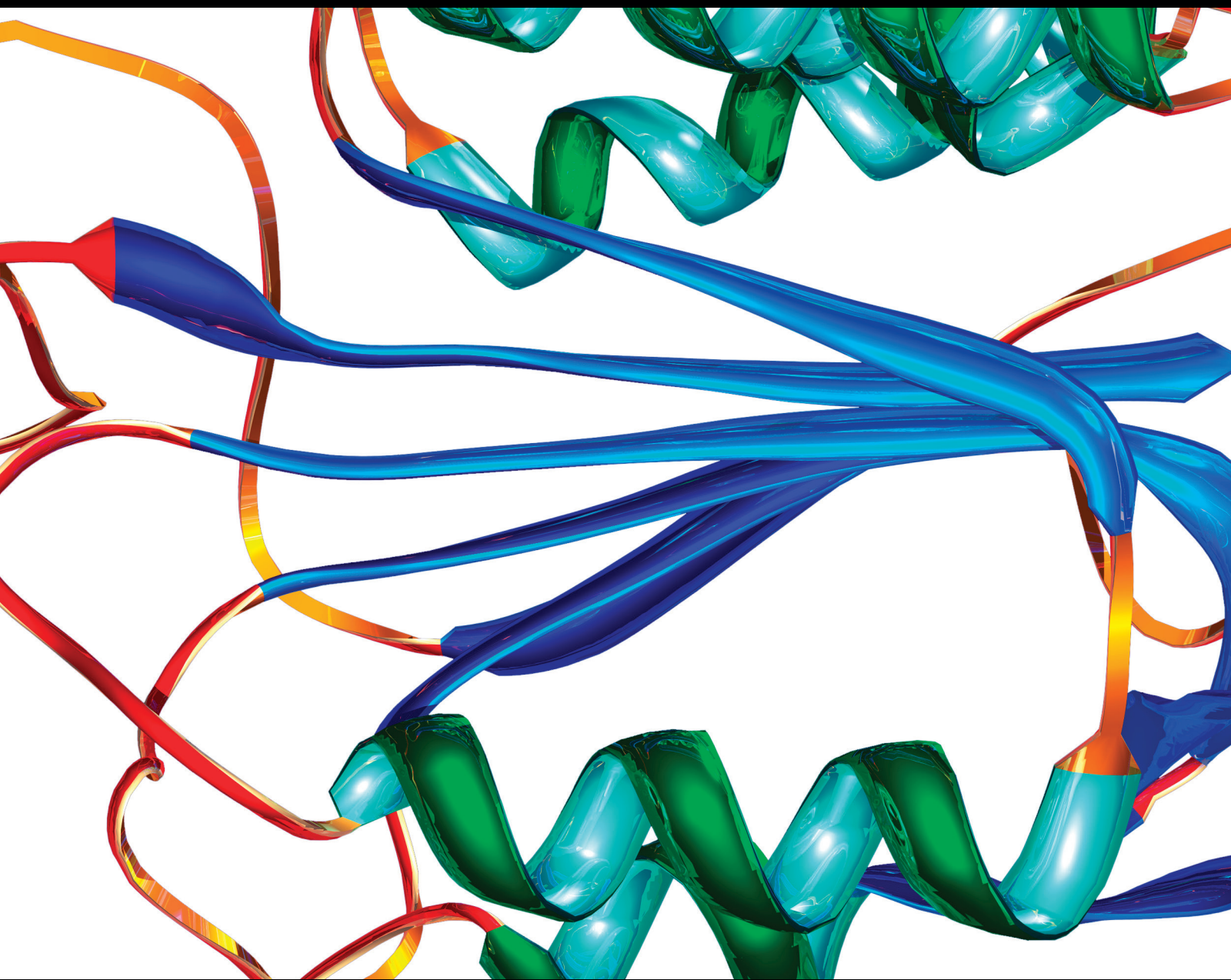
Disease Markers

Find the Essence through the Phenomena: Cardiovascular Diseases and Biomarkers

Lead Guest Editor: Shipeng Wei
Guest Editors: Agata M. Bielecka-Dabrowa, Zhongjie Shi, and Ospan A. Mynbaev



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Editorial

Find the Essence through the Phenomena: Cardiovascular Diseases and Biomarkers

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Biomarkers are often measured and evaluated to determine normal biological processes, pathological processes, or pharmacological responses to a therapeutic intervention. Our current issue focuses on new advances in cardiovascular diseases and biomarkers. The goal of this special issue is to summarize new biomarkers in risk prediction, screening, diagnosis, progression, and prognosis of cardiovascular diseases; measurement relevance of biomarkers addressing specificity, sensitivity, and biological variation of these biomarkers; and the role of biomarkers in evaluation and guidance of drug therapies. The importance of these biomarkers has been raised to the level that they have never been to before. Not simply because more research work has been done, and also because people realize that they can provide some information that could not be obtained by other means.

J. Tian et al. reported prognostic association of circulating neutrophil count with no-reflow in patients with ST-segment elevation myocardial infarction (STEMI) following successful primary percutaneous coronary intervention (PCI). A circulating neutrophil count $\geq 9.14 \times 10^9$/L is independently associated with no-reflow in patients with acute STEMI following primary PCI. J. Budzianowski et al. further discussed the importance of hematological indices in patients with acute coronary syndrome (ACS), including white blood cells (WBC), neutrophil to lymphocyte ratio (NLR), red cell distribution width (RDW), and platelet indices, such as platelet to lymphocyte ratio (PLR), mean platelet volume (MPV), and platelet distribution width (PDW) in the setting of ACS. M. Rabajdová et al. reported the detection of pathological changes in the aorta during thoracic aortic aneurysm progression. It is significantly associated with increased mRNA and protein levels of inflammatory cytokines (CRP and IL-6). A. Caraba et al. reported vitamin D status, disease activity, and endothelial dysfunction in early rheumatoid arthritis (RA) patients. They found that in early RA patients with moderate and high disease activity, low serum level of vitamin D is associated with disease activity, increased insulin resistance, and endothelial dysfunction. A. Kolaszko et al. reported the role of parathyroid hormone (PTH) and vitamin D serum concentrations in patients with cardiovascular diseases. They found that PTH serum concentration in contrast to 25-hydroxyvitamin D (25(OH)D), but not phosphorus, and Ca^{2+}, significantly elevates among the patients with heart failure and shows significant correlation with their clinical status expressed by the New York Heart Association (NYHA) classification. T. Chen et al. reported a correlation between serum gamma-glutamyl transferase (GGT), serum ferritin (SF), and the rate of CKD and found that GGT and SF synergistically influence the rate of CKD. In other words, patients with abnormal SF and GGT may have higher risk to develop cardiovascular diseases.

Other serum markers, like troponin, N-terminal pro-brain natriuretic peptide (NT-proBNP), and apelin, are

relatively new markers coming to the cardiovascular field, and more research work came out since then. R. Rajtar-Salwa et al. found that elevated troponin, but not NT-proBNP, is associated with increased risk of sudden cardiac death in hypertrophic cardiomyopathy (HCM) patients. Salska et al. also reported that pro-brain natriuretic peptide (pro-BNP) is not a marker of arrhythmia recurrence, whereas higher apelin concentration at admission indicates ineffectiveness of direct-current cardioversion or recurrence of arrhythmia within a month.

Gene polymorphism and mutation is gaining more and more spotlights nowadays. H. Song et al. found that interleukin-31 gene polymorphisms are tightly associated with dilated cardiomyopathy (DCM) susceptibility and contributes to worse prognosis in DCM patients in a Chinese population. J. Li et al. reported the protective role of cullin-3 single-nucleotide polymorphism rs17479770 in essential hypertension in male Chinese Han population. S. Sirotina et al. reported that a novel polymorphism in the promoter of the CYP4A11 gene is associated with susceptibility to coronary artery disease (CAD). M. A. Sazonova et al. reported that mitochondrial genome mutations are associated with myocardial infarction. It could be foreseen that in the future, gene screening will make treatment more individualized, such as early primary prophylaxis and more aggressive treatment as secondary prophylaxis for gene-positive patients.

Looking back at what has been accomplished by our colleagues before us and looking ahead what we need to accomplish to get our research to the next level, we believe that we will find more through the surface and eventually get to the core of it, the essence of science.

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Research Article

Apelin and Atrial Fibrillation: The Role in the Arrhythmia Recurrence Prognosis

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Apelin is a novel peptide of wide expression and multiple biological functions including the crucial role in cardiovascular homeostasis. The apelin role in the pathophysiology of heart rhythm disorders is considered, although the reports are scarce so far. The purpose of this study is to investigate the potential utility of apelin as a marker of arrhythmia recurrence after direct-current cardioversion (DC). The prospective, observational study included 60 patients (aged 41–86; 30% female) with nonvalvular, persistent atrial fibrillation from the group of 204 consecutive patients scheduled for DC during the 12-month period (from May 2010 to May 2011) in the Cardiology Clinic Medical University of Lodz, Poland. The study group was divided into SCD (successful DC), 45 (75%) patients, and NDC (nonsuccessful DC), 15 (25%) patients. Within the SCD group, the subgroups were distinguished depending on the time sinus rhythm maintenance after DC: up to 7 days (SDC-7), 11 patients; 7 to 30 days (SDC-30), 12 patients; over 90 days (SDC-90), 22 patients. Patients were evaluated during the hospitalization and within the 3-month follow-up period. The apelin level was determined within the plasma samples collected at the admission, using the commercially available enzyme-linked immunosorbent assay (ELISA) Kit for apelin-36. It was found that the median value of initial apelin in the subset of patients from groups NDC + SDC-7 + SDC-30 is significantly higher than from group SDC-90 ($p = 0.0463$); there was no relationship between NDC and SCD overall. Neither of the compared subgroup pairs revealed statistically significant correlation between the proBNP concentration and the DC effectiveness in our population. In conclusion, in our study, proBNP was not a marker of arrhythmia recurrence whereas higher apelin concentration at the admission indicated patients in whom DC was not effective or they had an arrhythmia recurrence within a month-period observation.

1. Introduction

Atrial fibrillation (AF) is the most frequent sustained cardiac arrhythmia in adults, which affects over 1% of the general population and becomes an important cause of the mortality and life quality deterioration [1, 2]. It causes significant morbidity and premature mortality, with the risk of death doubled, regardless of the other factors [3].

The therapeutic strategy in atrial fibrillation should be planed individually and include thromboembolism prophylaxis and the rhythm restoration or control [1]. One of the available therapy methods is the electric cardioversion, which is an effective way to restore the sinus rhythm [4]. There is a substantial risk of arrhythmia recurrence after cardioversion. It is estimated as 10% per year in patients after the first diagnosed AF incident. It increases by 5% each year [1]. The most important AF-recurrence risk factors include age, AF duration before the cardioversion, the number of previous relapses, the left atrium enlargement or dysfunction, coronary artery disease, or the mitral valve defect [1].

The new markers including biochemical substances of a potential role in the atrial fibrillation risk stratification are investigated. The researches are intended to deal with the improvement of the AF-associated clinical risk and/or recurrence rate assessment. The usefulness of such substances as troponin, natriuretic peptides, the inflammation,

renal function, and coagulation markers was proved [5]. It was proved that lone AF in patients with normal left ventricle ejection fraction is associated with elevated BNP/proBNP serum level; moreover, its level decreases within the sinus rhythm maintenance after DC and increases in case of arrhythmia recurrence. Some researcher however indicates that baseline BNP serum concentration is not associated with the risk of AF recurrence [6, 7].

Among other substances, the role of apelin in atrial fibrillation became an object of interest. Apelin is a novel peptide that was first isolated and named by Tatemoto et al. in 1998 [8]. It was named after the APJ endogenous ligand, as it was identified as the endogenous ligand for the previously recognised human APJ "orphan" receptor [8].

Apelin is encoded by a gene located on band q25-26.1 of the chromosome X and is synthesized as a pre-pro-apelin precursor peptide consisting of 77 amino acids, which is subsequently transformed into active isoforms consisting of 12 to 36 amino acids. Apelin applies its various regulating functions by acting on a transmembrane G protein-coupled APJ receptor, which indicates significant similarity to the angiotensin receptor (AT1) [9, 10].

Both apelin and APJ receptor are widely expressed in numerous cells and tissues of the human body including the central nervous system and peripheral tissues [9]. Apelin is also detectable in human plasma. Within cardiovascular system, it is present in the vascular smooth muscle cells, endocardial and endothelial cells of the heart chambers, and vascular endothelial cells of many vessels—coronary arteries, large conduit vessels, and small arteries and veins (e.g., kidney, lung, and adrenal gland vessels) [9]. It plays a role both in patophysiological and cardioprotective pathways. It has been proved that apelin has multiple functions within the cardiovascular homeostasis affecting the angiogenesis, vascular tone, and body fluid homeostasis or acting as a positive inotropic factor [8]. In human, it was shown that in early stages of the heart failure, plasma apelin level, parallel to BNP, rises and decreases with the progress of the disease [11].

As the apelin is produced and secreted in adipose tissue, it is also considered as an adipokine. As such, it may affect many of the metabolic pathways, especially in carbohydrate metabolism, where apelin/APJ system is interdependent with insulin. It also plays a role in the pathophysiology of the obesity, diabetes mellitus, and atherosclerosis [12].

Taking into account the discussed issue, it is important that apelin has also an influence on electrophysiological function of atrial myocytes, shortening the action potential, which was demonstrated in vitro on animal model [13].

Recently, in individual reports, it was shown the mean apelin level is significantly reduced in patients with isolated atrial fibrillation and normal heart in echocardiography, when compared to the healthy control [14], and it is also decreased in patients with other supraventricular tachyarrhythmias [15]. Moreover, in these patients, the maintenance of the sinus rhythm after cardioversion increases the apelin level [16]. Up to date, only one study evaluated the impact of the marker level for the risk of arrhythmia recurrence [17].

The aim of this study is to evaluate the potential impact of initial apelin-36 on the risk of AF recurrence after DC.

2. Materials and Methods

The prospective, observational study included 60 patients with nonvalvular persistent atrial fibrillation from the group of 204 consecutive patients scheduled for electric direct-current cardioversion (DC) during the 12-month period (from May 2010 to May 2011) in the Cardiology Clinic Medical University of Lodz, Poland. Patients were included after the exclusion of negative factors and signing an informed consent of the participation form. Patients were evaluated during hospitalization and at the 3-month follow-up period. The study was approved by the local Bioethics Committee.

The exclusion criteria were as follows:

(i) atrial flutter or atrial tachycardia; persistent atrial fibrillation with the duration of less than four weeks

(ii) INR < 2.0 within the four weeks prior to DC

(iii) electrolyte abnormalities; chronic kidney disease, defined as GFR < 60 ml/min/1.73 m^2 or creatinine concentration > 1.5 mg/dl

(iv) an acute or chronic inflammatory diseases; immunosuppressive therapy; antibiotics, steroids, or nonsteroid drugs intake

(v) active endocrine disorders; active cancer

(vi) alcohol abuse

(vii) a history of the rheumatic fever

(viii) a history of the cardiac surgery ever or any surgery within the past 6 months

(ix) state after the pacemaker or cardioverter-defibrillator implantation

(x) a history of the ineffective DC

(xi) symptoms of heart failure in the ≥II hemodynamic scale according to the New York Heart Association (NYHA)

(xii) data from echocardiography: intracardiac thrombus, left ventricle ejection fraction (LVEF) < 45%, the left atrium dimension > 55 mm measured in the parasternal long axis (LAX), left or right atrium longitudinal or transverse dimension > 70 mm measured in the four-chamber projection (dimension), valvular defects (moderate and severe aortic or mitral stenosis, valvular regurgitation > II degree).

In each patient, the detailed data on medical history were obtained. Patients underwent the physical examination, electrocardiogram (ECG), and transthoracic echocardiography. At the admission, the fasting laboratory tests including peripheral blood count, electrolytes, blood glucose, urea and creatinine, INR, and serum brain natriuretic

propeptide (proBNP) were performed. In each case, an additional blood sample of 5.2 ml was collected, immediately centrifuged, and stored at temperatures below −70°C as recommended by the reagent manufacturer.

The apelin (isoform apelin-36) level was determined within the plasma samples using the commercially available enzyme-linked immunosorbent assay (ELISA) Kit for apelin-36 (Uscn Life Science Inc.). The assay employs a monoclonal antibody for human apelin-36 and is based on competitive inhibition enzyme immunoassay technique—detection range was 14.25–3000 pg/ml and intra- and interassay CVs were <10% and <12%, respectively.

Electric cardioversion was performed under the short-term general anesthesia after obtaining patients consent. The procedure was carried out subject to the confirmation of the AF wave in the cardiomonitor registration, using the transthoracic electrodes and the diphase energy of 200 J. If there was no sinus rhythm restoration, the DC was repeated with increasing energy of 300 and 360 J up to three repetitions.

