria and uACR the difference was not statistically significant (Table 3). After a year of treatment, uNGAL concentrations increased in 10 patients (33%), but in 20 patients (67%) there was a considerable decrease in uNGAL (Figure 1). In turn, uNCR increased in 9 patients (30%) and decreased in 21 (70%). Albuminuria increased in 12 (40%) and uACR increased in 11 (73%), while it decreased in the remaining patients.

Patients whose eGFR values decreased during the study had higher initial concentrations of uNGAL and lower control concentrations of HDL-cholesterol and of triglycerides than patients whose eGFR values did not change or increased (Figures 2(a)–2(c)). The change in eGFR (defined as the difference between eGFR values at the end of the study and eGFR values at the beginning) was positively correlated with control concentrations of total cholesterol (R = 0.43; p = 0.022) and the initial and control concentrations of HDL-cholesterol (R = 0.39; p = 0.042 and R = 0.48; p = 0.010, resp.). The change in eGFR was negatively correlated with the initial values of uNCR

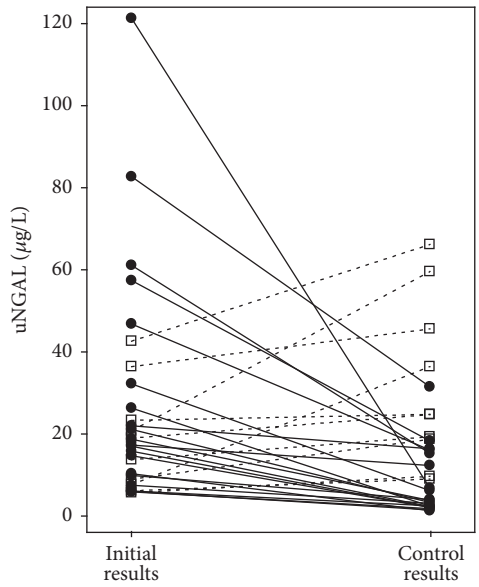

Figure 1: Urinary NGAL concentrations at the beginning of the study (initial results) and after 1 year of nephroprotective treatment (control results) among 30 DKD patients with available follow-up data. Closed circles and solid lines represent patients with decreasing uNGAL; open squares and dashed lines represent patients with increasing uNGAL. For abbreviations, see Table 1.

($R = -0.38$; $p = 0.036$) and the control concentration of uNGAL ($R = -0.51$; $p = 0.004$). The differences between the concentrations of uNGAL after 12 months and the initial concentrations correlated with, analogically assessed, changes in the concentrations of urinary albumin ($R = 0.42$; $p = 0.026$). A similar correlation was observed between the changes in uNCR and uACR ($R = 0.48$; $p = 0.011$). In most patients, a decrease in the values of both markers was observed during the study (Figure 3). In comparison with patients whose uACR values decreased, patients whose uACR values increased during 12 months had higher control values of HbA_1 and higher leukocyte count (Figures 2(d) and 2(e)). The increase in uNGAL concentrations was associated with higher control concentrations of HbA_{1c} (Figure 2(f)). Additionally, diabetes duration correlated positively with the changes in albuminuria ($R = 0.40$; $p = 0.033$) and uNCR ($R = 0.46$; $p = 0.010$).

The increase in uNCR was significantly more frequent ($p = 0.048$) in hypertensive patients (9 out of 23 patients, 45%) than in patients with no hypertension (0 out of 7 patients). No correlations were observed between the increase or decrease in the values of kidney function markers and the presence of other comorbidities or medications applied. Furthermore, no correlations were observed between the changes in the values of kidney function markers and the age or gender of patients.

4. Discussion

DKD remains one of the most serious complications of diabetes. Its late recognition and inadequate treatment may lead to end-stage renal disease and the need for renal replacement therapy. However, although DKD is progressive and irreversible, there are studies indicating that early recognition of the disease and initiation of nephroprotective treatment may slow down its progression.

In T2DM patients, kidney function must be evaluated in a comprehensive manner. The evaluation should take into account not only GFR and albuminuria indicating the possible damage to the filtration membrane, but also the function of renal tubules. To assess the function of renal tubules, we analyzed the changes in uNGAL and uNCR measured in a first morning urine sample during a 12-month observation of patients suffering from T2DM and CKD stages G1 and G2, with accompanying normal or moderately increased albuminuria (A1 or A2), according to KDIGO criteria [20].

Patients recruited for our study had medical conditions and symptoms typical for the T2DM related metabolic syndrome and insulin resistance. These was excess weight or obesity (more than 78% of patients), hypertension (more than 77% of patients), or dyslipidemia requiring drug treatment (40% of patients). Taking into account cardiovascular disease observed in the ARETAEUS1 study, a study examining the clinical profile of the Polish population with T2DM of short duration, the patient group in our study was a representative sample [21].