DC was considered effective provided that the sinus rhythm was obtained during the first three electrical discharges. The heart rate was monitored on the screen of the cardiomonitor, and additionally, there was the ECG recording confirming the sinus rhythm required.

DC was considered ineffective if it could not restore the sinus rhythm after the three consecutive electrical discharges of increasing energy according to the scheme above. The ineffectiveness was confirmed by the presence of the AF wave in ECG.

Patients after successful DC were prospectively observed within the 3-month period weather the sinus rhythm was maintained. Clinical observation included 24-hour Holter monitoring performed on the next day, four weeks, and three months after the DC. In 7 days after DC, ambulatory ECG was performed. Patients reporting the symptoms suggesting the AF recurrence underwent additional resting ECG recording. As the confirmation of the arrhythmia recurrence, the resting ECG or Holter-ECG displaying the AF wave (including the paroxymal AF) was considered. The observation was completed after three months in patients who maintained the sinus rhythm or earlier in case of the recurrence of AF. The further treatment was recommended individually.

To develop the statistical analysis of the obtained data, STATISTICA 10.0 (StatSoft Inc., USA) was used. The compliance of the quantitative variables with normal distribution was checked using the Shapiro-Wilk test. To examine the relations between quality features, the χ^2 test, χ^2 test with Yates' correction, Fisher's exact test, or V2 test were performed. The variance homogeneity was proved using Levene's test. For the quantitative variable comparisons, Student's t-test and Mann–Whitney test were applied. Statistical significance assumed the results where the probability level (p) was lower than 0.05.

The study group consisted of patients undergoing DC in our center within the planned time period. The statistical power analysis was not performed because apelin is a parameter with not-normal distribution and nonparametric statistical tests were used.

3. Results and Discussion

3.1. Study Group Characteristics. The study group comprised 60 patients; it was divided depending on the effectiveness of cardioversion into SCD (successful DC), 45 (75%) patients, and NDC (nonsuccessful DC), 15 patients. Within the SCD group, the subgroups were distinguished depending on the time sinus rhythm maintenance after successful DC: up to 7 days (SDC-7); 7 to 30 days (SDC-30); over 90 days (SDC-90). The amount of each subgroup was SDC-7, 11 patients (18.34% of the total and 22.23% among patients in whom cardioversion was effective); SDC-30, 12 patients (20% of the total and 26.67% among patients in whom cardioversion was effective); SDC-90, 22 patients (36.67% of the total and 48.89% among patients in whom cardioversion was effective). These divisions were aimed at distinguishing patients in whom DC was effective in the long-term against the others.

Men predominated the study population accounting for 70%. The percentage of men in the various subgroups was within the range of 54.55% (SDC-7) to 80.00% (NDC). The youngest participant was 41 years and the oldest 86 years (mean 61.8 years).

The characteristics of the study population divided into subgroups is presented as shown in Table 1.

3.2. FA Duration. Atrial fibrillation duration in study population ranged from 1 to 24 months (mean 7.83). The longest average duration of arrhythmias was observed in the NDC group 9.93 (± 7.95) and the shortest 6.08 (± 6.07) in the SDC-30 group (see Table 1). In the study group, there was no relationship between the duration of arrhythmia and DC effectiveness ($p = 0.590$); moreover, it was found that the tested subgroups did not differ significantly concerning the FA duration.

Two thirds of respondents (66.67%) did not underwent electric cardioversion in the past. The majority (60%) of the remaining group had a DC only once. The maximum number of procedures was 9 in 1 patient. Comparing the numbers of patients, who underwent or not DC in the past within the study, only the subgroups SDC-7 (9.09% of patients had DC in the past) and SDC-90 (50% of patients had DC in the past) differed significantly ($p = 0.0273$).

There was no difference between the effectiveness of DC depending on the previous effective cardioversion ($p = 0.5306$). Moreover, there was no difference in apelin concentration depending on the previous DC ($p = 0.8166$).

3.3. Echocardiographic Parameters. It was examined whether there is a relationship between atrial dimensions assessed in echocardiography and the effectiveness of electrical cardioversion. The average values and median longitudinal and transverse dimensions of the left atrium (LA) and the right atrium (RA) are presented in Table 1.

Statistical analysis showed no significant difference in longitudinal dimension of the left or right atrium between any of the compared subgroups of patients differing in DC efficiency.

Table 1: Study population characteristic.

	N (%)	Men (%)	FA duration (months); mean (±SD)	Previous DC (%)	Arterial hypertension *N* (%)	Diabetes mellitus *N* (%)	Coronary artery disease *N* (%)	Atrial dimensions							
								LA long. (cm)		LA trans. (cm)		RA long. (cm)		RA trans. (cm)	
								Mean (±SD)	Median (min–max)	Mean (±SD)	Median (min–max)	Mean (±SD)	Median (min–max)	Mean (±SD)	Median (min–max)
NDC	15 (25%)	12 (80%)	9.93 (±7.95)	4 (26.67%)	10 (66.67%)	3 (20%)	3 (20%)	6.3 (±0.38)	6.4 (5.80–6.58)	4.74 (±0.71)	4.7 (3.80–6.60)	5.81 (±0.45)	5.8 (5.20–6.70)	4.59 (±0.49)	4.4 (3.90–5.70)
SDC	45 (75%)	30 (66.67%)	7.13 (±5.95)	16 (35.56%)	26 (57.78%)	2 (4.4%)	2 (4.4%)	6.12 (±0.52)	6.2 (4.80–7.0)	4.86 (±0.54)	4.9 (3.30–6.10)	5.78 (±0.59)	5.9 (4.00–6.80)	4.52 (±0.71)	4.6 (3.00–5.65)
SDC-7	11 (18.32%)	6 (54.55%)	7.91 (±5.86)	1 (9.09%)	6 (54.55%)	3 (27.27%)	2 (18.18%)	6.05 (±0.62)	6 (4.80–7.0)	4.64 (±0.60)	4.8 (3.30–5.40)	5.92 (±0.36)	5.9 (5.40–6.50)	4.1 (±0.67)	4.1 (3.00–5.10)
SDC-30	12 (20%)	8 (66.67%)	6.08 (±6.07)	4 (33.33%)	6 (50%)	0	1 (8.33)	6.05 (±0.40)	6.2 (5.24–6.50)	4.75 (±0.40)	4.8 (3.94–5.50)	5.63 (±0.57)	5.7 (4.60–6.40)	4.74 (±0.59)	4.9 (3.50–5.50)
SDC-90	22 (36.67%)	16 (72.73%)	7.32 (±6.12)	11 (50%)	14 (63.64%)	0	0	6.19 (±0.54)	6.35 (5.24–7.0)	5.04 (±0.53)	5.11 (3.94–6.10)	5.79 (±0.68)	5.95 (4.00–6.80)	4.61 (±0.72)	4.7 (3.50–5.65)

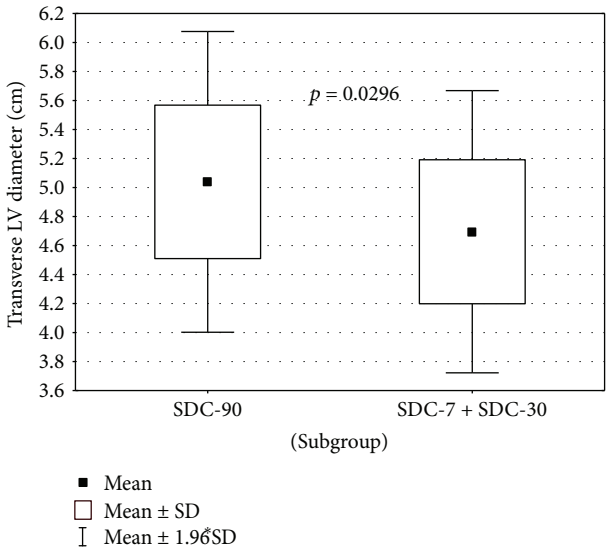

Figure 1: LA transverse diameter in subgroups SDC-90 versus SDC-7 + SDC-30.

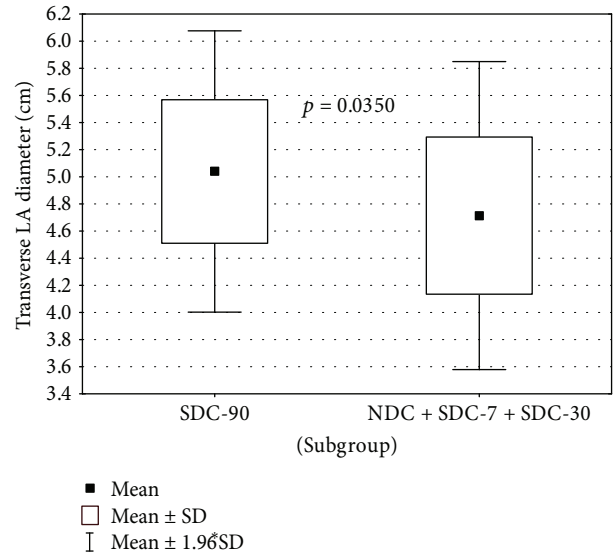

Figure 2: LA transverse diameter in subgroups SDC-90 versus NDC + SDC-7 + SDC-30.

Interestingly, it has been found that an SDC-7 + SDC-30 subgroup compared to the SDC-90 subgroup differs significantly concerning the value of the transverse dimension LA ($p = 0.0296$). Patients in the SDC-90 group had larger transverse LA dimension in comparison with SDC-7 + SDC-30 group. A statistically significant difference in LA transverse diameter was also found between subgroups NDC + SDC-7 + SDC-30 and SDC-90 ($p = 0.0350$). Here, consistently, the average size of LA was significantly higher in SDC-90 versus NDC + SDC-7 + SDC-30. These dependencies were illustrated in Figures 1 and 2.

It was also shown that the transverse RA dimension in SDC-30 group was significantly higher than in SDC-7 group ($p = 0.0234$). This stands also for the comparison between SDC-90 and SDC-7 groups, where the difference was close to statistical significance ($p = 0.0578$).

All the patients from the general study population had normal left ventricle function with ejection fraction of over 55%.

3.4. Comorbidity. Most of the observed patients were overweight or obese. BMI ranged from 23.74 to 44.98 (mean 30.70). Only 7 subjects had a normal body weight, 25 (41.67%) were overweight (BMI 25.0–30.0), and 28 were obese (BMI > 30.0), including two patients with morbid obesity (BMI > 40). The subgroups did not significantly differ concerning the body mass.

Eight patients (13.34%) had hyperthyroidism treatment currently or in the past, but at the moment of cardioversion, all were evaluated as euthyroid.

It was examined whether there is a correlation between the effectiveness of the DC and arterial hypertension occurrence in study population. Hypertension was diagnosed in 36 (60%) patients, with the greatest percentage of HA (66.67% and 63.84%) in the NDC and SDC-90 subgroup of SCD. There were no statistically significant correlation between the incidence of arterial hypertension and the effectiveness of treatment with DC.

Then the relation between DC efficiency and diabetes mellitus was subjected. Diabetes occurred in the total of 6 (10%) patients. A statistically significant difference in the incidence of diabetes among SDC-7 and SDC-90 ($p = 0.0302$) was observed. Diabetes was diagnosed in 27.27% in the SDC-7 subgroup but none of the patients from SDC-90 subgroup. The same proportion of people diagnosed with and without diabetes was observed for subgroups SDC-7 and SDC-30. This correlation, however, was not statistically significant ($p = 0.0932$).

Another coexisting disease entity was coronary artery disease, which appeared in a total of 5 (8.35%) patients, therein none of the patients in SDC-90 group. None of the patients had medical history of myocardial infarct. There were no statistically significant correlation between the incidence of coronary artery disease and the effectiveness of DC treatment in study population.

None of the patients had a medical history of the transient ischemic attack (TIA) or stroke.

The data on numbers and percentages of patients with known comorbidity in different study subgroups are presented in Table 1.

3.5. Apelin and proBNP Concentration in relation to DC Effectiveness. In a subsequent step of analysis, we sought the relationship between the apelin concentration and the effectiveness of electrical cardioversion. Mean and median values of plasma apelin in each group are shown in Table 2. It was found that the median value of apelin in the subset of patients from groups NDC + SDC-7 + SDC-30 is significantly higher than from group SDC-90 ($p = 0.0463$). This dependency was presented in Figure 3.

As to the concentration of apelin, proBNP was examined. Mean and median proBNP values in each group are shown in

Table 2: Apelin and proBNP serum concentration in study subgroups.

Subgroup	Apelin concentration (pg/ml)					proBNP concentration (pg/ml)				
	Mean	Median	Min.	Max.	SD	Mean	Median	Min.	Max.	SD
NDC	2736.61	2756	2243	2975	213.73	619.11	609.3	102	1284	373.24
SDC	2581.49	2662	1026	3000	373.86	733.28	636.3	70.78	2416	548.36
SDC-7	2672.64	2804	1597	3000	404.89	813.21	832.2	285.4	1711	395.91
SDC-30	2650.42	2664	2402	2974	146.03	526.98	412.3	102	1333	371.66
SDC-90	2490	2522	1026	2987	440.14	805.85	673.55	70.78	2416	670.72
SDC-7 + SDC-30	2661.04	2695	1597	3000	292.07	663.87	636.3	102	1711	402.1
NDC + SDC-7 + SDC-30	2688.33	2710.5	1597	3000	265.78	646.2	621.85	102	1711	386.44

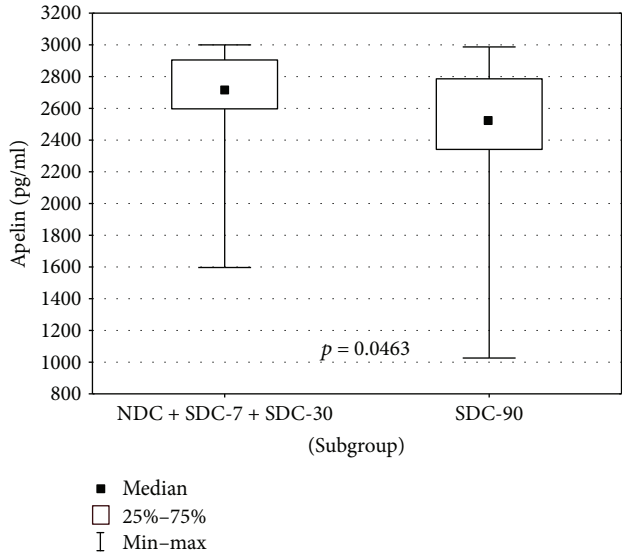

Figure 3: Apelin concentration in SDC-90 versus NDC + SDC-7 + SDC-30.

Table 2. Neither of the compared subgroup pairs revealed statistically significant correlation between the proBNP concentration and the DC effectiveness in our population. In Table 3, there were collected the p values for all conducted comparisons.

Table 3: Apelin and proBNP serum level: p values for all conducted comparisons.

Compared subgroups	p value for apelin	p value for proBNP
NDC versus SDC	0.1252	0.6633
SDC-7 versus SDC-30	0.1858	0.0881
SDC-7 versus SDC-90	0.0793	0.3961
SDC-30 versus SDC-90	0.4027	0.3634
SDC-7 + SDC-30 versus SDC-90	0.1162	0.9547
NDC + SDC-7 + SDC-30 versus SDC-90	**0.0463**	0.8121

4. Discussion

The results of our study confirm the important role of apelin in the pathophysiology of cardiac arrhythmias. It was shown that the median, initial apelin level was significantly lower in patients who maintained sinus rhythm during the 3-month follow-up after the successful cardioversion, compared with patients in whom cardioversion was ineffective or who presented an arrhythmia recurrence. It has been shown that apelin affects the physiology of the circulatory system, including regulation of atrial cardiomyocytes action potential [13]. These reports are scarce, but they have become the base to search for apelin level variations in the abnormal heart rhythms. It has been documented that apelin plasma level is reduced in patients with isolated atrial fibrillation [14] and other supraventricular arrhythmias [16]. It is investigated whether apelin, among other biochemical markers, has its potential in predicting the risk of atrial fibrillation recurrence after electric cardioversion. So far, to our best knowledge, only one paper has been published on the subject. Falcone et al. [17] have achieved the opposite results, indicating an increased risk of AF recurrence in patients with lower baseline apelin.

Moreover, in our study group, there were no statistically significant differences in the values of proBNP depending on the DC efficiency, which is consistent with the results of some studies, for example, Shin et al. [6] and Tveit et al. [18]. However, some of the authors indicate the proBNP predictive value in predicting the FA recurrence [17, 19].

Discrepancies in the obtained results may result from the difference in the duration of AF, which was higher in patients from our study group. In addition, our study also included patients in whom DC was not effective. The variability in blood serum apelin concentration in patients with cardiac dysfunctions, including arrhythmia and heart failure, cannot be clearly predicted. It is due to the fact that apelin is involved both in pathophysiological and cardioprotective mechanisms. In heart failure, the level of apelin rises in the initial stage of the disease parallel with BNP and decreases in the advanced stage—those observations, in our opinion, might also be applied to patients with AF [11].

Despite the numerous data on the apelin multiple role in the physiology and pathophysiology of the cardiovascular system, from animal and human studies, the marker still remains poorly understood. The variability of its concentration in various pathophysiological conditions may be both a

cause and a consequence of the phenomenon; therefore, their interpretation is not obvious.

There is a number of conditions, in which the apelin level variation has been proved including cardiovascular and metabolic disorders. It is difficult to separate these factors that undoubtedly affect the level of apelin in the group of cardiac patients.

At the same time, those conditions may also affect the course of atrial fibrillation, including the effectiveness of the cardioversion. Inability to eliminate all factors that may additionally affect the apelin level is undoubtedly the study limitation. However, we were able to reduce those effects as presented beneath.

Study groups did not differ significantly in terms of AF duration, and patients with a very large (>70 mm) atria dimensions were excluded. Examined patients did not differ significantly within the longitudinal atria dimensions, and their transverse atria dimensions were even larger in the subgroup with the sinus rhythm maintenance over 90 days.

Numerous studies demonstrated significantly higher plasma apelin level in obese patients [12, 20, 21]. Most of our study population was overweight or obese, but subgroups were not significant in between different concerning BMI.

In addition, apelin serum level was found to be decreased in human with primary hypertension [22, 23], coronary artery disease [24, 25], advanced heart failure [26], and increased in type 2 diabetes mellitus and impaired glucose tolerance subjects [27–29]. In our population, none of the patients had heart failure with symptoms above the first hemodynamic NYHA class, all had normal left ventricular systolic function in echocardiography. In addition, there was no statistical association between the arterial hypertension or coronary artery disease occurrence and the effectiveness of cardioversion; the largest proportion of HA patients occurred in the SDC-90 subgroup. Among comorbidities, only diabetes showed a statistically significant difference in the incidence between the SDC-7 and SDC-90 group and was more frequent in the SDC-7.

5. Conclusion

In conclusion, our data confirm the contribution of apelin to the atrial fibrillation pathophysiology. Conversely, compared to what has been previously documented, in our group initial, serum apelin level was significantly higher in patients who remained in the sinus rhythm over 90 days after the DC as compared to patients with the AF recurrence during follow-up and those in whom cardioversion was not effective. Furthermore, there were no differences in the concentration of proBNP depending on the DC efficiency.

A lower initial apelin serum concentration may indicate a group of patients with a more advanced stage of hemodynamic disorders caused by atrial fibrillation.

Basing on the currently available data, apelin cannot serve as an independent prognostic marker for the failure of the sinus rhythm maintenance strategy, but in the future, in addition to other biochemical and echocardiographic indices, it may be helpful in distinguishing a group of patients at risk of arrhythmia recurrence.

Abbreviations

SDC: Successful cardioversion
NDC: Nonsuccessful cardioversion
SDC-7: Sinus rhythm maintenance within 7 days
SDC-30: Sinus rhythm maintenance 7 to 30 days
SDC-90: Sinus rhythm maintenance over 90 days.

Conflicts of Interest

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

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Research Article

Mitochondrial Genome Mutations Associated with Myocardial Infarction

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Myocardial infarction is one of the clinical manifestations of coronary heart disease. In some cases, the cause of myocardial infarction may be atherosclerotic plaques which occurred in the human aorta. The association of mtDNA mutations with atherosclerotic lesions in human arteries was previously detected by our research group. In this study, we used samples of white blood cells collected from 225 patients with myocardial infarction and 239 control persons with no health complaints. DNA was isolated from the blood leukocyte samples. Then, PCR fragments of DNA were obtained. They contained the investigated regions of 11 mitochondrial genome mutations (m.5178C>A, m.3336T>C, m.652delG, m.12315G>A, m.14459G>A, m.652insG, m.14846G>A, m.13513G>A, m.1555A>G, m.15059G>A, m.3256C>T). According to the obtained results, three mutations of the human mitochondrial genome correlated with myocardial infarction. A positive correlation was observed for mutation m.5178C>A. At the same time, a highly significant negative correlation with myocardial infarction was observed for mutation m.14846G>A. One single-nucleotide substitution of m.12315G>A had a trend towards negative correlation. These mutations can potentially be useful for creating molecular/cellular models for studying the mechanisms of myocardial infarction and designing novel therapies. Moreover, these mutations can possibly be used for diagnostic purposes.

1. Introduction

Myocardial infarction is one of the clinical manifestations of coronary heart disease. In this serious disease, some myocardial contractile cells die. Subsequently, these cells are replaced by connective tissue. The death of cells is a consequence of coronary heart disease. At the same time, metabolism disturbance occurs and irreversible changes in cells develop [1]. In most cases, acute myocardial infarction occurs due to coronary artery thrombosis in the area of an atherosclerotic plaque [1, 2]. In particular, myocardial infarction can occur in patients with atherosclerosis, arterial hypertension, and coronary heart disease. The prime risk factors for developing MI are obesity, lack of motor performance, and smoking. The clinical picture of MI is distinguished by a great variety. That is why it is difficult to make the right diagnosis. The patient may have chest discomfort or irregular heartbeat. Sometimes there is a complete absence of pain. With atypical cases of myocardial infarction, there is abdominal pain, laborious breathing, or dyspnea [3, 4].

At the present time, there are no reliable algorithms for the early prognosis of myocardial infarction, which would determine an increased individual predisposition to this disease and its risk factor, atherosclerosis. The focus of further researches for the determination of the causes of myocardial infarction development should be

Table 1: Primers for PCR.

Mutation	Primers	Size of PCR fragment
m.5178C>A	F: bio-GCAGTTGAGGTGGATTAAAC (4963–4982) R: GGAGTAGATTAGGCGTAGGTAG (5366–5345)	383 bp
m.3336T>C	F: bio-AGGACAAGAGAAATAAGGCC (3129–3149) R: ACGTTGGGGCCTTTGCGTAG (3422–3403)	294 bp
m.652delG	F: TAGACGGGCTCACATCAC (621–638) R: bio-GGGGTATCTAATCCCAGTTTGGGT (1087–1064)	467 bp
m.12315G>A	F: bio-CTCATGCCCCCATGTCTAA (12230–12249) R: TTACTTTTATTTGGAGTTGCAC (12337–12317)	108 bp
m.14459G>A	F: CAGCTTCCTACACTATTAAAGT (14303–14334) R: bio-GTTTTTTTAATTTATTTAGGGGG (14511–14489)	209 bp
m.652insG	F: TAGACGGGCTCACATCAC (621–638) R: bio-GGGGTATCTAATCCCAGTTTGGGT (1087–1064)	467 bp
m.14846G>A	F: bio-CATTATTCTCGCACGGACT (14671–14689) R: GCTATAGTTGCAAGCAGGAG (15120–15100)	450 bp
m.13513G>A	F: CCTCACAGGTTTCTACTCCAAA (13491–13512) R: bio-AAGTCCTAGGAAAGTGACAGCGAGG (13825–13806)	335 bp
m.1555A>G	F: TAGGTCAAGGTGTAGCCCATGAGGTGGCAA (1326–1355) R: bio-GTAAGGTGGAGTGGGTTTGGG (1704–1684)	379 bp
m.15059G>A	F: bio-CATTATTCTCGCACGGACT (14671–14689) R: GCTATAGTTGCAAGCAGGAG (15120–15100)	450 bp
m.3256C>T	F: bio-AGGACAAGAGAAATAAGGCC (3129–3149) R: ACGTTGGGGCCTTTGCGTAG (3422–3403)	294 bp

transferred to the interaction of environmental, ecological, and molecular-genetic risk factors, as well as to the search of new methods and approaches to early diagnosis of individual predisposition. Molecular-genetic factors predisposing to the development of myocardial infarction have not been studied enough.

In a number of published articles, there has been a report of mutations and polymorphisms of the nuclear genome associated with a risk factor for atherosclerosis, such as myocardial infarction [5–9]. However, these data do not cover the full range of variability of myocardial infarction clinical manifestations.

The results obtained by our group suggest, however, that mitochondrial genome mutations can also be associated with myocardial infarction. We have previously demonstrated that mtDNA mutations were present in cells from atherosclerotic aortas and arteries [10–14]. In this work, we evaluated the association of these mutations with myocardial infarction as a risk factor for atherosclerosis.

Unlike nuclear genome mutations, in the analysis of mitochondrial genomes, there occurs not the determination of homo- and heterozygotes by mutation, but the detection of the heteroplasmy level of mtDNA mutations (the ratio of mutant copies of the mitochondrial genome to the total number of DNA molecules in the mitochondria). The method of determining the heteroplasmy level of mitochondrial genome mutations based on pyrosequencing technology was previously developed by our laboratory scientists [10, 15–18]. It should be noted that the developed method is the most accurate in assessing the heteroplasmy level of mitochondrial genome mutations [10, 15]. In pyrosequencing, a short DNA fragment (6–10 bp) containing the investigated mutation is studied [19, 20]. Therefore, the probability of mistakes in determining the heteroplasmy level of mutations is minimal [21–23].

2. Materials and Methods

A total of 464 subjects were enrolled in the Cardiology Research Complex MH RF and Moscow State University clinic. All study participants were aged between 40 and 55 years. The investigated sample included 225 patients with myocardial infarction and 239 control persons with no health complaints. The work was conducted in accordance with the Declaration of Helsinki. The study protocol has been approved by the Ethics Community of Cardiology Research Complex MH RF, and all subjects gave written informed consent upon enrollment.

DNA was isolated from the blood leukocyte samples of study participants. The phenol-chloroform extraction method, developed by the authors of the article [24–26] based on the Maniatis technology [27], was used.

Then, PCR fragments of DNA were obtained, which contained the investigated regions of 11 mitochondrial genome mutations (m.5178C>A, m.3336T>C, m.652delG, m.12315G>A, m.14459G>A, m.652insG, m.14846G>A, m.13513G>A, m.1555A>G, m.15059G>A, m.3256C>T).

Primers for PCR and the size of PCR fragments are listed in Table 1 [10].

Biotinylation of one of the primers for PCR was carried out with the aim of pyrosequencing the amplicon.

Each 30 μl PCR reaction contained 0.4–0.6 μg mitochondrial DNA, 16.6 μM $(NH_4)_2SO_4$, 0.3 pM of each primer, 200 μM of each deoxyribonucleotriphosphate, 67 mM Tris-HCl (pH 8.8), $MgCl_2$ (1.5 mM for m.14846G>A, m.15059G>A, and m.14459G>A; 2.5 mM for the rest of the investigated mutations), and 3 units of *Taq* polymerase [10].

In PCR, the following annealing temperatures were used for the primers [10]:

(1) For mutations m.5178C>A, m.652delG, and m.652insG—60°C

(2) For mutations m.3336T>C, m.14846G>A, m.13513G>A, m.15059G>A, and m.3256C>T—55°C

(3) For mutations m.12315G>A, m.14459G>A, and m.1555A>G—50°C

As an apparatus for PCR, "PTC DNA Engine 200" was used.

The association of these mutations with atherosclerotic lesions in human arteries was previously established [10–13, 17]. The PCR fragments were analyzed on the automated pyrosequencing device PSQTMHS96MA (Biotage, Sweden) to determine the heteroplasmy level of mtDNA mutations [10].

Primers for pyrosequencing are listed in Table 2 [10].

The results were analyzed using the software package SPSS 22.0 [28]. Bootstrap analysis was used. Correlation was considered statistically significant at the level of $p \leq 0.05$. The results at the significance level of $p \leq 0.2$ were considered to show a tendency toward statistical significance.

Table 2: Primers for pyrosequencing.

Mutation	Primer
m.5178C>A	ATTAAGGGTGTTAGTCATGT (5200–5181)
m.3336T>C	TGCGATTAGAATGGGTAC (3354–3337)
m.652delG	CCCATAAACAAATA (639–651)
m.12315G>A	TTTGGAGTTGCAC (12328–12316)
m.14459G>A	GATACTCCTCAATAGCCA (14439–14456)
m.652insG	CCCATAAACAAATA (639–651)
m.14846G>A	GCGCCAAGGAGTGA (14861–14848)
m.13513G>A	AGGTTTCTACTCCAA (13497–13511)
m.1555A>G	ACGCATTTATATAGAGGA (1537–1554)
m.15059G>A	TTTCTGAGTAGAGAAATGAT (15080–15061)
m.3256C>T	AAGAAGAGGAATTGA (3300–3286)

3. Results and Discussion

For all the study participants, age and demographic characteristics were determined (Tables 3 and 4). The data in Table 4 is presented as an average value with the standard deviation indicated (in parentheses).

According to Table 3, the age of conventionally healthy participants ranged from 29 to 75 years. At the same time, the age of patients with myocardial infarction ranged from 43 to 87 years. The average age of conventionally healthy study participants was 13 years less than the age of patients with myocardial infarction.

It is noteworthy that women predominated in the group of conventionally healthy study participants. At the same time, men predominated in the group of patients with myocardial infarction.

Significant differences between conventionally healthy study participants and patients with myocardial infarction were found only for risk factors such as sex and age (Table 4). It is worth mentioning that the tendency to the occurrence of such differences was found for smoking frequency. Perhaps, by increasing the sample, these differences will become reliable.

For the present investigation, the 11 mitochondrial genome mutations were taken, for which, in preliminary studies, a connection with atherosclerosis was found [10–12, 17, 18]. First, we examined 42 mtDNA mutations, for which an association with various pathologies was found [10]. We investigated lipofibrous plaques and areas of normal aortic intima. Therewith, 11 mitochondrial genome mutations associated with atherosclerosis were detected. It was decided to investigate the identified mutations in a sample of patients with myocardial infarction.

An evaluation of Spearman correlation of the investigated mtDNA mutations with myocardial infarction is presented in Table 5.

The coefficient of correlation was necessary for us to identify the direction of linkage of mtDNA mutations with myocardial infarction. If the connection was positive, the mutations were associated with myocardial infarction. If it was negative, mutations showed an antipathological effect. A positive correlation was observed for mutation m.5178C>A. At the same time, a highly significant negative correlation with myocardial infarction was observed for mutation m.14846G>A. One single-nucleotide substitution of m.12315G>A had a trend towards negative correlation ($p \leq 0.1$).

For the found three mitochondrial genome mutations, an analysis of the odds ratio to be associated with the occurrence of myocardial infarction or to have a protective effect from this pathology was made. According to the obtained data, the probability of the occurrence of myocardial infarction in carriers of the mitochondrial genome mutation m.5178C>A was 2.8-fold higher than that in the study participants in which this mutation is absent. At the same time, the probability of the occurrence of this pathology in carriers of mutation m.14846G>A and in carriers of mutation m.12315G>A was 2.4-fold lower and 1.15-fold lower, respectively, than that in the study participants without these mutations.

Therefore, the mtDNA mutation m.5178C>A was a risk factor for the occurrence of myocardial infarction. At the same time, mutations m.14846G>A and m.12315G>A had a protective effect concerning this pathology.

The three mutations that had a positive or negative correlation with myocardial infarction were located in the coding region of the mitochondrial genome. Mutations m.5178C>A and m.14846G>A were localized in the genes encoding the second protein subunit of NADH

Table 3: Age characteristics of the study participants.

Study participants	Age			Standard deviation
	Minimum (years)	Mean (years)	Maximum (years)	
Conventionally healthy	29	52	75	8.5
Patients with myocardial infarction	43	65	87	8.3

Table 4: Demographic characteristics of the study participants.

Parameter	Conventionally healthy study participants	Patients with myocardial infarction	Significance of differences
Sex, m/f	109 : 130	135 : 90	0.008*
Age, years	52 (8.5)	65 (8.3)	0.027*
Body mass index, kg/m^2	23.5 (4.3)	29.1 (5.2)	0.43
Systolic blood pressure, mmHg	123 (19)	142 (25)	0.21
Diastolic blood pressure, mmHg	81 (15)	87 (23)	0.35
Smoking, %	19	41	0.12

*Significant differences between conventionally healthy study participants and patients with myocardial infarction.

Table 5: Spearman correlation of 11 mtDNA mutations with myocardial infarction.