According to the latest ADA 2016 and ESC 2016 guidelines, T2DM patients should change their dietary habits, maintain regular physical activity, and reduce their body weight [19]. Most patients in our study group were able to lose weight due to pharmacological treatment, as well as changes in diet and lifestyle.

According to the SHARP study, in order to reduce the risk of CVD in CKD patients, statins should be used [22]. Moreover, statins play a significant role in nephroprotective treatments [23]. In our study, 40% of patients were treated with statins. In accordance with ESC 2016 guidelines, none of patients was treated with fibrates, even though hypertriglyceridemia is a most pronounced lipid disorder in T2DM patients [24].

From the clinical point of view, it is important to observe that in most patients glycemic control, measured with HbA_{1c}, improved after 12 months. This, in turn, had probably a direct influence on the reduction of albuminuria and the uACR values. Albuminuria is a recognized risk factor both for the progression of DKD and for cardiovascular disease [3]. Consequently, for nephrologists, reduction of albuminuria is crucial in the treatment of T2DM [19]. However, it has to be remembered that increased albuminuria (30–300 mg/g) is the first indicator of DKD only in a part of T2DM patients. In about 30% of DKD patients, progressive reduction in GFR is not accompanied by increased excretion of urinary albumin [25].

Since the assessment of kidney function based on eGFR and albuminuria is far from being satisfactory, and it allows only monitoring the function of the glomerular filtration membrane, we have made an attempt to assess the kidney function in T2DM patients with respect to possible damage to renal interstitium, by measuring uNGAL. uNGAL excretion

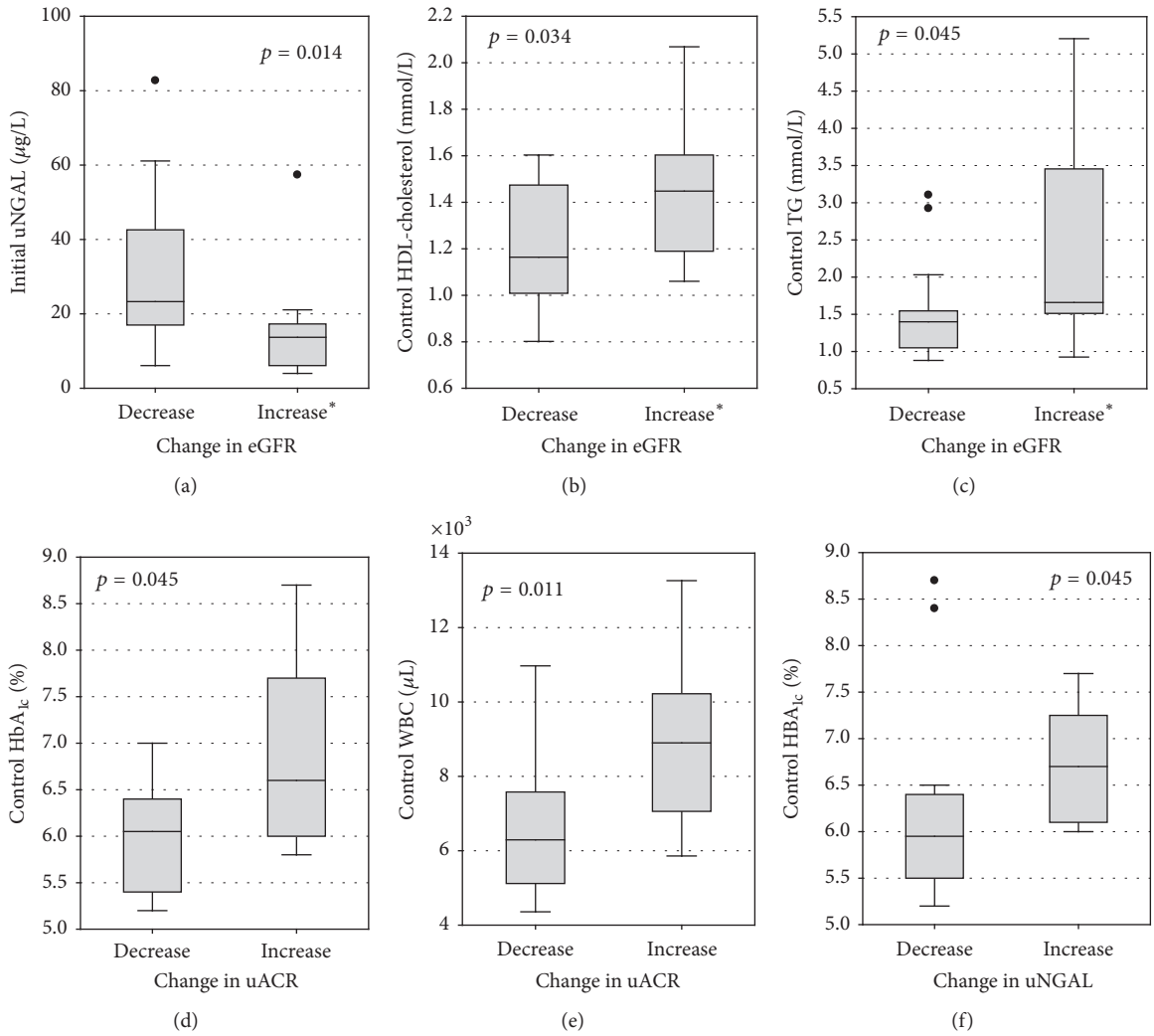

Figure 2: Statistically significant differences in laboratory test results between patients with different direction of change in the studied markers of kidney function. The change in the marker of kidney function was defined as the difference between the control value (after 12 months of treatment) and the initial value (at the beginning of the study). *Increase or no change in eGFR. TG, triglycerides; see Tables 1 and 3.