Mutation	Correlation coefficient	Significance
m.5178C>A	0.109	0.045**
m.3336T>C	0.051	0.198
m.652delG	0.053	0.242
m.12315G>A	−0.096	0.065*
m.14459G>A	0.064	0.187
m.652insG	−0.045	0.229
m.13513G>A	0.069	1.174
m.14846G>A	−0.127	0.001**
m.1555A>G	−0.059	0.191
m.15059G>A	0.079	0.116
m.3256C>T	0.075	0.111

**$p \leq 0.05$; *$p \leq 0.1$.

dehydrogenase and cytochrome B, respectively. Mutations in these genes can therefore lead to mitochondrial respiratory chain enzyme dysfunction. Mutation m.12315G>A was localized in the transport RNA gene Leu (recognition codon CUN). It can possibly lead to defects in the transport RNA and can affect protein synthesis, which, in turn, may result in deficiencies of mitochondrial respiratory chain enzymes. Therefore, the described mutations can eventually lead to energy deficiency in affected cells, which may play a role in pathological processes, including myocardial infarction.

It is necessary to note that for this research, we used the method of quantitative assessment of the heteroplasmy level of mtDNA mutations, developed by us on the basis of pyrosequencing technologies in 2007 [10, 18, 29]. Based on the threshold heteroplasmy level of the mutation, associated with myocardial infarction, we detected the significance of the differences in this parameter between patients with myocardial infarction and conventionally healthy participants in the study. At the same time, two groups of scientists from Japan investigated the frequency of the occurrence of m.5178C>A in the Japanese sample of patients and in healthy people using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis with the restriction enzyme *Alu*I [30, 31]. They found that the frequency of occurrence of the 5178C allele is higher in the group of patients with myocardial infarction than in healthy people. Unfortunately, it is impossible to determine the heteroplasmy level of mutation, using the PCR-RFLP method. In consequence of this, Japanese scientists could not take into account patients who have a not very high threshold heteroplasmy level of mutation m.5178C>A linked to myocardial infarction.

According to a generally accepted opinion of scientists around the world, polymorphisms do not lead to pathologies, unlike mutations. The mtDNA mutation m.5178C>A, according to our data, was associated with atherosclerosis [11, 17]. In the present investigation, we have found a link of this mutation with myocardial infarction. Therefore, as a pathological variant of the mitochondrial genome mutation m.5178C>A, in our articles, we name it a "mutation" and not a polymorphism.

It is noteworthy that mutation m.14846G>A, according to the literature, leading to a progressive exercise of intolerance, proximal limb weakness, and attacks of myoglobinuria, showed a protective effect on myocardial infarction at a high level of significance [32]. This can mean that the molecular mechanisms which lead to exercise intolerance, proximal limb weakness, and attacks of myoglobinuria protect the heart from the occurrence of myocardial infarction.

According to data from the literature, mutation m.12315G>A turned out to be associated with mitochondrial myopathy, ophthalmoplegia, ptosis, limb weakness, sensorineural hearing loss, and pigmentary retinopathy [33, 34].

At the same time, in our study, m.12315G>A showed a tendency to have a protective effect on myocardial infarction. It can also indicate that the molecular mechanisms which lead to the occurrence and development of mitochondrial myopathy, ophthalmoplegia, ptosis, limb weakness, sensorineural hearing loss, and pigmentary retinopathy protect from myocardial infarction.

It may also be suggested that the differences between the Russian and the Japanese samples are connected with undersampling. We plan to expand our sample.

It is necessary to note, for a number of diseases, for example, cystic fibrosis, that a gradient in the spread of some mutations from west to east has been found. Supposedly in this case, we are dealing with a similar gradient in the spread of some mitochondrial genome mutations. This is confirmed by the fact that the two articles in which it is stated that mutation m.5178C>A is associated with a lower frequency of its occurrence in patients with myocardial infarction, compared to healthy people, belong to Japanese research groups [30, 31].

To answer this question, we plan to get in our further studies, with an increase in the size of our sample. Perhaps, with mutation m.12315G>A, we will get very significant differences between patients with myocardial infarction and conventionally healthy study participants.

4. Conclusion

In the present study, we report on three mutations of the human mitochondrial genome that correlated with myocardial infarction. A positive correlation was observed for mutation m.5178C>A. At the same time, a highly significant negative correlation with myocardial infarction was observed for mutation m.14846G>A. One single-nucleotide substitution of m.12315G>A had a trend towards negative correlation ($p \leq 0.1$).

Therefore, the mtDNA mutation m.5178C>A was a risk factor for the occurrence of myocardial infarction. At the same time, mutations m.14846G>A and m.12315G>A had a protective effect concerning this pathology.

These mutations can potentially be useful for creating molecular/cellular models for studying the mechanisms of myocardial infarction and designing novel therapies. Moreover, these mutations can possibly be used for diagnostic purposes.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Research Article

A Novel Polymorphism in the Promoter of the *CYP4A11* Gene Is Associated with Susceptibility to Coronary Artery Disease

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Enzymes CYP4A11 and CYP4F2 are involved in biosynthesis of vasoactive 20-hydroxyeicosatetraenoic acid and may contribute to pathogenesis of coronary artery disease (CAD). We investigated whether polymorphisms of the *CYP4A11* and *CYP4F2* genes are associated with the risk of CAD in Russian population. DNA samples from 1323 unrelated subjects (637 angiographically confirmed CAD patients and 686 age- and sex-matched healthy individuals) were genotyped for polymorphisms rs3890011, rs9332978, and rs9333029 of *CYP4A11* and rs3093098 and rs1558139 of *CYP4F2* by using the Mass-ARRAY 4 system. SNPs rs3890011 and rs9332978 of *CYP4A11* were associated with increased risk of CAD in women: OR = 1.26, 95% CI: 1.02–1.57, $P = 0.004$, and $Q = 0.01$ and OR = 1.45, 95% CI: 1.13–1.87, $P = 0.004$, and $Q = 0.01$, respectively. Haplotype G-C-A of *CYP4A11* was associated with increased risk of CAD (adjusted OR = 1.41, 95% CI: 1.12–1.78, and $P = 0.0036$). Epistatic interactions were found between rs9332978 of *CYP4A11* and rs1558139 of *CYP4F2* ($P_{\text{interaction}} = 0.025$). In silico analysis allowed identifying that SNP rs9332978 is located at a binding site for multiple transcription factors; many of them are known to regulate the pathways involved in the pathogenesis of CAD. This is the first study in Europeans that reported association between polymorphism rs9332978 of *CYP4A11* and susceptibility to coronary artery disease.

1. Introduction

Coronary artery disease (CAD) is a common cardiovascular disorder (CVD), a major cause of mortality and disability in Russia and worldwide [1, 2]. CAD is a multifactorial polygenic disorder resulting from complex interactions between multiple genetic and environmental factors [3, 4]. Advances in molecular genetic and biochemical techniques have improved our understanding of the metabolic disorders causing CVD and coronary atherosclerosis, and the identification of candidate genes responsible for CAD susceptibility is now an area of intense research interest. Genome-wide association studies (GWAS) have provided powerful tools to dissect genetic determinants of complex multifactorial disorders and to identify new potential genes that may increase the risk of coronary artery disease. Meta-analyses of the largest GWAS conducted on coronary artery disease have identified a number of genes associated with disease

susceptibility in different populations and provided insights into the molecular basis of the disease [5, 6].

Genetically determined alterations in the metabolism of arachidonic acid (AA) have been implicated in the pathogenesis of CVD such as hypertension, atherosclerosis, and coronary artery disease [7–10]. Arachidonic acid is metabolized by various enzymes such as cyclooxygenases, lipoxygenases, and cytochrome P450 monooxygenases producing a variety of bioactive substances such as prostanoids, leukotrienes, hydroxyeicosatetraenoic acids (HETEs), and epoxyeicosatrienoic acids (EETs) [11, 12]. EETs are products of cytochrome P450 epoxygenases that realize their cardiovascular effects through activating receptor-mediated signaling pathways and ion channels and possess vasodilatory, angiogenic, and anti-inflammatory properties in the cardiovascular system [7, 8, 13, 14]. 20-HETEs (20-hydroxyeicosatetraenoic acids) are vasoactive eicosanoids which are derived from the ω-hydroxylation of AA by members of the CYP4 gene family and known to be involved in the regulation of vascular tone and sodium transport in the kidney [10, 15]. 20-HETEs possess multifaceted effects on cardiovascular functions including those implicated to the pathogenesis of CVD: stimulation of smooth muscle contractility, migration, and proliferation, as well as activation of endothelial cell dysfunction, angiogenesis, and inflammation [9, 15]. Cytochrome P450 4A11 and 4F2 are the major 20-HETE-producing CYP4 isoforms in humans [16, 17] which also participate in the metabolism of several drugs including those used for therapy of CVDs [18].

Various studies have revealed that single nucleotide polymorphisms (SNPs) in *CYP4A11* and *CYP4F2* have an impact on expression or catalytic activity of these enzymes, thereby contributing to the molecular basis of cardiovascular disorders including CAD [17, 19–24]. Many of these studies have discovered an association between some of these SNPs and the susceptibility to hypertension and coronary artery disease, making *CYP4A11* and *CYP4F2* reasonable candidate genes for altering the risk of CVD. However, the results of these studies were variable and sometimes contradictory that may arise from differences in ethnic backgrounds, effects of environmental factors, or inconsistent inclusion criteria. A huge portion of the studies conducted in Asian populations have considered the *CYP4F2* and *CYP4A11* genes as candidates for pharmacogenetic investigations of drugs. A limited number of studies investigated the contribution of these genes to the development of hypertension [17, 25, 26], and no studies investigated the relationship of these genes with CAD susceptibility in European populations. The purpose of this study was to investigate whether common single nucleotide polymorphisms in the *CYP4A11* and *CYP4F2* genes are associated with susceptibility to coronary artery disease in Russian population.

2. Methods

2.1. Study Subjects. The study was approved by the Ethical Review Committee of Kursk State Medical University, and the participants who were recruited gave written informed consent. A total of 1323 unrelated Russian subjects including 637 patients with coronary artery disease and 686 healthy controls were enrolled from the Cardiology Divisions of Kursk Regional Clinical Hospital and Kursk Emergency Hospital as well as from the Regional Cardiovascular Centre during a period between 2012 and 2015. All recruited patients had clinical signs or a history of CAD (angina or myocardial infarction) and angiographically confirmed coronary artery stenosis of >50%. CAD patients had no clinical signs and/or histories of congenital heart disease, cardiomyopathy, malignancy, connective-tissue disorder, chronic inflammatory disease, and liver or kidney disease. The control group included blood donors, healthy volunteers without any chronic disease, and also hospital-based patients having no clinical evidence for CAD or a history of cerebrovascular/peripheral vessel disease. These subjects were recruited over several periods in the framework of our previous studies [27–29]. Demographic and clinical data of the study participants are shown in Table 1. As can be seen from Table 1, the study groups were matched with respect to both sex and age ($P > 0.05$). A percentage of positive family history of CAD, hypertension, and diabetes was significantly higher in the case group versus that in the healthy controls. Biochemical parameters (blood lipids and fasting glucose) were available from 347 subjects of the control group. Significant differences between the groups were seen regarding the lipid parameters and blood glucose concentration (Table 1).

2.2. Selection of Single Nucleotide Polymorphisms. Six common SNPs such as rs3890011, rs1126742, rs9332978, and rs9333029 of the *CYP4A11* gene and rs3093098 and rs1558139 of the *CYP4F2* gene were selected for the study based on their known functional relevance, haplotype tagging properties, and previously reported associations with cardiovascular diseases [21, 22, 24]. The functionality of the selected SNPs and their haplotype properties were assessed in silico by the SNP Function Prediction tool developed by Xu and Taylor [30] and available online at the SNPinfo Web Server (https://snpinfo.niehs.nih.gov/snpinfo/). SNP rs1126742 of *CYP4A11* was excluded from the study because of insufficient genotyping call rate (<70%) for this polymorphism.

2.3. Genotyping. Genomic DNA was isolated from 5 ml of peripheral blood samples obtained from all study participants using standard phenol/chloroform procedure. Polymerase chain reaction (PCR) was performed on the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, USA). SNP genotyping was performed using a MALDI-TOF mass spectrometry iPLEX platform (Agena Bioscience Inc., San Diego, CA, USA) at the Core Facility "Medical Genomics" in the Research Institute of Medical Genetics (Tomsk, Russia). Blind replicates were included for quality control. Genotype data on SNPs rs9332978 and rs9333029 of *CYP4A11* were not available from two CAD patients and two healthy controls, respectively.

2.4. Data Analysis. An association analysis between SNPs and disease risk could detect a difference of 2–6% in the genotype

Table 1: Demographic and clinical data of the study participants.

Baseline characteristics	Controls, $n = 686$	CAD patients, $n = 637$	P value
Age, mean ± SD	58.8 ± 7.6	59.5 ± 10.3	0.16
Males	404 (58.9)	401 (63.0)	0.13
BMI (kg/m^2), mean ± SD	27.9 ± 10.2	28.8 ± 9.4	0.10
Hypertension	0 (0.0)	842 (90.2)	—
Diabetes	0 (0.0)	79 (8.5)	—
Fasting blood glucose (mmol/L)[1]	5.2 ± 0.7	8.1 ± 0.5	**<0.0001**
TC (mmol/L)[1]	4.2 ± 0.3	6.2 ± 0.4	**<0.0001**
HDL-C (mmol/L)[1]	1.2 ± 0.2	1.1 ± 0.3	**<0.0001**
LDL-C (mmol/L)[1]	3.1 ± 0.3	4.3 ± 0.6	**<0.0001**
TG (mmol/L)[1]	1.5 ± 0.4	1.7 ± 0.3	**<0.0001**
Smokers (ever/never)[2]	273 (41.5)	251 (43.5)	0.48
Positive family history of CAD	150 (23.8)	323 (34.6)	**<0.0001**
Positive family history of hypertension	100 (15.9)	273 (29.2)	**<0.0001**
Positive family history of diabetes	24 (3.8)	127 (13.6)	**<0.0001**

[1]The biochemical parameters were available from 347 subjects of the control group. [2]Data on smoking status were not available from 60 CAD patients and 28 controls. SD: standard deviation; BMI: body mass index (age and BMI were normally distributed and were analyzed by Student's t-test); TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; TG: triglyceride. Other data are expressed as frequencies and percentages and were evaluated by the χ^2 test. Bolded is statistically significant P value.

distributions between the cases and controls assuming 81–92% statistical power and a 5% type I error ($\alpha = 0.05$) on the basis of the sample sizes of 637 CAD patients and 686 healthy controls. Allele frequencies were estimated by the gene counting method, and the chi-square test was used to assess significant departures from Hardy–Weinberg equilibrium (HWE). Categorical variables were also compared by using the chi-square test. Allele, genotype, and haplotype frequencies in the study groups were evaluated by the SNPassoc package for R [31] and the SNPStats software [32]. The strength of the association of the SNPs with the occurrence of coronary artery disease was measured by multiple logistic regression analysis to calculate odds ratios (OR) with 95% confidence intervals (CI) and adjusted for confounding factors. Epistatic interactions between SNPs (log-likelihood ratio test (LRT)) were analyzed by the SNPassoc package for R [31], assuming codominant, dominant, and recessive models, and adjusted for age, gender, and hypertension. Haplotypes of *CYP4A11* and *CYP4F2* were estimated in the entire groups of CAD patients and controls by the SNPStats software. P value ≤ 0.05 was set to be statistically significant. As an adjustment for multiple testing, false discovery rate-(FDR-) based Q value was calculated for each SNP using the method proposed by Benjamini and Hochberg [33] and implemented in the FDR calculator available online at http://www.sdmproject.com/utilities/?show=FDR. Significance of the associations was assessed by a 0.20 threshold of Q value, as previously suggested [34]. The regulatory potential of the studied SNPs was evaluated by the SNP Function Prediction tool [30] using the TRANSFAC database on potential transcription factor recognition sites (BIOBASE Corporation, Wolfenbuettel, Germany) as well as by using the rSNPBase database of curated regulatory SNPs (http://rsnp.psych.ac.cn) [35].

3. Results

3.1. Association Study between the CYP4A11 and CYP4F2 SNPs and CAD Risk. The genotype and allele frequencies of *CYP4A11* and *CYP4F2* SNPs are shown in Table 2. A significant departure from Hardy–Weinberg equilibrium (HWE) was observed for SNP rs9333029 of *CYP4A11*: no individuals with homozygous genotype GG were identified among the study participants. Notably, frequencies of genotypes AA and AG were compatible with those reported in various European populations, and genotype GG is also uncommon among Europeans (the 1000 Genomes Project, http://www.internationalgenome.org). Allele and genotype frequencies of other SNPs were similar with those observed in other European populations. As can be seen from Table 2, polymorphism rs9332978 of *CYP4A11* was found to be associated with increased risk of coronary artery disease at codominant genetic model after adjustment for confounding factors. In particular, the increased risk of CAD was associated with a carriage of variant allele C ($P = 0.002$, $Q = 0.01$) and genotypes T/C and CC ($P = 0.008$, $Q = 0.04$). In addition, allele G of rs3890011 showed a significant association with the risk of CAD ($P = 0.02$, $Q = 0.05$). These associations remain significant after adjustment for multiple testing using the FDR method. Table 3 shows gender-stratified distributions of genotypes and alleles for the studied SNPs in the case and control groups. The rs3890011 and rs9332978 polymorphisms of *CYP4A11* were associated with the increased risk of coronary artery disease exclusively in females ($P = 0.004$, $Q = 0.01$).

3.2. Interactions between SNPs of CYP4A11 and CYP4F2. We performed a log-likelihood ratio test to look for epistatic interaction between SNPs (Table 4). As can be seen from

Table 2: Genotype and allele frequencies for SNPs of *CYP4A11* and *CYP4F2* in patients with CAD and healthy controls.

Gene, polymorphism	Genotype, allele	Controls, $n = 686$ n (%)[1]	CAD patients, $n = 637$ n (%)[1]	OR (95% CI)[2]	P value	Q value
CYP4A11, C>G (rs3890011)	C/C	404 (58.9)	338 (53.1)	1.00		
	C/G	236 (34.4)	244 (38.3)	1.21 (0.91–1.58)	0.086	0.22
	G/G	46 (6.7)	55 (8.6)	1.40 (0.92–2.23)		
	G	328 (23.9)	354 (27.8)	1.21 (1.02–1.48)	0.02	0.05
CYP4A11, T>C (rs9332978)	T/T	542 (79)	459 (72.3)	1.00		
	T/C	134 (19.5)	158 (24.9)	1.42 (1.09–1.84)	0.008	0.04
	C/C	10 (1.5)	18 (2.8)	2.26 (1.04–4.95)		
	C	154 (11.2)	194 (15.3)	1.44 (1.16–1.81)	0.002	0.01
CYP4A11, A>G (rs9333029)	A/A	526 (76.9)	477 (74.9)	1.00		
	A/G	158 (23.1)	160 (25.1)	1.09 (0.78–1.59)	0.43	0.72
	G/G	0 (0.0)	0 (0.0)	—		
	G	158 (11.5)	160 (12.6)	1.04 (0.80–1.43)	0.42	0.70
CYP4F2, A>G (rs3093098)	A/A	479 (69.8)	448 (70.3)	1.00		
	A/G	186 (27.1)	172 (27)	0.97 (0.76–1.27)	0.89	0.91
	G/G	21 (3.1)	17 (2.7)	0.85 (0.43–1.68)		
	G	228 (16.6)	206 (16.2)	0.96 (0.80–1.17)	0.76	0.76
CYP4F2, G>A (rs1558139)	G/G	200 (29.1)	192 (30.1)	1.00		
	G/A	336 (49)	312 (49)	0.98 (0.73–1.28)	0.91	0.91
	A/A	150 (21.9)	133 (20.9)	0.94 (0.70–1.29)		
	A	636 (46.4)	578 (45.4)	0.97 (0.80–1.18)	0.61	0.76

[1]Absolute number and percentage of individuals/chromosomes with particular genotype/allele. [2]Odds ratio with 95% confidence intervals adjusted for age, gender, BMI, hypertension, diabetes, and smoking.

Table 4, SNPs rs3890011 ($P = 0.035$) and rs9332978 ($P = 0.004$) of *CYP4A11* showed significant individual effects on CAD risk at a dominant genetic model. Notably, we found epistatic interactions between rs9332978 of *CYP4A11* and rs1558139 of *CYP4F2* (recessive model, $P_{\text{interaction}} = 0.025$) as well as between rs3093098 and rs1558139 of the *CYP4F2* gene (overdominant model, $P_{\text{interaction}} = 0.047$).

3.3. Analysis of Haplotypes and Linkage Disequilibrium between SNPs. The three SNPs of the *CYP4A11* gene and the two SNPs of *CYP4F2* were used to establish five haplotypes. The patterns of estimated haplotypes and their frequencies in the case and control groups are shown in Table 5. Four haplotypes of *CYP4A11* gene and three haplotypes of *CYP4F2* with a frequency > 1% have been identified in the study patients. As can be seen from Table 5, the overall distribution of the haplotypes of *CYP4A11* was significantly different between the CAD patients and the healthy controls ($P = 0.019$). The frequency of the H2 (G-C-A) haplotype was significantly higher in the CAD patients than that in the healthy controls (OR = 1.42, 95% CI: 1.13–1.79, $P = 0.003$). Thus, the common G-C-A haplotype was thought to be a susceptibility haplotype in CAD patients. No significant difference in the *CYP4F2* haplotype frequencies was found between the case and control groups ($P > 0.05$). Table 6 shows pairwise linkage disequilibrium coefficients among the SNPs of *CYP4A11*. Polymorphism rs9332978 in the promoter of *CYP4A11* was in strong linkage disequilibrium to the intronic polymorphism rs3890011 ($D' = 0.974$, $P < 0.0001$). A strong linkage disequilibrium was also found between SNPs rs3890011 and rs9333029 of *CYP4A11* ($D' = 0.955$, $P < 0.0001$). Furthermore SNPs rs9332978 and rs9333029 are also in linkage disequilibrium but with a lesser degree ($D' = 0.565$, $P < 0.0001$).

3.4. In Silico Analysis of SNPs. Table 6 shows the results of bioinformatic analysis for the regulatory potential of the studied SNPs. The SNP Function Prediction tool allowed identifying putative transcription factor binding sites (TFBS) at SNP rs9332978 of *CYP4A11* and SNP rs3093098 of *CYP4F2*. In particular, 27 and 7 TFBSs were identified to possess the potential impact on the gene expression through a binding site located at SNP rs9332978 in the proximal promoter of *CYP4A11*, as reported by the TRANSFAC database and rSNPBase, respectively. As can be seen from Table 6 (detailed information on all transcription factor binding sites identified is listed in Supplementary Tables 1 and 2), polymorphism rs3890011 of *CYP4A11* has the regulatory potential and an experimentally proven eQTL (i.e., locus controlling transcript levels of the gene). Moreover, SNPs rs3093098 and rs1558139 of *CYP4F2* fall into RNA binding protein-mediated regulation sites.

4. Discussion

4.1. Variation in the CYP4A11 Gene and CAD Susceptibility. *CYP4A11* and *CYP4F2* are highly polymorphic genes which

Table 3: Genotype frequencies for SNPs of the *CYP4A11* and *CYP4F2* genes in patients with CAD and healthy controls stratified by gender.

Gene, polymorphism	Genotype	Males, n (%)[1]					Females, n (%)[1]				
		Controls, $n = 404$	CAD patients, $n = 401$	P value	Q value	$_{adj}$OR (95% CI)[2]	Controls, $n = 282$	CAD patients, $n = 236$	P value	Q value	$_{adj}$OR (95% CI)[2]
CYP4A11, C>G (rs3890011)	C/C	228 (56.4)	224 (55.9)			1.00	176 (62.4)	114 (48.3)			1.00
	C/G	144 (35.6)	144 (35.9)	0.98	0.98	1.01 (0.76–1.37)	92 (32.6)	100 (42.4)	0.004	0.01	1.62 (1.14–2.39)
	G/G	32 (7.9)	33 (8.2)			1.04 (0.61–1.77)	14 (5.0)	22 (9.3)			2.66 (1.29–5.46)
CYP4A11, T>C (rs9332978)	T/T	313 (77.5)	297 (74.1)			1.00	229 (81.2)	162 (69.2)			1.00
	T/C	86 (21.3)	96 (23.9)	0.45	0.98	1.18 (0.85–1.62)	48 (17.0)	62 (26.5)	0.004	0.01	1.85 (1.21–2.82)
	C/C	5 (1.2)	8 (2.0)			1.69 (0.52–5.24)	5 (1.8)	10 (4.3)			3.09 (1.02–9.35)
CYP4A11, A>G (rs9333029)	A/A	300 (74.4)	302 (75.3)			1.00	226 (80.4)	175 (74.2)			1.00
	A/G	103 (25.6)	99 (24.7)	0.77	0.98	0.98 (0.62–1.47)	55 (19.6)	61 (25.8)	0.12	0.20	1.44 (0.89–2.76)
	G/G	0 (0.0)	0 (0.0)			—	0 (0.0)	0 (0.0)			—
CYP4F2, A>G (rs3093098)	A/A	277 (68.6)	278 (69.3)			1.00	202 (71.6)	170 (72.0)			1.00
	A/G	112 (27.7)	115 (28.7)	0.33	0.98	1.02 (0.74–1.39)	74 (26.2)	57 (24.2)	0.54	0.54	0.93 (0.61–1.84)
	G/G	15 (3.7)	8 (2.0)			0.55 (0.24–1.35)	6 (2.1)	9 (3.8)			1.72 (0.61–5.97)
CYP4F2, G>A (rs1558139)	G/G	124 (30.7)	124 (30.9)			1.00	76 (27.0)	68 (28.8)			1.00
	G/A	194 (48.0)	188 (46.9)	0.95	0.98	0.96 (0.72–1.38)	142 (50.4)	124 (52.5)	0.46	0.54	1.02 (0.64–1.85)
	A/A	86 (21.3)	89 (22.2)			1.04 (0.72–1.56)	64 (22.7)	44 (18.6)			0.77 (0.44–1.34)

[1]Absolute number and percentage of individuals with particular genotype. [2]Odds ratio with 95% confidence intervals adjusted for age, BMI, hypertension, diabetes, and smoking.

Table 4: Epistatic interactions between the *CYP4A11* and *CYP4F2* genes in CAD (gene-gene interactions are evaluated by SNPassoc package for R [31]).

SNPs	Genetic models	*CYP4A11* (rs3890011)	*CYP4A11* (rs9332978)	*CYP4A11* (rs9333029)	*CYP4F2* (rs3093098)	*CYP4F2* (rs1558139)
CYP4A11 (rs3890011)	Codominant	0.086	0.459	0.725	0.598	0.435
	Dominant	**0.035**	0.712	0.450	0.319	0.533
	Recessive	0.180	0.159	—	0.986	0.594
	Overdominant	0.152	0.879	0.168	0.899	0.844
CYP4A11 (rs9332978)	Codominant	0.933	**0.008**	0.396	0.194	0.226
	Dominant	0.768	**0.004***	0.885	0.156	0.512
	Recessive	0.608	0.063	—	—	**0.025**
	Overdominant	0.824	**0.019**	0.935	0.307	0.686
CYP4A11 (rs9333029)	Codominant	0.477	0.371	0.433	0.902	0.216
	Dominant	0.534	0.398	0.433	0.835	0.098
	Recessive	—	—	—	—	—
	Overdominant	0.899	0.441	0.433	0.743	0.134
CYP4F2 (rs3093098)	Codominant	0.849	0.837	0.866	0.894	0.096
	Dominant	0.627	0.592	0.711	0.775	0.152
	Recessive	0.675	0.703	—	0.661	—
	Overdominant	0.808	0.718	0.838	0.897	**0.047**
CYP4F2 (rs1558139)	Codominant	0.890	0.923	0.918	0.806	0.912
	Dominant	0.715	0.807	0.718	0.668	0.733
	Recessive	0.701	0.764	—	0.686	0.717
	Overdominant	0.998	0.913	0.940	0.991	0.989

The upper part of the matrix contains the *P* values for epistatic interactions evaluated by log-likelihood ratio (LRT) test. The diagonal contains the *P* values from LRT for the crude effect of each SNP. The lower triangle contains the *P* values from LRT comparing the two-SNP additive likelihood to the best of the single-SNP models. Bolded are statistically significant *P* values for SNP-SNP interactions (∗ most significant *P* value for a particular model). *P* values are adjusted for age and gender.

Table 5: Estimated haplotype frequencies of *CYP4A11* in CAD patients and controls.

Haplotypes[1]		Controls	CAD patients	OR (95% CI)[2]	*P* value
SNPs C>G (rs3890011), T>C (rs9332978), and A>G (rs9333029) of *CYP4A11*					
H1	C-T-A	0.7549	0.7158	1.00	—
H2	G-C-A	0.1060	0.1433	1.41 (1.12–1.78)	**0.0036**
H3	G-T-G	0.1087	0.1146	1.15 (0.88–1.50)	0.30
H4	G-T-A	0.0207	0.0135	0.67 (0.37–1.22)	0.19
Global haplotype association *P* value: **0.021**					
SNPs A>G (rs3093098) and G>A (rs1558139) of *CYP4F2*					
H1	A-A	0.4636	0.4525	1.00	—
H2	A-G	0.3703	0.3858	1.05 (0.91–1.27)	0.49
H3	G-G	0.1662	0.1605	0.97 (0.78–1.24)	0.85
Global haplotype association *P* value: 0.72					

[1]Rare haplotypes with frequency < 0.01 are not shown. [2]Odds ratio with 95% confidence intervals adjusted for age, gender, BMI, diabetes, and hypertension. Bolded is statistically significant *P* value.

became attractive candidates for association studies of cardiovascular diseases. A number of these studies have been done in Asian populations, and no studies investigated the contribution of the genes to coronary artery disease susceptibility in Europeans. In addition, the results of these studies were variable and sometimes contradictory, thus justifying the need for further investigations of the relationship between *CYP4A11* and *CYP4F2* gene polymorphisms and CAD risk in independent racial and ethnic groups.

SNP T8590C (rs1126742) is the most extensively investigated polymorphism in the *CYP4A11* gene that has been shown to be associated with the level of blood pressure and hypertension susceptibility [17, 36] as well as with endothelial dysfunction in the coronary arteries in patients with

Table 6: Bioinformatic analysis for the regulatory potential of the studied SNPs.

SNP	Allele	Location	SNP Function Prediction (FuncPred)[1]			Regulatory annotations on SNPs (rSNPBase)[2]							Transcription factors potentially related with SNP	
			TFBS	miRNA	Regulatory potential	rSNP	LD-proxy of rSNP ($r^2 > 0.8$)	Proximal regulation	Distal regulation	miRNA regulation	RNA binding protein- mediated regulation	eQTL	TRANSFAC database	rSNPBase
rs3890011	C/G	Intron	No	No	Yes	No	Yes	No	No	No	No	Yes	—	—
rs9332978	T/C	Promoter	Yes	No	No	Yes	Yes	Yes	No	No	No	No	AIRE, ATF6, CDPCR3, CEBPA, CEBPDELTA, CEBPGAMMA, CEBP, CRX, FAC1, GRE, IPF1, MYOGNF1, OCT1, OCT4, OCT, PAX3, PAX6, PAX8, PLZF, POU3F2, PPARG, S8, SP3, SREBP1, SREBP, TAXCREB, ZF5	FOXA1, HNF4A, ARID3A, CEBPB
rs9333029	A/G	Intron	No	No	No	No	Yes	No	No	No	No	Yes	—	—
rs3093098	A/G	Intron	Yes	No	No	Yes	Yes	Yes	Yes	No	Yes	No	AP4, CACD, DR4, ETF, GABP, GRE, HAND1E47, HIC1, MYOGNF1, PAX3, PLZF, PPARA, RFX, SP1, SP3, SPZ1, SZF11, TAXCREB, TBX5, TEL2, VDR, WT1, ZF5	Pol2
rs1558139	G/A	Intron	No	No	Yes	Yes	Yes	Yes	No	No	Yes	Yes	—	—

[1]Data predicted by the SNP Function Prediction tool, National Institute of Environmental Health Sciences (https://snpinfo.niehs.nih.gov/snpinfo/). TFBS: transcription factor binding site; ND: no data. [2]Data obtained at rSNPBase, a database of curated regulatory SNPs (http://rsnp.psych.ac.cn). rSNP, rSNPBase identified regulatory SNPs; LD-proxy of rSNP ($r^2 > 0.8$), SNP in strong LD with rSNPs; proximal regulation, SNP involved in proximal transcriptional regulation; distal regulation, SNP involved in distal transcriptional regulation; miRNA regulation, SNP within mature miRNA; RNA binding protein mediated regulation, SNP involved in RNA binding protein-mediated post-transcriptional regulation; eQTL, SNP with experimental eQTL evidence. TRANSFAC is the database on potential transcription factor recognition sites (BIOBASE Corporation, Wolfenbuettel, Germany).

CAD in Europeans [37]. Another SNP rs3890011 in intron of *CYP4A11* was also a subject of investigations in cardiovascular disorders in various populations of the world. In particular, a study in Chinese population did not identify the link of the rs3890011 polymorphism with blood pressure variation and hypertension susceptibility [38]. However, Fu with coworkers have revealed a significant association between SNP rs3890011 and the risk of CAD, but the association occurred only in males [24]. This study did not observe an association between a promoter polymorphism rs9332978 of *CYP4A11* and CAD risk. The authors also reported that the functional effect of the rs3890011 polymorphism is related to neighboring functional SNPs (possibly rs9332978), potentially affecting the structure and/or catalytic activity of the enzyme [24].