increases significantly in the epithelial cells of the ascending limb of the loop of Henle in renal tubules in a response to ischemia or toxins [26]. It is assumed that NGAL may play a significant role in pathophysiology of kidney adaptation to the destructive influence of diabetic environment on renal tubules [14].

In this study, following the implemented treatment, a statistically significant decrease in uNGAL and uNCR was obtained after 12 months. The observed tendency for decreasing uNGAL and uNCR values may be a confirmation of the positive influence of multifactorial treatment of T2DM patients, aimed at inhibiting the progression of renal tubules damage. During a 3.5-year observation of T2DM patients, Nielsen et al. [27] observed a positive correlation of uNCR with HbA_{1c}. Similarly, we observed a decrease in HbA_{1c} during a 12-month study, accompanied by a decrease in tubular proteinuria (uNCR values).

The observed significant decrease in eGFR confirms the observation that in people over the age of 30, the decline in GFR values is a physiological process (the annual rate of decrease in GFR is 0.75–1 mL/min/1.73 m^2) [28, 29], further accelerated to 2.3–5.4 mL/min/1.73 m^2 in DKD [30]. In our study, the decrease in eGFR values may also be a consequence of blocking the RAAS in most patients. Such treatment aims at lowering the glomerular filtration pressure, which in the initial phase of DKD leads also to a clinically insignificant decrease in eGFR. However, when applied for a longer time, such treatment has nephroprotective and cardioprotective effects, independently of blood pressure values [3]. According to ADA standards, all patients with uACR greater than 30 mg/g, irrespective of eGFR values, should be treated with angiotensin converting enzyme inhibitors (ACEI) and angiotensin II receptor blockers. In such patients, it is necessary to monitor the serum creatinine and kalemia.

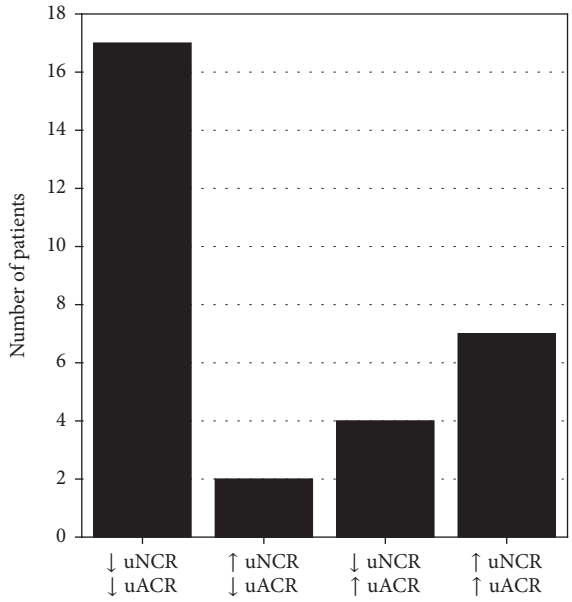

Figure 3: Numbers of patients in whom either a decrease or an increase in the values of uNCR and uACR was observed after 12 months of treatment in comparison with initial values. ↓: decrease in the value of a given marker as assessed after 12 months of the study; ↑: increase in the value of a given marker as assessed after 12 months of the study; For abbreviations, see Table 1.

This treatment is not recommended in primary prevention of DKD with normotension, normal range of albuminuria, and normal eGFR [19]. Treatment with ACEI leads to the reduction in hemoglobin concentration [31, 32]. In this study the decrease in hemoglobin concentrations (statistically significant, but clinically insignificant), confirms, together with the hypotensive effect, the effectiveness of ACEI.

Other authors, in 12-month follow-up of DKD patients, observed the increase in uNGAL [33], as well as inverse correlations between uNGAL, uACR, and GFR in T2DM patients [15–17], which is contrary to our results. The discrepancies may result from different patient care and from racial differences in populations studied (all patients in our study were of Caucasian race).