The present study was designed to investigate whether common SNPs rs3890011, rs1126742, rs9332978, and rs9333029 of *CYP4A11* and rs3093098 and rs1558139 *CYP4F2* are associated with the risk of CAD in Russian population. The present study has revealed that polymorphisms rs3890011 and rs9332978 are both associated with the risk of CAD; however, the association occurred only in females. Interestingly, these SNPs represent a part of common functional haplotype of *CYP4A11*, as it has been demonstrated by our and some other studies [23, 39]. In particular, haplotype G-G-T (rs9332978, rs3890011, rs1126742) was found to be moderately associated with the CAD risk in Chinese Han population, whereas SNP rs9332978 alone did not show a significant association with disease risk [39]. We found that the high CAD risk haplotype G-C-A (rs3890011, rs9332978, and rs9333029; risk alleles are underlined) in our patients coincides with a part of haplotype G-C-T (rs3890011, rs9332978, rs1126742) reported as the disease risk haplotype in the study of Fu with coworkers [39]. It seems reasonable to say that the functional effect of this haplotype on enzyme's activity could be related with variation in the proximal promoter of *CYP4A11*, that is, with SNP rs9332978. At least, this suggestion may be supported by the study in Japanese population that reported a relationship between the SNP rs9332978, expression of *CYP4A11*, and the hypertension risk [40]. Sugimoto et al. [40] observed that the −845GG genotype is associated with lower promoter activity when compared with −845AA genotype, and allele −845G was positively correlated to hypertension susceptibility. Additionally, the authors [40] supposed that the rs9332978 polymorphism falls into DNA binding site for an unidentified protein and/or potential transcription factor. The bioinformatic analysis for the regulatory potential of investigated polymorphisms allowed us identifying putative transcription factor binding sites at SNP rs9332978 of the *CYP4A11* gene. Interestingly, it was found that the DNA binding site located at this SNP may be regulated by numerous transcription factors representing the pathways being involved into the molecular mechanisms of coronary atherosclerosis. In particular, CEBPB is an important transcription factor binding to promoter regions of multiple inflammatory response genes, synergistically upregulating and sustaining their expression after inflammatory stimulation [41, 42]. HNF4A (hepatocyte nuclear factor 4 alpha) is a transcriptionally controlled transcription factor that binds to DNA sites required for the transcription of alpha 1-antitrypsin, apolipoprotein CIII, transthyretin genes, and HNF1-alpha (data obtained from the UniProtKB). In accordance with Gene Ontology descriptions (GO, http://www.geneontology.org), HNF4A has the potential to coregulate genes involved in blood coagulation, lipid homeostasis (positive regulation of cholesterol homeostasis), cell proliferation, and other important biological processes which play a role in the pathogenesis of CAD. Altogether, these data make SNP rs9332978 of *CYP4A11* a subject of great interest for further research of molecular pathogenesis of coronary artery disease.

4.2. Possible Role of SNP rs9332978 of CYP4A11 in the Pathogenesis of CAD. Literature data on a relationship between decreased 20-HETEs and pathogenesis of cardiovascular disease are extremely limited. Nevertheless, based on available publications, the UniProtKB database and Gene Ontology descriptions, we proposed the mechanisms by which loss-of-function polymorphisms rs9332978 and rs1126742 of *CYP4A11* are associated with reduced 20-HETE synthase activity and increased risk of coronary artery disease (Figure 1). 20-HETE is a potent endogenous agonist of PPARα (peroxisome proliferator-activated receptor alpha) [43]. The peroxisome proliferator-activated receptors are ligand-activated transcription factors belonging to the nuclear hormone receptor superfamily. Bioinformatic analysis allowed us identifying a binding site at SNP rs9332978 for transcription factor PPARG as a potent coregulator of *CYP4A11* gene expression (Table 6). Importantly, PPARα is a major regulator of intra- and extracellular lipid metabolism [44]. PPARα and PPARG serve as physiological sensors of lipid levels whereby dietary fatty acids can modulate lipid homeostasis [45]. PPARα activation can increase the levels of HDL-C through increasing concentration of apo A-I and A-II and through stimulating the reverse cholesterol transport pathway [46]. Hence, a deficiency of 20-HETE may reduce the hypolipidemic effects of PPARα, leading to decreased HDL-C and hypercholesterolemia. Moreover, activation of PPARα can exert anti-inflammatory effects, suppressing the acute-phase response and decreasing the release of proinflammatory cytokines [47]. In addition, 20-HETE was found to be a potent, dose-dependent inhibitor of platelet aggregation and biosynthesis of thromboxane A2, most probably by antagonizing the prostaglandin H2/thromboxane A2 (PGH2/TXA2) receptor [48], thereby leading to increased formation of thrombi.

However, when interpreting genotype-phenotype correlation, it is important to keep in mind that it is difficult to predict the consequences of a change in the activity of the CYP4A11 enzyme, especially taking the dual role of 20-HETE in vascular and renal homeostasis into consideration [12, 15, 49]. Undoubtedly, this means that our suggestions on the involvement of these SNPs in CAD pathogenesis require experimental confirmation. In addition, we cannot rule out the possibility of complex interactions between different polymorphic genes and their comprehensive contribution to the levels of 20-HETE in the heart and coronary

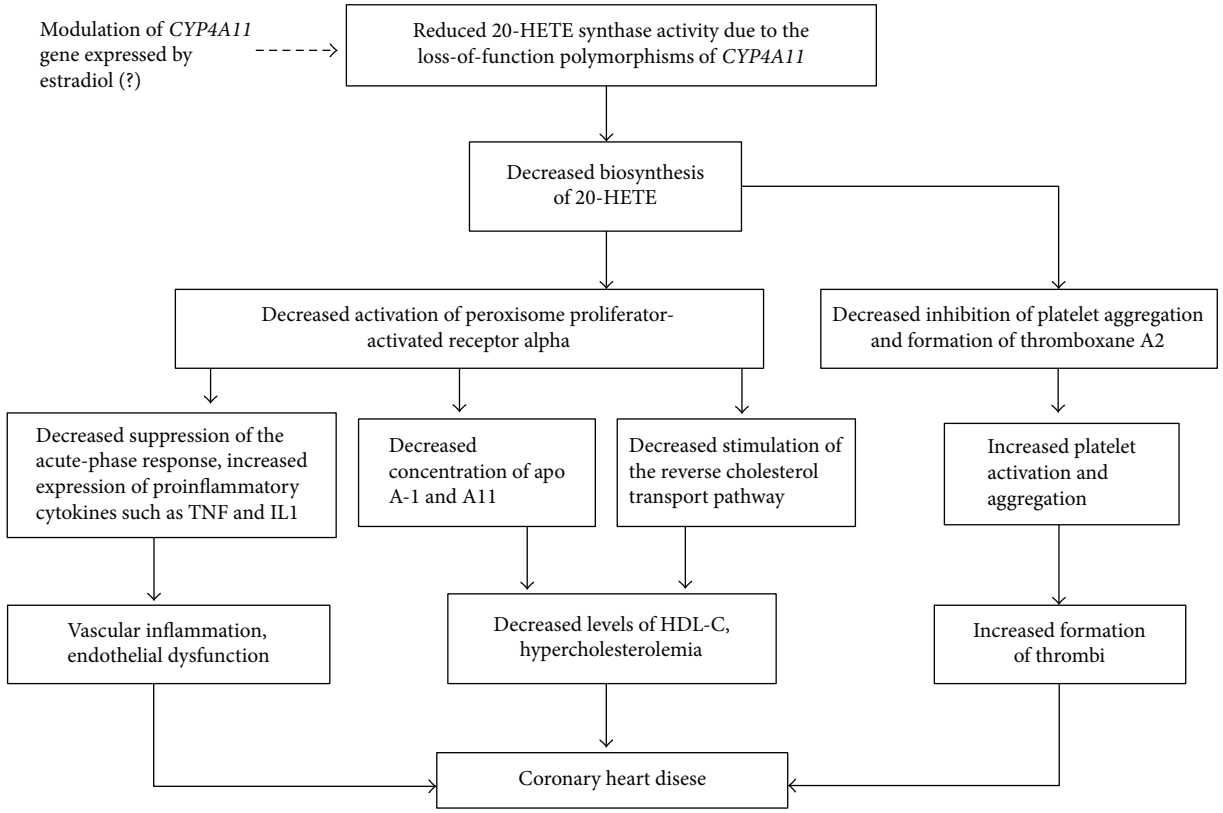

Figure 1: Proposed mechanisms by which the loss-of-function polymorphisms of the *CYP4A11* gene are involved in the pathogenesis of coronary heart disease (see the text for details).

arteries in patients with CAD. In this context, our interesting finding was an epistatic interaction between SNP rs9332978 of *CYP4A11* and SNP rs1558139 of *CYP4F2* (this polymorphism was associated with the risk of essential hypertension in Japanese population [19]) in CAD patients suggesting that gene-gene interactions could be involved into the regulation of 20-HETE metabolism and jointly contribute to the development of coronary artery disease.

4.3. Gender-Specific Relationship between SNP rs9332978 of CYP4A11 and Risk of CAD. Interestingly, the effects of rs9332978 of the *CYP4A11* gene on CAD risk in our study were evident only in women. This finding supports the hypothesis that the interaction of sex hormones with expression cytochrome P450 enzymes involved in the 20-HETE metabolism could have a role in well-established sex dimorphism in the risk of cardiovascular disease [50]. It can be assumed that association between rs9332978 of *CYP4A11* and CAD susceptibility in women may be related with the inhibitory effect of estradiol on the *CYP4A11* expression in the carriers of the variant genotypes, leading to reduced synthesis of 20-HETE and increasing the disease risk through the mechanisms described above. Biosynthesis of 20-HETE is regulated in age- and sex-dependent manner [15, 49, 51], and CYP4A11 itself has a catalytic activity for the metabolism of estrogens such as 17beta-estradiol and estrone [52, 53]. This means that estrogens may represent important modifiers of CYP4A11-medicated metabolism of 20-HETE. The study of White with coworkers provided evidence that polymorphism in the *CYP4A11* gene is related with disorders underlying coronary atherosclerosis, and this relationship is also sex specific [54]. In particular, polymorphism rs1126742 of *CYP4A11* was found to be associated with HDL-C and C-reactive protein in women [54]. This relationship may be explained by the effects of PPARα agonists which are known to improve lipid metabolism disorder, and this capacity appears to be modulated by estrogens [55]. White with coworkers proposed that the effect of the loss-of-function allele of *CYP4A11* on decreased formation of PPARα agonists could be magnified in women [54]. SNP rs9332978 of *CYP4A11* is located within a binding site for transcription factor FOXA1 (forkhead box A1 or hepatocyte nuclear factor 3 alpha). Interestingly, FOXA1 modulates the transcriptional activity of nuclear hormone receptors and is involved in positive regulation of intracellular estrogen receptor signaling pathway [56]. Apparently, female hormones have the potential to bind with specific sites at the promoter of *CYP4A11* and therefore could be responsible for sex-specific alterations in the expression of CYP4A11, thereby affecting the production of 20-HETE. The mechanisms whereby estrogens exert their regulatory effects on CAD through the modulation of *CYP4A11* gene expression remained to be elucidated in further studies.

There are several limitations to address in the context of the current results. First, we did not measure 20-HETE levels in the study patients, and possible alterations in the

metabolism of arachidonic acid in CAD could not be established in our study. Further studies are needed to clarify the effects of the investigated polymorphisms of *CYP4A11* on arachidonic acid metabolism and/or 20-HETE production. Second, other polymorphisms in the *CYP4A11* gene such as those located in intronic regions or distal promoter regions not investigated in this study might also be associated with disease susceptibility. Unfortunately, insufficient genotyping call rate (<70%) for polymorphism rs1126742 (T8590C) of *CYP4A11* did not allow the inclusion of this SNP into the study. In order to describe the complete haplotype structure of the *CYP4A11* gene, it is necessary to expend a spectrum of polymorphisms in future studies. Third, potential interactions of genetic polymorphisms of *CYP4A11* and *CYP4F2* with environmental conditions such as food and dietary elements were unexplored in the study, thus not allowing any conclusion to be drawn with respect to sex-specific associations between the genes and disease risk.

5. Conclusions

The present study identified that polymorphism rs9332978 of *CYP4A11* could be a novel marker of genetic susceptibility to coronary artery disease, at least in Europeans. Moreover, our study provided additional evidence that *CYP4A11* is an important susceptibility gene for coronary artery disease despite the fact that different polymorphisms of the gene showed association with disease risk in various populations. Although the molecular mechanisms underlying the development of coronary artery disease in women with the rs9332978 polymorphism of *CYP4A11* remain to be determined, the results of the present study support the hypothesis that variation in the *CYP4A11* gene is an important determinant associated with the risk of coronary artery disease in gender-specific manner. Further efforts should be made to address the function of the studied SNPs of *CYP4A11* in arachidonic acid metabolism in order to determine the effect of the polymorphisms on the production of 20-HETE in the coronary arteries in CAD patients. Nevertheless, the association between SNP rs9332978 of *CYP4A11* and the risk of coronary artery disease provides insights into the molecular basis of disease pathogenesis and suggests possible avenues in developing novel drugs for pharmacological intervention in the metabolism of 20-hydroxyeicosatetraenoic acids in patients with CVD. Further pharmacogenomic studies are needed to substantiate the contribution of CYP4A11 polymorphisms in the pathogenesis of coronary artery disease and to assess the dual role of 20-hydroxyeicosatetraenoic acids in cardiovascular homeostasis as a promising target for vascular medicine in the future.

Conflicts of Interest

The authors declared no conflict of interest.

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Supplementary Materials

Supplementary Table 1: Transcription Factor Binding Sites for SNP rs9332978 of *CYP4A11*. Supplementary Table 2: Transcription Factor Binding Sites for SNP rs9332978 of *CYP4A11*. *(Supplementary Materials)*

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Research Article

The Role of Parathyroid Hormone and Vitamin D Serum Concentrations in Patients with Cardiovascular Diseases

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25-hydroxyvitamin D (25(OH)D) plays a crucial role in human homeostasis. Its deficiency (vitamin D deficiency—VDD), being common in European population, combined with elevated concentration of parathyroid hormone (PTH), represents a vicious cycle of mechanisms leading to heart failure (HF). Despite several papers published in that field, the effect of VDD and PTH concentration on cardiovascular system remains unequivocal; thus, the aim of the study was to compare these data among HF and non-HF patients being prospectively enrolled into the study during hospital stay in the cardiology ward. Patients with HF had higher PTH concentration (85.0 ± 52.6 versus 64.5 ± 31.7, $p \leq 0.02$) compared to non-HF patients. Mean PTH values were associated with the clinical status expressed by the New York Heart Association class (NYHA class) ("0"—66.04, "I"—56.57, "II"—72.30, "III"—85.59, and "IV"—144.37 pg/ml, $p \leq 0.00004$). Interestingly, neither 25(OH)D (31.5 versus 29.7 ng/ml, $p \leq$ ns) nor phosphorus (P) (1.23 versus 1.18 mmol/l, $p \leq$ ns) nor total calcium (Ca^{2+}) concentration (2.33 versus 2.37 mmol/l, $p \leq$ ns) differed among the groups. Reassuming PTH serum concentration in contrary to 25(OH)D, P and Ca^{2+} are significantly raised among the patients with HF and shows significant relationship with the clinical status expressed by the NYHA class.

1. Introduction

A lipid-soluble vitamin D has huge impact on human homeostasis [1]. It is produced during sun exposure and delivered via nutrients (including oily fish and egg yolks), as well as dietary supplements [2]. Vegetable sources provide ergocalciferol (D2), and animal sources provide cholecalciferol (D3). Ergocalciferol and cholecalciferol represent similar metabolic flux being transported to the liver by a vitamin D-binding protein (DBP) and then submitted for hydroxylation at the C25 position by specific hydroxylase. 25-hydroxyvitamin D is the main circulating form of vitamin D [3]. Low serum 25-hydroxyvitamin D (25(OH)D) concentrations are a predominant cause of a negative calcium balance and secondary hyperparathyroidism (SHPT).

SHPT is a frequently occurring entity in patients suffering from heart failure (HF). Since parathormone (PTH) is an important regulator of bone and mineral metabolism, its biological activity is regulated by oscillations in serum calcium concentrations with subsequent reactions on several pathways. The main purpose of those mechanisms is to raise serum concentration of calcium due to enhancement of renal and intestinal reabsorption and osteoclast activation leading to bone resorption. PTH rise in HF clinical setting is related to the impairment of acid-based homeostasis, diuretic-induced calcium loss, and vitamin D deficiency (VDD). Moreover, increased PTH concentrations are related to the impaired hemodynamic state expressed by reduced stroke volume and increased pulmonary capillary wedge pressure being commonly observed in HF patients during right heart catheterization [4].