The authors of ESC 2016 guidelines stress the potential benefits of introducing novel urinary biomarkers in selected patient populations, as these may contribute to improved assessment of cardiovascular risk [3]. Clinical trials aimed at finding the optimal biomarker pass through successive phases [34]. The authors of this study have observed that, at the time of the first visit, patients with cardiovascular disease had higher values of uACR and uNCR than patients without cardiovascular disease. Both uACR and uNCR were independently associated with cardiovascular complications, irrespective of classical cardiovascular risk factors. No new incidents of cardiovascular disease were diagnosed in the study group during the 12-month follow-up. During this time significant decrease in both uNGAL and uNCR and no increase in albuminuria were observed. Our results indicate that renal complications of T2DM, both those involving glomeruli and tubules, are significantly associated with cardiovascular complications of diabetes. Thus, the measurement of uNCR may be helpful in the clinical prediction of CVD in T2DM patients.

The major limitation of our study is the small number of patients enrolled. Therefore, we cannot draw any definitive conclusions. However, the results seem promising and should be validated in larger studies.

5. Conclusions

On the basis of a 12-month observation of early-phase DKD patients it can be concluded that multifactorial nephroprotective treatment, focused primarily on the improvement of glycemic control, has a positive effect on the function of renal tubules as reflected by the diminishing concentrations of uNGAL and uNCR. Additionally, uNCR may be considered as independent predictor of the increased risk of CVD in the population studied.

Competing Interests

The authors declare no conflict of interests.

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Review Article

Platelets miRNA as a Prediction Marker of Thrombotic Episodes

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The blood platelets are crucial for the coagulation physiology to maintain haemostatic balance and are involved in various pathologies such as atherosclerosis and thrombosis. The studies of recent years have shown that anucleated platelets are able to succeed protein synthesis. Additionally, mRNA translation in blood platelets is regulated by miRNA molecules. Recent works postulate the possibility of using miRNAs as biomarkers of atherosclerosis and ischemic episodes. This review article describes clinical studies that presented blood platelets miRNAs expression profile changes in different thrombotic states, which suggest use of these molecules as predictive biomarkers.

1. Background

According to the World Health Organization statistics, cardiovascular disease causes nearly half of the deaths in developed countries. A worrying trend is the increase in the incidence of young people despite the growing awareness of healthy life style, the role of physical activity, and proper diet [1]. Myocardial infarction (MI) in persons under the age of 45 years accounts for 6% to 10% of this type of incidents. Unlike older patients, half of young patients have single-vessel coronary disease, and, in up to 20%, the cause is not related to atherosclerosis. One of the important risk factors for MI was family history of disease [2]. "CONFIRM" study conducted from 2003 to 2009 shows that positive family history of MI is the strongest clinical predictor of future myocardial infarction in young patients [3]. All of these evidences indicate the need for a detailed analysis of the genetic basis of the pathogenesis of thrombosis. It has been shown that miRNAs regulate the biological response of platelets: change of their shape and secretion of granules content [4]. miRNA profiling has been shown to be more accurate than mRNA expression profiling in characterizing the differentiation of multiple human cancers [5]. That postulates the possibility of using platelets miRNAs as predictive biomarkers of thrombotic events. This review article describes clinical studies that presented blood platelets miRNAs expression profile changes in different thrombotic states, which suggest use of these molecules as predictive biomarkers.

2. The Role of Platelets in Thrombotic Events

The blood platelets are crucial for the coagulation physiology to maintain haemostatic balance and are involved in various pathologies such as atherosclerosis and thrombosis. Due to a large number of specific membrane receptors blood platelets are high reactive cells, readily activated by many physiological and nonphysiological agonists. The signaling pathways *via* specific receptors are dependent on the type of agonists but they always lead to physiological responses expressed as platelet activation [6]. The expression of multiple membrane receptors, both constitutive and activation-dependent, mediates platelet adhesion and aggregation at sites of vascular injury. In primary haemostasis activation of blood platelets leads to formation of platelet plug that seals the breach in the vessel wall and prevents excess blood loss [7, 8]. Subsequently, the activated platelets facilitate secondary haemostasis which supports the formation of a fibrin clot by carrying coagulation factors and providing a catalytic surface

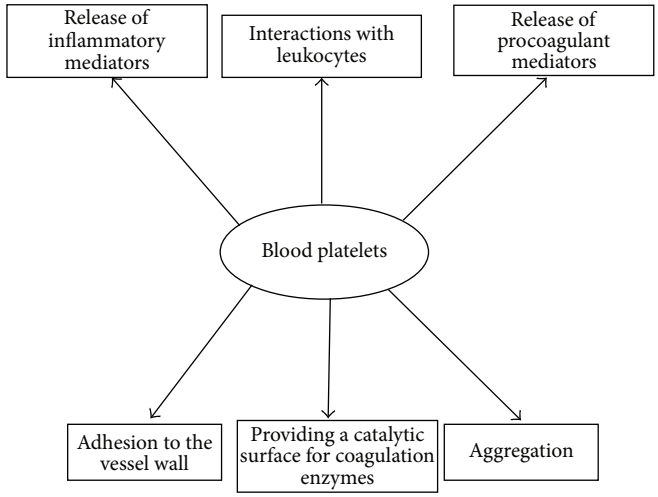

Figure 1: Scheme presented role of blood platelets in hemostasis.

for the major interactions of the coagulation cascade [9–11] (Figure 1).

The platelet activation mediated by a complex series of intracellular processes involved in haemostasis, thrombosis, and inflammation is one of the most important risk factors in the cardiovascular system disturbance, associated with the occurrence of thromboembolic complications [12]. Thromboembolic complications leading to ischemic acute coronary syndromes, stroke, and deep vein thrombosis are the reason of death or chronic conditions that limit the quality of life and generate high costs of therapy and care.

The acute coronary syndrome (ACS) refers to group of clinical symptoms compatible with acute myocardial ischemia and includes unstable angina (UA), non-ST-segment elevation myocardial infarction (NSTEMI), and ST-segment elevation myocardial infarction (STEMI) [13]. It is well known that acute coronary syndromes with different clinical manifestations have a common pathophysiology, which is associated with coronary artery thrombosis [14]. The platelets are known to play a fundamental role in pathogenesis of ACS. Platelets are able to form pathogenic, occlusive intracoronary thrombus, leading to acute ischemic events [15]. The platelet adhesion and aggregate formation are critical events that occur in ACS. The patients with ACS exhibit increased reactivity and aggregation of blood platelets inside coronary circulation, which results in partial or complete obstruction of the coronary artery [16].

The platelets contribute to acute thrombosis with a multiple step mechanism: the first is adhesion of platelets to the endothelium. The interaction occurs between the constituents of the exposed subendothelium, including collagen, von Willebrand factor, fibronectin, and specific platelet surface membrane receptors. Thereby, platelets overcome the high blood shear forces and attach themselves to the target endothelium site. The binding of fibrinogen and selected matrix proteins containing Arg-Gly-Asp (RGD) sequences to integrin αIIbβ3 (the most important and abundant platelet integrin) mediates stable platelet adhesion, aggregation, and thrombus formation. The further activation process occurs with a specific conformational change that induces the onset of multiple internal signaling networks. The hyperreactive platelets accelerate the formation of an intracoronary thrombus, leading to a cascade of clinical events [17]. The other distinctive feature of platelet activation is the release of the platelet microparticles (PMPs) by these cells. PMPs are the most plentiful cell microparticles found in the circulation [18–20] and as circulating sources of tissue factor (TF) that is a transmembrane protein involved in thrombin generation are potential mediators of blood coagulation. Moreover, a formation of platelet microparticles is associated with the exposure of phosphatidylserine at PMPs outer membrane surface. The mechanisms of interaction of PMPs with various cells may involve a membrane fusion, endocytosis, or interaction of microparticles with cell membrane receptors to stimulate cellular signaling events [20].

A recent study evaluated the association between hyperreactivity of platelets to adenosine diphosphate (ADP) and outcomes in patients with stable cardiovascular disease [21]. According to other researchers, the platelets not only play a role in the formation of coronary artery thrombosis, but also may be involved in the initiation and propagation of atherosclerosis, potentially through interaction of activated platelets with endothelial cells and leukocytes or through the release of various, stimulating inflammation mediators [22]. The resting or activated blood platelets, apart from the integrin receptors for adhesive proteins, possess on their surface the additional molecules responsible for interaction with other cells, such as ICAM-2 (intercellular adhesion molecule-2), JAM-A, JAM-C (junctional adhesion molecule) and PECAM-1 (platelet endothelial cell adhesion molecule-1), P-selectin, CD40/CD40L molecules, complement receptors, receptors for immunoglobulins (FcR), and Toll-like receptors [23].

The platelet activation leads to exocytosis of granule constituents and release of an arsenal of potent inflammatory and mitogenic substances into the local microenvironment, thereby altering chemotactic, adhesive, and proteolytic properties of endothelial cells. These platelet-induced modifications of the endothelial phenotype support chemotaxis, adhesion, and transmigration of monocytes to the site

of inflammation. Platelets contain three types of specific secretory granules, such as dense granules, α-granules, and lysosomes [24], which after platelet activation release a variety of mediators: adhesion proteins (e.g., fibrinogen, fibronectin, vWF, thrombospondin, vitronectin, P-selectin, GPIIb-IIIa, receptor complex GPIba-V-IX, and collagen receptor GP VI), growth factors (e.g., PDGF, TGF-β, EGF, and bFGF), chemokines (RANTES, platelet factor 4 [PF4; CXC chemokine ligand 4 (CXCL4)], epithelial neutrophil-activating protein 78 [ENA-78; CXCL5]), cytokine-like factors (e.g., IL-1β, CD40L, and β-thromboglobulin), and coagulation factors (e.g., factors V, XI, XIII, PAI-1 [plasminogen activator inhibitor], α2-antiplasmin, TFPI [tissue factor pathway inhibitor], antithrombin, plasminogen, and protein S). These proteins act in a concerted and fine-regulated manner, influencing widely differing biologic functions such as cell adhesion, cell aggregation, chemotaxis, cell survival and proliferation, coagulation, and proteolysis. All of these molecules accelerate inflammatory processes and cell recruitment [25–28].