It is worth to emphasize that 1,25-dihydroxyvitamin D and 25-hydroxyvitamin D have, beyond their important role in calcium and phosphorus metabolism, diverse effects on

the immune system [5]. Vitamin D has been reported to protect tissue from myocardial and cerebral ischemia [6, 7]. Many evidence suggests a critical role of vitamin D in blood pressure (BP) regulation; 25(OH)D deficiency stimulates renin-angiotensin-aldosterone system (RAAS) and promotes hypertension [8]. Worldwide studies have shown an association between VDD and tissue inflammation, endothelial dysfunction, arterial stiffness, atherosclerotic plaque formation (coronary heart disease [CAD], peripheral arterial disease), left ventricular hypertrophy, atrial fibrillation, metabolic syndrome, and diabetes. VDD is also associated with an increased risk of death, heart failure, and myocardial infarction (MI) in postmenopausal women, as well as with an increased risk of stroke, MI, and sudden cardiac death or/and death related to other heart diseases among diabetic patients with chronic kidney disease (CKD) [9–18].

VDD is common in European population due to indoor lifestyle and sun avoidance. In Central Europe (CE) effective vitamin D synthesis occurs from April to October only if at least twenty percent of skin is exposed to sunlight for the minimal time of fifteen minutes daily. Sunscreens limit the synthesis in 90%; additionally, VDD is frequently related to smoking, obesity, renal and liver failure, inappropriate eating habits, and drug intake. VDD in obese patients may result from low outdoor activity, inappropriate eating habits, and sequestration of fat-soluble cholecalciferol in adipose tissue [1, 19–22].

VDD combined with raised PTH represents a vicious cycle of mechanisms on one hand leading to HF and in the other hand worsening the prognosis of HF. It is worth to mention that myocardial fibrosis and hypertrophy, excessive adrenergic stimulation, calcium cell overload, oxidative stress enhancement, and FGF-23 formation have huge impact on impaired survival [23].

2. Aim of the Study

The aim of the study is to assess and compare the serum intact PTH and 25(OH)D in HF and non-HF patients being prospectively enrolled into the study during hospital stay in the cardiology ward.

3. Material and Methods

3.1. General Information. This cross-sectional study was designed to asses serum intact PTH, 25(OH)D, total calcium (Ca^{2+}), and phosphorus (P) concentrations in addition to routine laboratory examinations and medical history obtained on admission in 140 consecutive patients hospitalized in a clinical ward of cardiology form November 15 to December 31, 2014. The study complies with the Declaration of Helsinki and was approved by the local ethics committee (number of approval KNW/0022/KB1/40/I/12/13), and all patients gave informed consent prior to enrollment.

3.2. Inclusion and Exclusion Criteria. Inclusion criteria were as follows: impaired systolic function with left ventricular ejection fraction (LVEF) below 50% according to the Simpson method in the echocardiographic examination or patient diagnosed and/or treated due to HF prior to admission, CAD diagnosed on the base of invasive/noninvasive testing performed before admission, or if patient was previously submitted for coronary intervention. Exclusion criteria were as follows: lack of consent, under eighteen years of age, vitamin D supplementation, or CKD stage four or higher. Invasive coronary angiography (CAG) was found positive for CAD if at least one coronary artery of at least two millimeters was significantly stenosed.

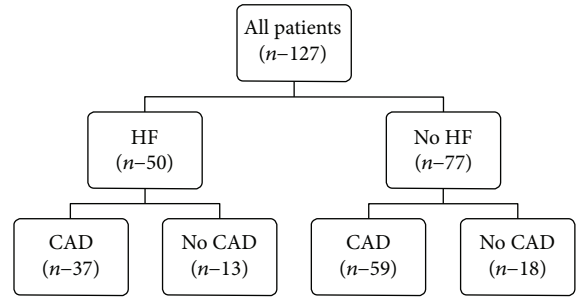

Figure 1: Subgroups of patients. HF: heart failure; No HF: heart failure absent; CAD: coronary artery disease present; No CAD: coronary artery disease absent.

3.3. Group Formation. Finally, 127 patients were enrolled into the study. Demographic and clinical data were recorded on admission to the hospital. Patients were requested to characterize their daily profile of sun exposure during the last four months as "above" or "below" seven hours a week (one hour per day).

Patients were post hoc divided into four groups depending on CAD and/or HF occurrence. HF patients were divided into two groups according to the etiology of HF. Ischemic is defined as impaired systolic function with significant narrowings in coronary tree (IHF) and nonischemic (N-IHF) is defined as impaired systolic function without significant narrowings in coronary tree.

Patients with CAD but without HF were depicted as (CAD N-HF) and formed the third group. Patients not fulfilling inclusion criteria of CAD nor HF were depicted as (N-HF&N-CAD) and referred to the fourth group.

3.4. Diagnostic Techniques and Further Definitions. Serum concentrations of intact PTH and 25(OH)D were assessed using the immunoenzymatic assay technique with the Micro-Vue Intact PTH ELISA and 25(OH)-Vitamin D Xpress ELISA Kit, respectively. Serum P and Ca^{2+} concentration was assessed using the enzymatic method as described by Spinreact S.A., Spain, and the colorimetric Arsenazo III method as described by Biomaxima S.A., Poland.

Arterial hypertension was diagnosed if systolic and diastolic blood pressures exceeded 140 and/or 90 mmHg or if the patient already received antihypertensive treatment. Diabetes was diagnosed according to the American Diabetes Association (ADA) criteria or in patients already receiving antidiabetic medication. The estimated glomerular filtration

Table 1: Patient characteristic divided depending on heart failure occurrence.

	HF present (n – 50)	HF absent (n – 77)	p
Age	61.5 (57.0–74.0)	65.3 (57.0–74.0)	ns
Male (%)	36 (72)	43 (56)	ns
BMI	28.1 (24.3–30.9)	28.5 (25.4–31.2)	ns
Hypertension	38 (76)	61 (79)	ns
Dyslipidemia	32 (64)	38 (49)	ns
Diabetes	15 (30)	27 (35)	ns
Atrial fibrillation	14 (28)	8 (10)	≤0.02
Urgent admission	22 (44)	34 (44)	ns
Sun exposure >7 h/week	26 (52)	50 (65)	ns
Coronary artery disease	39 (78)	59 (77)	ns
EDV (ml)	146.2 ± 55.9	86.3 ± 20.9	≤0.001
LVEF (%)	28.7 ± 11.7	55.9 ± 4.7	≤0.001
ICD	12 (24)	1 (1)	≤0.001
CRT	2 (4)	0 (0)	ns
DDDR	2 (4)	13 (17)	≤0.06
VVIR	2 (4)	5 (5)	ns
OHT	2 (4)	0 (0)	ns
DEATH (during hospitalization)	2 (4)	2 (3)	ns

HF: heart failure; BMI: body mass index; >7 h/week: more than seven hours per week; EDV: end-diastolic volume; LVEF: left ventricular ejection fraction; ICD: implantable cardioverter-defibrillator; CRT: cardiac resynchronization therapy; DDDR: dual chamber permanent pacemaker; VVIR: single chamber permanent pacemaker; OHT: orthotopic heart transplantation.

Table 2: Clinical and laboratory findings depending on heart failure occurrence.

	HF present (n – 50)	HF absent (n – 77)	p
eGFR (ml/min/1.73^2)	76.1 ± 26.1	82.9 ± 22.6	≤0.09
Creatinine (mmol/l)	90.0 ± 30.9	81.1 ± 48.5	≤0.01
ALT (IU/l)	30.5 ± 21.4	31.2 ± 22.6	ns
AST (IU/l)	35.5 ± 34.2	27.1 ± 14.3	ns
HGB (g/dl)	13.5 ± 1.5	13.6 ± 1.5	ns
HCT (%)	41.3 ± 6.9	41.8 ± 6.0	ns
RBC (10^6/ul)	4.47 ± 0.6	4.46 ± 0.5	ns
PLT (10^3/ul)	203.8 ± 66.6	210.0 ± 53.8	ns
WBC (10^3/ul)	7.5 ± 2.6	6.8 ± 2.2	ns
25(OH)D (ng/ml)	31.5 ± 8.94	29.7 ± 10.2	ns
Ca^{2+} (mmol/l)	2.33 ± 0.13	2.37 ± 0.1	ns
P (mmol/l)	1.23 ± 0.17	1.18 ± 0.2	ns
PTH (pg/ml)	85.0 ± 52.6	64.5 ± 31.7	≤0.02

HF: heart failure; eGFR: estimated glomerular filtration rate; ALT: alanine aminotransferase; AST: aspartate aminotransferase; HGB: hemoglobin; HCT: hematocrit; RBC: red blood cells; PLT: platelets; WBC: white blood cells; 25(OH)D: 25-hydroxyvitamin D; Ca^{2+}: total calcium; P: phosphorus; PTH: parathyroid hormone.

rate (eGFR) was calculated on the basis of the Modification of Diet in Renal Disease (MDRD) formula.

For the design of the study and patient characteristic, please refer to Figure 1 and Table 1.

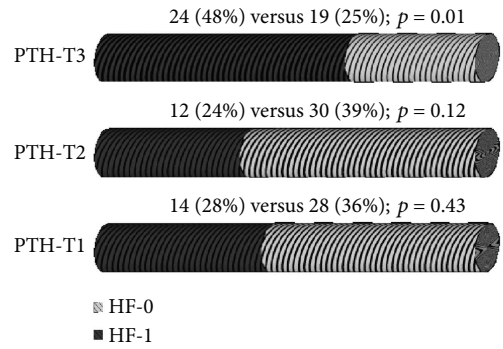

Figure 2: Incidence of HF divided according to tertiles of PTH serum concentration. HF: heart failure; PTH: parathyroid hormone; T: tertile.

4. Statistical Analysis

Distributions of the examined parameters were analyzed using the Shapiro-Wilk test. Values were presented as the mean 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 normal distribution were compared using student t-test. Post hoc analyses between the groups were performed by the use of factorial ANOVA test with the Bonferroni correction. Variables with abnormal distribution were compared using the Mann–Whitney U test. Categorical variables of abnormal distribution were compared using

Table 3: Laboratory findings among all examined groups.

Parameter	N-IHF $n-13$	HF IHF $n-37$	p	IHF $n-37$	CAD CAD N-HF $n-59$	p	N-IHF $n-13$	No CAD N-HF & N-CAD $n-18$	p
	Versus			Versus			Versus		
25(OH)D (ng/ml)	29.25	32.29	ns	32.29	29.47	ns	29.25	31.00	ns
Ca^{2+} (mmol/l)	2.36	2.32	ns	2.32	2.37	ns	2.36	2.37	ns
P (mmol/l)	1.21	1.23	ns	1.23	1.18	ns	1.21	1.18	ns
PTH (pg/ml)	85.26	84.91	ns	84.91	64.24	≤0.06	85.26	62.67	ns
eGFR (ml/min/1.73^2)	67.38	79.14	ns	79.14	81.15	ns	67.38	87.88	ns
Creatinine (mmol/l)	96.00	87.89	ns	87.89	84.11	ns	96.00	71.06	ns
ALT (IU/l)	35.23	28.84	ns	28.84	29.73	ns	35.23	37.47	ns
AST (IU/l)	38.69	34.41	ns	34.41	26.32	ns	38.69	30.53	ns
HGB (g/dl)	13.52	13.48	ns	13.48	13.51	ns	13.52	13.80	ns
HCT (%)	42.44	40.86	ns	40.86	41.36	ns	42.44	42.97	ns
RBC (10^6/ul)	4.61	4.42	ns	4.42	4.43	ns	4.61	4.53	ns
PLT (10^3/ul)	169.62	215.78	≤0.04	215.78	212.29	ns	169.62	202.88	ns
WBC (10^3/ul)	6.68	7.79	ns	7.79	7.04	≤0.04	6.68	5.87	ns

N-IHF: nonischemic heart failure; IHF: ischemic heart failure; CAD N-HF: coronary artery disease without heart failure; N-HF&N-CAD: without coronary artery disease nor heart failure; 25(OH)D: 25-hydroxyvitamin D; Ca^{2+}: total calcium; P: phosphorus; PTH: parathyroid hormone; eGFR: estimated glomerular filtration rate; ALT: alanine aminotransferase; AST: aspartate aminotransferase; HGB: hemoglobin; HCT: hematocrit; RBC: red blood cells; PLT: platelets; WBC: white blood cells.

Table 4: Clinical findings among all examined groups.

n (%) Parameter	N-IHF $n-13$	HF IHF $n-37$	p	IHF $n-37$	CAD CAD N-HF $n-59$	p	N-IHF $n-13$	No CAD N-HF & N-CAD $n-18$	p
	Versus			Versus			Versus		
Age (years)	51.54	65.00	≤0.001	65.00	66.37	ns	51.54	62.82	≤0.03
BMI	25.83	28.91	ns	28.91	28.43	ns	25.83	29.02	ns
EDV (ml)	163.08	140.32	ns	140.32	87.03	≤0.001	163.08	84.82	≤0.001
LVEF (%)	24.62	30.11	ns	30.11	55.25	≤0.001	24.62	58.53	≤0.001
Male	8 (62)	28 (76)	ns	28 (76)	34 (58)	ns	8 (62)	8 (47)	ns
HT	5 (38)	33 (89)	≤0.001	33 (89)	49 (83)	ns	5 (38)	12 (71)	ns
DYSLIP	4 (31)	28 (76)	≤0.01	28 (76)	31 (53)	≤0.04	4 (31)	7 (41)	ns
Diabetes	3 (23)	12 (32)	ns	12 (32)	24 (41)	ns	3 (23)	3 (18)	ns
AF	4 (31)	10 (28)	ns	10 (28)	5 (8)	≤0.03	4 (31)	3 (18)	ns
Urgent	4 (31)	18 (49)	ns	18 (49)	28 (47)	ns	4 (31)	6 (35)	ns
SE > 7 h/w	7 (54)	19 (51)	ns	19 (51)	41 (69)	ns	7 (54)	9 (53)	ns
ICD	5 (38)	7 (19)	ns	7 (19)	0 (0)	≤0.001	5 (38)	1 (6)	ns
CRT	1 (8)	1 (3)	ns	1 (3)	0 (0)	ns	1 (8)	0 (0)	ns
DDDR	0 (0)	2 (5)	ns	2 (5)	8 (14)	ns	0 (0)	5 (29)	ns
VVIR	1 (8)	1 (3)	ns	1 (3)	2 (3)	ns	1 (8)	2 (12)	ns
OHT	2 (15)	0 (0)	ns	0 (0)	0 (0)	ns	2 (15)	0 (0)	ns
Death	0 (0)	2 (5)	ns	2 (5)	2 (3)	ns	0 (0)	0 (0)	ns

N-IHF: nonischemic heart failure; IHF: ischemic heart failure; CAD N-HF: coronary artery disease without heart failure; N-HF&N-CAD: without coronary artery disease nor heart failure; BMI: body mass index; EDV: end-diastolic volume; LVEF: left ventricular ejection fraction; HT: hypertension; DYSLIP: dyslipidemia; AF: atrial fibrillation; urgent: urgent admission; SE > 7 h/week: sun exposure exceeding seven hours per week; ICD: implantable cardioverter-defibrillator; CRT: cardiac resynchronization therapy; DDDR: dual chamber permanent pacemaker; VVIR: single chamber permanent pacemaker; OHT: orthotopic heart transplantation.

Chi-squared test with Yates correction. Quantified Spearman's rho correlation coefficients were used to assess the linear correlations between LVEF and PTH among all specified groups. Differences between the values were considered statistically significant if $P \leq 0.05$. Analyses were performed using Statistica 10 with medical package (Statsoft Inc.).

5. Results

Patients with HF had higher incidence of atrial fibrillation (AF) and were characterized by significant negative remodeling of the left ventricle expressed by lower LVEF and higher end-diastolic volume (EDV). Incidence of implantable cardioverter-defibrillator (ICD) was significantly elevated in the HF group and reached borderline significance as far as frequency of dual chamber permanent pacemaker (DDDR) was concerned. The remaining parameters depicted in Table 1 did not show statistically significant differences.

Patients with HF had deteriorated renal function expressed by significantly increased serum creatinine concentration and dropped eGFR of borderline statistical significance. PTH in HF patients was significantly elevated. There was no incidence of PTH-dependent hypercalcemia in examined group of patients. The rest of the findings are depicted in Table 2 and did not show statistical significance.

The number of patients with the PTH serum concentration values in the third tertile was significantly raised among HF versus non-HF patients—data are presented in Figure 2.

Patients in N-IHF group were younger, had lower occurrence rate of hypertension and dyslipidemia, and had significantly decreased platelet blood count compared to IHF group of patients. Patients in CAD N-HF group had decreased white blood count and EDV and increased LVEF compared to IHF group of patients with trend towards significance considering PTH serum blood concentration. Data are depicted in Tables 3 and 4.