3. Protein Synthesis in Platelets

In the human body about 1×10^{11} platelets are made daily as a result of complex processes of differentiation, maturation, and fragmentation of megakaryocytes. Mature and fully differentiated megakaryocytes are equipped with all elements necessary for the production of platelets [29, 30]. It has been known for a long time that platelet proteins may have different origins: some are synthesized in megakaryocytes, and some derive directly from blood plasma [31]. However, the studies completed in recent years have shown that anucleated platelets are able to succeed protein synthesis. Both, platelet-specific granules and other organelles, such as numerous mitochondria and mRNA molecules, allow the synthesis of proteins in platelets [32]. The activated platelets produce many proteins which release and/or expression is not observed in resting cells. This observation has become a prerequisite for studies on the ability of platelets to protein synthesis [33]. Despite lack of nucleus, platelets have stable mRNA transcripts with a long life correlated with platelet lifespan. They contain a very small amount of the mRNA, which is approximately 2×10^{-15} g and this is about 12 500 times less than in nucleated cells [34]. The characteristics of platelets transcriptome by cDNA microarray analysis showed that the platelets contain thousands of base pairs coded information pieces derived from megakaryocytes. About 5000 mRNA transcripts in the blood platelets have been described so far, which represent a half of the amount of transcripts detected in megakaryocytes [35]. In 1989 Roth et al. [36] have discovered polyadenylated mRNA molecules in blood platelets. SAGE (Serial Analysis of Gene Expression) method also shows the presence of noncoding 3′-untranslated (3′-UTRs) regions in platelets mRNA, which are longer and more complicated in comparison to the same region in mRNA of other eukaryotic cells [37]. 3′-UTRs regions are located after coding sequence and play a role in posttranscriptional regulation. 3′-UTRs regions are signal for process called polyadenylation. In this process the enzyme called poly-A polymerase adds adenine nucleotides to the 3′ end of mRNA forming poly-A tail which is 100–250 adenine residues long. The presence of poly-A tail causes that mRNA molecules are more stable and prevents them against intracellular degradation. Additionally, the platelets' mRNA 3′-UTRs regions have also a large number of sequences rich in adenine and uracil ARE (ang. AU-rich elements) [33, 37] which regulate mRNA degradation by inhibition of shortening of poly-A tail [38]. According to Booyse and Rafelson Jr., the blood platelets' mRNA is more stable and resistant to degradation in comparison to the mRNA contained in other mammalian cells [39]. They demonstrated that blood platelets contain very stable mRNA transcripts with long lifespan correlated with platelet lifetime [40]. This is especially important in the case of anucleated platelets, which are not able to restore their mRNA pool.

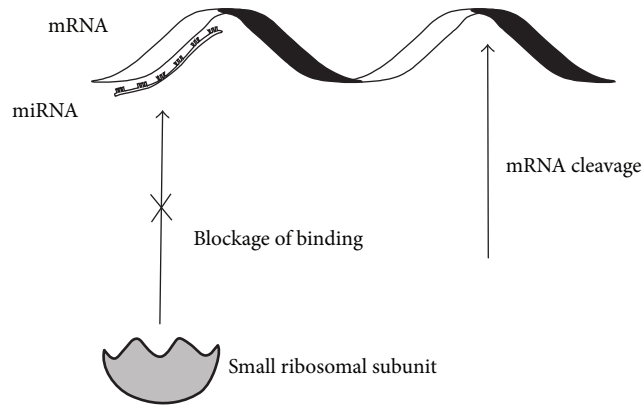

Figure 2: Role of miRNA in translation process.

4. The Role of miRNA in Platelet Protein Synthesis

The study carried out in 2009 by Landry et al. [41] shows that mRNA translation in blood platelets is regulated by miRNA molecules, which often hybridizes to the mRNA sequences localized in 3′-UTRs regions. MicroRNAs belong to class of small noncoding RNAs (21–24 nucleotides) and normally they negatively regulate the target mRNA expression at the posttranscriptional level. The miRNAs are generated from hairpin structures maturated by the RNAse III ribonucleases Drosha/Dicer to mature miRNAs. The mature miRNA molecules are incorporated into the complex containing Argonaute 2 protein (Ago-2). The mature miRNAs act as posttranscriptional regulators of gene expression by base pairing with mRNAs, thereby causing exonucleolytic mRNA decay or translational repression [42] (Figure 2). The mature miRNA together with the RNA-induced silencing complex (RISC) hybridizes to the mRNA sequence located 3′-UTR. The gene regulation by RISC complex is guided by sequence complementarity between the "seed region" (nucleotides 2–7 and 8) of the microRNA and the 3′-UTR of the mRNA. Recently, it has been also demonstrated that miRNAs may act as positive regulators in some cases. It is estimated that 1–4% genes in the human genome are miRNAs, and a single

miRNA can regulate as many as 200 mRNAs. There is increasing evidence suggesting that miRNAs play critical roles in many key biological processes, such as cell growth, tissue differentiation, cell proliferation, embryonic development, and apoptosis. miRNAs play also important roles in cellular signaling network, cross-species gene expression variation and coregulation with transcription factors [43].

5. Platelets miRNA and Thrombotic Complications

The mutation of miRNAs, dysfunction of miRNA biogenesis, and dysregulation of miRNAs and their targets may result in various diseases. Currently, it has been reported that more than 70 diseases are associated with miRNAs (http://cmbi.bjmu.edu.cn/hmdd) [5]. Many studies have reported a great number of miRNA-disease associations and shown that the mechanisms of miRNAs involved in diseases are very complex. miRNA profiling has been shown to be more accurate than mRNA expression profiling in characterizing the differentiation of multiple human cancers.

Landry et al. [41] in 2009 first described 219 different types of miRNA in blood platelets expression profiles of which were different among platelets and megakaryocytes. Additionally, differential platelet miRNA profiles compared to neutrophils, were observed, what suggest the lack of leukocyte contribution to the platelet miRNA signals. The three most abundant miRNAs in blood platelets were miR-223, let-7c, and miR-19a. In support of this assertion platelets contain also pre-miRNA molecules and known protein components of the pre-miRNA processing complex, that is, RNase Dicer and TRBP2, as well as Ago-2, the core component of miRNA effector complexes. Moreover, RNase Dicer and TRBP2 form a complex which in platelets is catalytically active in pre-miRNA processing into miRNA like as megakaryocytes. It indicates the possibility of precursor miRNA maturation in the platelets. In contrast, the nuclear microprocessor components Drosha and DGCR8 were not detected in blood platelets, which is consistent with their enucleate nature. The same study [41] demonstrates that platelets have functional protein complexes of miRNA (miR-223) and Ago-2, and these complexes specifically regulate expression of the functionally important platelet purinergic P2Y12 adenosine diphosphate receptor. Furthermore, there is an evidence which suggests that miRNA (miR 28) can modulate expression of the c-mpl thrombopoietin (Tpo) platelet receptor [44].

In 2011, Osman and Fälker [45] have identified 281 transcripts, of which 228 were mature miRNA and 53 minor miRNA. Six of these miRNAs (miR-15 a, miR-339-3 p, miR-365, miR-495, miR-98, and miR-361-3 p) were up- or downregulated in activated human platelets. The changes in the levels of some miRNAs in platelets were associated with thrombin stimulation response.

Nagalla et al. [46] detected in platelets 284 miRNA transcripts, 74 of which showed various expression depending on the platelet reactivity. However, only the expression of 7 miRNA (miR-19b, miR-34b, miR-190, miR-320a, miR-320b, miR-320c, and miR-320d) showed a strong correlation with the degree of platelet response to adrenaline. The most abundant miRNA in platelets is miR-223 followed by miR-126. The miR-96, miR-200b, miR- 495, miR-107, and miR-223 are critically involved in platelet reactivity, aggregation, secretion, and adhesion [47].

In 2012 Plé et al. [54] have discovered more than 492 different mature miRNA transcripts in platelets. The in vitro study demonstrated that human blood platelets are able to uridylate miRNA molecules, which indicates the presence in platelets of the uridyltransferase enzyme TUT4. Additionally, in this study authors detected numerous miRNA isoforms (isomiRs) resulting from imprecise maturation caused by ribonucleases Drosha/Dicer. This study unveils the existence of very varied and multifaceted microRNA pathway in human platelets which suggest important role of miRNA in blood platelets functioning.

In study performed by Sondermeijer group [48] 214 miRNA molecules were identified, which have different expression levels in blood platelets obtained from patients with premature coronary artery disease in comparison to healthy donors of blood platelets. After biostatistics analysis six miRNAs (miR340*, miR615-5p, miR545:9.1, miR451, miR454*, and miR624*) remained significantly and more than 1.5-fold upregulated whereas miR-12801 was remained significantly and more than 1.5-fold downregulated. Two independent cohort studies indicate that two miRNAs (miR624* and miR340*) are significantly upregulated in patients with coronary artery disease (CAD) as compared to healthy controls. The authors recommend using these molecules as potential blood platelets diagnostic markers of coronary artery disease.

The changes in blood platelets miRNA expression profiles were also observed in patient after acute coronary syndrome. In blood platelets of patients with STEMI the most downregulated miRNAs were miR186-5p and miR185-5p, whereas miR127-3p and miR221-3p were upregulated in these cells. While in blood platelets from patients with NSTEMI the most downregulated miRNAs were miR20a-5p and miR942, the most upregulated miRNA molecules were miR483-5p and miR146a-5p [49].

Another circulation pathology, where platelet miRNAs show different expression profiles, is atrial fibrillation. This condition is notoriously associated with heart failure (HF) which possesses a very negative prognosis. MiRNAs have different expression profiles in platelets of patients with systolic HF compared to controls without cardiac disease. MiR-150 expression level was more than 3-fold lower in blood platelets obtained from patients with HF with atrial fibrillation [50].

The clinical evidences suggest that vesicle-associated membrane protein 8 (VAMP8)/endobrevin, a critical v-SNARE involved in platelet granule secretion, may be associated with clinical arterial thrombosis. The studies performed on five independent patient populations demonstrated an association between myocardial infarction and the rs1010 SNP in VAMP8 [55, 56]. Additionally, the blood platelet hyperreactivity was also correlated with increased levels of mRNA for VAMP8. The VAMP8 expression was found to

Table 1: Changes in blood platelets miRNA levels in thrombotic states.

MicroRNA level changes	Disease	Reference
↑ miR340*	Premature coronary artery disease	[48]
↑ miR615-5p		
↑ miR545:9.1		
↑ miR451		
↑ miR454*		
↑ miR624*		
↓ miR-12801		
↑ miR340*	Mature coronary artery disease	
↑ miR624*		
↓ miR186-5p	Acute coronary syndrome (STEMI)	[49]
↓ miR185-5p		
↑ miR127-3p		
↑ miR221-3p		
↓ miR20a-5p	Acute coronary syndrome (NSTEMI)	
↓ miR942		
↑ miR483-5p		
↑ miR146a-5p		
↑ miR-150	Heart failure with atrial fibrillation	[50]
↓ miR-154	Sickle cell disease	[51]
↓ miR-329		
↓ miR-376		
↑ miR-144	Diabetes mellitus type 2 patients with ischemic stroke	[52, 53]
↓ miR-146a		
↓ miR-223	Diabetes mellitus type 2 patients without ischemic stroke	

be regulated by the platelet miRNA-miR-96. MicroRNA-96 can bind to the 3′-UTRs region of VAMP8 mRNA which was detected in platelets. Various levels of miR-96 were also presented in blood platelets with differing reactivity. The mean miR-96 level was found to be 2.6-fold higher in the hyporeactive subjects than in the hyperreactive subjects [57].

In physiological coagulation process the potent agonist of blood platelets is thrombin, a major enzyme generated in coagulation cascade [58]. The thrombin-activated platelets change their shape, secrete the contents of their granules, and finally aggregate [59]. The receptors belonging to the family of Protease-Activated Receptor (PAR) are responsible for blood platelets response to the thrombin. PAR are members of seven-transmembrane G protein-coupled receptor family. On the human platelets surface receptors PAR-1 and PAR-4 are present [60]. Edelstein et al. [61] demonstrated different expression level of miR-376c correlated with different PAR-4 reactivity.

The changes in platelet miRNAs expression were also observed in the rabbit atherosclerotic plaque model. The study showed that, in comparison to normal control animals, miR-126 and miR-223 levels in platelets of atherosclerotic plaque of rabbit were reduced. Moreover, the levels of these miRNAs were correlated with plaque morphology [62].

The changes in platelet miRNA expression were found also in patients with sickle cell disease (SCD) with state of hypercoagulability resulting prothrombotic predisposition. The forty differentially expressed miRNAs were identified in platelet of SCD patients with a risk of thromboembolic complications. From 24 downregulated miRNA molecules, 14 came from three miRNA families: miR-154, miR-329, and miR-376 which are localized in 14q32 region [51].

The blood platelet miRNAs can be also a risk factor and biomarkers for ischemic stroke. The miR-144 level in platelets is higher in diabetes mellitus type 2 (T2DM) patients with ischemic stroke. The platelet miR-223 expression decreases in this group in comparison to T2DM patients without thromboembolic complications [52]. In other study performed by Duan et al. [53] the expressions of platelet miR-223 and miR-146a in patients with diabetes mellitus and ischemic stroke were significantly lower than in healthy donors. Additionally, the expression level of these two miRNAs was correlated with blood platelet activation rates.

6. Conclusions

The genomics and proteomics and innovative research methods, based on the molecular analysis and closely related to bioinformatics, have become in recent years the basis of diagnostic tests designed to determine the predisposition of human to morbidity of several diseases. A recent works postulates the possibility of using miRNAs as biomarkers of atherosclerosis and ischemic episodes [4, 63] (Table 1). The miRNAs present in platelets may exert important regulatory

functions in synthesis of proteins, which are involved in platelet activation pathways associated with platelet hyperactivity leading to thrombus formation. Many studies demonstrated different platelet miRNA expression profile patterns between patients with ischemic episodes and controls. The modern laboratory diagnostic is not limited to the identification of early stages of coronary obstruction but thanks to molecular methods is capable of detecting a predisposition to the illness. The detection of specific changes in platelet miRNA expression profiles associated with hyperactivity of platelets may have important implications in the prevention of embolic incidents.

Competing Interests

The authors report no conflict of interests.

Acknowledgments

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