N-IHF patients were younger, had significantly decreased LVEF, and had increased EDV compared with N-HF&N-CAD group of patients. Dyslipidemia, AF, and ICD implantation in IHF patients were observed more frequently compared to CAD N-HF group of patients. The remaining parameters did not show statistical significance among the groups—data were presented in Tables 3 and 4.

The mean PTH concentration was significantly increased in patients in worse clinical state expressed by the NYHA class; results were reported in Figure 3 and in Table 5.

PTH and LVEF showed statistically significant correlation in all patients, in HF and in IHF patients. In N-IHF patients, only borderline significance was reported. There was no significant correlation in patients without HF. Data were presented in Table 6.

PTH concentration showed statistically significant correlation with EDV, LVEF, serum creatinine concentration, eGFR, and N-terminal probrain natriuretic peptide serum concentration (NT-proBNP). Data were presented in Table 7.

PTH concentration was significantly elevated in patients treated with loop diuretics (LD), with the highest values observed in HF patients. Data was presented in Table 8.

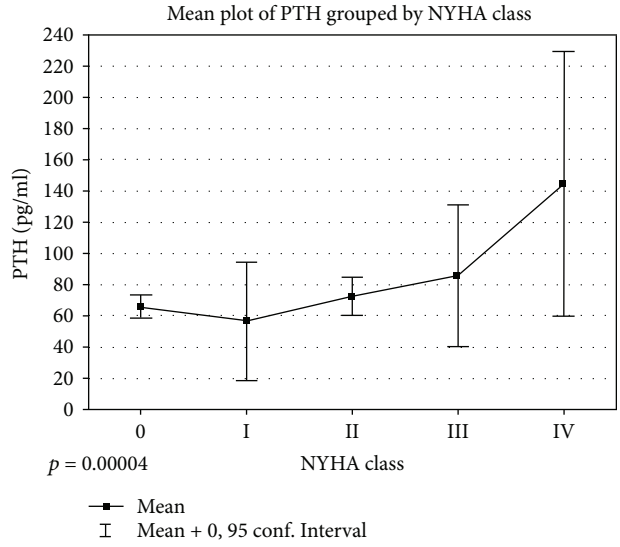

Figure 3: Mean plot of PTH grouped by NYHA class. NYHA: New York Heart Association; PTH: parathyroid hormone.

Neither PTH nor 25(OH)D concentrations were dependent on self-reported sun exposure in HF and N-HF patients.

6. Discussion

The presented study, according to our best knowledge, is the first to compare vitamin D, PTH, calcium, and phosphorus serum concentration in cardiology ward patients with and without HF taking into account its etiology (ischemic versus nonischemic). There were no differences in vitamin D concentration among the groups although it oscillated between lower normal limits.

Self-reported sunlight exposure is a novel approach. The cut-off value was set up on one hour per day what we believe provides optimal vitamin D synthesis.

Patients with HF had higher rates of PTH, irrespectively of its etiology what remains in line with previously published studies [23–27]. PTH concentration in HF correlates with disease severity, expressed by decreased LVEF, increased EDV, and functional NYHA class. It also correlates with lower eGFR and higher NT-proBNP concentration what is coherent with previous reports [28–31].

SHPT, being an exclusive cause of elevated PTH concentration in our study, is a common state in HF patients originating from several overlapping mechanisms. Increased aldosterone activity (compensatory activation of RAAS) and loop diuretic intake promote excessive urine calcium and magnesium loss. Renal dysfunction, due to chronic hypoperfusion, increases P retention and 25(OH)D activation disturbance (decreased activity of renal 1-alpha-hydroxylase induced by uremia), which stimulates PTH secretion. This is coherent with our findings: LD intake affected PTH concentration in all participants; its predominant influence was observed in HF patients.

25(OH)D insufficiency, common in HF, is also a cause of SHPT [29, 30]. There are reports showing independent

Table 5: PTH values (pg/ml) divided according to NYHA class.

NYHA	*N*	Mean	Median	Lower quartile	Upper quartile	SD	*p*
All	127	72.54	60.93	42.61	88.80	42.26	0.00004**
0	73	66.04	57.49	42.61	82.24	31.29	0.000001*
I	7	56.57	47.11	25.00	65.81	41.06	0.000043*
II	33	72.30	72.01	48.55	89.97	34.15	0.000017*
III	7	85.59	89.86	38.44	112.93	49.19	0.005274*
IV	7	144.37	117.97	62.70	203.98	91.66	—

**All effects tested by ANOVA; *Post hoc comparison versus NYHA IV by the use of Fischer LSD test. NYHA: New York Heart Association; SD: standard deviation.

Table 6: Correlation between PTH concentration and LVEF in different groups of patients.

Group of patients	Spearman Rho coefficients	*p*
All (*n* – 127)	−0.3479	≤0.00006
HF (*n* – 50)	−0.4168	≤0.0026
N-HF (*n* – 77)	0.0480	ns
N-IHF (*n* – 13)	−0.5409	≤0.0563
IHF (5)	−0.3883	≤0.0176

HF: heart failure; N-HF: without heart failure; N-IHF: nonischemic heart failure; IHF: ischemic heart failure.

Table 7: Correlation between PTH and subsequent parameters.

All patients	Spearman Rho coefficients	*p*
EDV	0.269	0.002205
LVEF	−0.290	0.000953
eGFR	−0.375	0.000014
creatinine	0.407	0.000002
NT-proBNP	0.449	0.016441

EDV: end diastolic volume; LVEF: left ventricle ejection fraction; eGFR: estimated glomerular filtration rate; NT-proBNP: N-terminal probrain natriuretic peptide.

Table 8: PTH concentration (pg/ml, mean ± SD) according to loop diuretic intake and HF occurrence.

Loop diuretic intake	(+)	(−)	*p*
All (*n* = 127)	(*n* = 46) 99.09 ± 53.78	(*n* = 81) 58.26 ± 26.89	0.00000015
HF (*n* = 50)	(*n* = 28) 105.77 ± 58.45	(*n* = 22) 60.15 ± 30.49	0.000452
N-HF (*n* = 77)	(*n* = 14) 84.06 ± 39.48	(*n* = 63) 57.63 ± 25.87	0.008666

HF patients: heart failure patients; N-HF patients: patients without heart failure. Loop diuretics: furosemide and torasemide.

association between higher PTH level and an exacerbated risk of HF in the general population [28]. SHPT increases bone turnover and the risk of fractures; it is also associated with cardiovascular calcifications and higher mortality risk. Treatment of severe SHPT in CKD is based on control of dietary phosphate intake, gastrointestinal absorption (chelating agents), vitamin D supplementation, and calcimimetic drug intake [32]. It is not fully elucidated whether treatment of SHPT in HF patients has beneficial effect on prognosis; thus, we think further studies are required in this field.

Polish Society of Endocrinology recommends at least 15 minutes of sun exposure of 15–20% of body surface daily, from April to October, in order to provide optimal 25(OH)D serum concentration [33]. In our analysis, the assumed criterion of optimal-declared sun exposure time was above 7 hours per week during the last 4 months, which was equivalent of one hour per day and outstrips common recommendations. The study was performed during November and December; thus, 25(OH)D concentration to a lesser degree depended on endogenous 25(OH)D synthesis.

There were no differences between examined groups in self-reported sunlight exposure; it also had no influence on vitamin D or PTH concentration. Against the odds, HF patients did not declare decreased sun exposure in comparison to the rest of the cardiology ward patients. Moreover, self-reported sunlight exposition did not affect 25(OH)D serum concentration: in group of higher sun exposure, 25(OH)D serum concentration was 30.73 ng/ml versus 29.89 ng/ml (p = ns). Partially, it could have resulted from the lack of skin synthesis in months preceding blood sampling, from a relatively small number of study participants or from patients' personal fitness overrating and disease negation.

Decreased serum 25(OH)D concentration in HF patients, correlating with disease severity, was reported by numerous authors [34]. In our research, mean 25(OH)D concentration did not differ among the groups which corroborates with our previous findings [31].

CE population, with regard to latitude, is prone to 25(OH)D deficiency especially during winter. Despite the fact that blood samples were collected in November and December, mean 25(OH)D concentration oscillated between normal limits in both groups. 25(OH)D deficiency requiring treatment (<20 ng/ml) occurred in 30 patients (23.6%), and suboptimal concentration (20–30 ng/ml) was stated in 24 participants (18.9%). The range of 25(OH)D concentration was 12.0–46.6 ng/ml in all patients. Recent large cross-sectional study reported by Płudowski et al. showed considerable prevalence of 25(OH)D deficiency in Polish volunteers, with mean concentration amounting 18.0 ± 9.6 ng/ml; deficiency (<20 ng/ml) occurred in 65.8% participants, and

suboptimal concentration (20–30 ng/ml) was stated in 24.1%. Sampling was performed in March and May and mean 25(OH)D concentration in late winter was 17.7 ± 10.1 ng/ml, in spring 18.3 0 ± 9.1 ng/ml, respectively [35]. Discrepancies between these values were most probably caused by different time of blood sampling, considering the half-life of 25(OH)D defined was twenty-five days, and lack of skin synthesis in winter could influence the findings. This so-called "seasonal effect" being coherent with our results was also reported by Tokarz et al. in the acute myocardial infarction group of patients [36]. It is worth to mention that 25(OH)D concentration during winter is based upon food intake and previous liver storage. Comparing to Płudowski et al. [35], our patients were older (63.8 versus 54.0 years), had higher BMI (28.3 versus 26.0), and were mostly men (62.2% versus 22.7%). Moreover, measurements were performed with laboratory assays provided by different companies. The problem of 25(OH)D standardization had been brought up in previously published studies. Immunoassays, being sensitive to 24,25-dihydroxyvitamin D, can overrate the serum 25(OH)D concentrations up to 5 ng/ml [37–40].

On the other hand, Gruson et al. showed that 1,25(OH)2D, but not 25(OH)D, correlates with NYHA functional class in chronic heart failure patients, especially that 1,25(OH)2D to PTH(1–84) ratio is a promising predictor of cardiovascular death in HF [41, 42]. There are some other reports showing that 25(OH)D deficiency was not related to HF in contrast to PTH [43, 44]. Being aware of limitations, we decided to asses 25(OH)D in our study because of easier access and lower costs than 1,25(OH)D assessment, as well as the wide range of other studies comparing 25(OH)D concentration in circulatory system.

Patients with AF had higher PTH concentration. In HF group, AF occurrence was more frequent, what is related to adverse remodeling of the myocardium but also may be affected by elevated PTH concentration, which is a proarrhythmic agent. An opposite causal link is also presumable: AF may promote PTH elevation [45]. In CAD group, higher number of white blood cells was observed when compared to patients without ischemia, with the highest values in IHF. It could be explained by the most excessive inflammatory state in advanced atherosclerosis, complicated by heart muscle damage. Statistically, significant difference between PLT count in IHF versus N-IHF (215.78 versus 169.62, $p \leq 0.04$) group of patients is related with unfavorable prognosis and was widely reported in the literature [46–48].

7. Limitations of the Study

The results of the study might have been biased on several pathways. The main limitation of this study is its cross-sectional design, self-reported data about sun exposure, and relatively small amount of patients; however, the study had a strictly pilot character. The researchers did not interfere with the management process; nevertheless, the data were acquired from a single cardiology center providing management to the population of approximately one million inhabitants.

8. Conclusions

PTH serum concentration is significantly raised in patients with HF irrespectively of its etiology and correlates with LVEF, NYHA functional class, renal function, and loop diuretics intake. Vitamin D serum concentration oscillates between lower normal limit in all patients and is not affected by the HF, CAD occurrence, or declared exposure to sun radiation.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Both Agnieszka Kolaszko and Grzegorz Kubiak equally contributed to this work. Agnieszka Kolaszko and Grzegorz Kubiak designed and conducted the study. Grzegorz Kubiak, Agnieszka Kolaszko, and Beata Morawiec performed the examinations and acquired the data. Agnieszka Kolaszko and Grzegorz Kubiak analyzed the data and wrote the manuscript. Grzegorz Kubiak performed the statistical analysis. Ewa Nowalany-Kozielska and Piotr Ceranowicz critically reviewed the manuscript and made substantial amendments.

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Research Article

Prognostic Association of Circulating Neutrophil Count with No-Reflow in Patients with ST-Segment Elevation Myocardial Infarction following Successful Primary Percutaneous Intervention

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Objective. The aim of the present study was to investigate the predictive value of neutrophil count for no-reflow in patients with ST-segment elevation myocardial infarction (STEMI) who underwent successful primary percutaneous intervention (PCI). *Methods.* We conducted a retrospective study of 361 patients diagnosed with acute STEMI between 2011 and 2015. All patients underwent successful PCI within 12 h from the onset of symptoms. Angiographic no-reflow was diagnosed based on a post-PCI thrombolysis in myocardial infarction flow grade ≤ 2 without mechanical obstruction. According to a neutrophil count cut-off determined by receiver operating characteristic curve analysis, patients were divided into two groups: group A (neutrophil count $< 9.14 \times 10^9$/L) and group B (neutrophil count $\geq 9.14 \times 10^9$/L). *Results.* Compared to patients in the normal reflow group, patients with no-reflow had higher neutrophil counts ($P < 0.05$). The incidence rate of no-reflow in group A (18, 9.3%) was significantly lower than that in group B (38). Multivariate logistic regression analysis revealed that a neutrophil count $\geq 9.14 \times 10^9$/L was independently predictive for no-reflow (odds ratio = 4.474, 95% confidence interval: 1.610–12.433, $P = 0.004$) after adjusting for potential confounders. *Conclusions.* A circulating neutrophil count $\geq 9.14 \times 10^9$/L is independently associated with no-reflow in patients with acute STEMI following primary PCI.

1. Introduction

ST-segment elevation myocardial infarction (STEMI) is known to be one of the leading causes of mortality worldwide. Primary percutaneous coronary intervention (PCI) is the most effective way of preventing the progression of myocardial necrosis and reducing mortality associated with STEMI. However, according to Kloner et al. [1] under some circumstance, restoration of arterial flow into the previously ischemic tissue either does not occur or is greatly impeded. Early and adequate restoration of the infarct-related artery (IRA) does not always result in optimal myocardial reperfusion [2]. This phenomenon is defined as "no-reflow" [1, 2]. No-reflow reduces the benefits of primary PCI in patients with acute STEMI. The size of the "no-reflow" zone is closely correlated with cardiac systolic function, myocardial remodeling, ventricular arrhythmias, cardiogenic shock, mortality during hospitalization, and worsened outcomes at

follow-up [3]. Currently, no single effective therapeutic approach is available for no-reflow, making prevention vital. Identifying patients at the greatest risk is the first step in the prevention of no-reflow [4]. It is necessary to detect available blood biomarkers and other clinical indices to predict the risk of no-reflow and reduce the incidence of this phenomenon in the early stage.

Myocardial ischemia/reperfusion injury is the most important pathological characteristic in the development of no-reflow [5]. Recent fundamental studies have shown that neutrophils become trapped in an area of myocardial ischemia reperfusion via the NF-κB cascade [6–9]. Trapped leukocytes are established as important inflammation mediators of cardiac ischemia/reperfusion injury [8, 10–12]. Furthermore, clinical studies have reported that neutrophil accumulation at the coronary culprit lesion site predicts mortality in patients with acute coronary syndrome (ACS)/acute myocardial infarction (AMI) [13]. We hypothesized that the trapped neutrophils are derived from the circulating blood in the context of ischemia-reperfusion. With the above in mind, the aim of the present study was to investigate the prognostic association of an easily detectable biomarker—the count of circulating neutrophils with angiographic no-reflow assessed by post-PCI thrombolysis in myocardial infarction (TIMI) flow grade. Therefore, the neutrophil count on admission was considered in the present study.

2. Methods

2.1. Patients. A total of 361 patients diagnosed with acute STEMI from January 2011 to December 2015 were enrolled retrospectively in the present study. All patients underwent successful primary PCI within 12 hours from the onset of symptoms. The Ethics Committee of Anzhen Hospital approved the study protocol (Beijing, China).

2.2. STEMI Diagnostic Criteria. The STEMI diagnostic criteria are as follows: (1) typical ischemic chest pain lasting for at least 30 min and not alleviated by resting or nitroglycerin; (2) ST-segment elevation ≥ 2 mm in at least two consecutive leads or the onset of left bundle branch block; and (3) an increase and/or a decrease in cardiac biomarker values (preferably troponin), with at least one value above the 99th percentile of the upper reference limit [14].

2.3. Inclusion Criteria. The inclusion criteria are as follows: (1) patients diagnosed with STEMI; (2) men and nonpregnant women between 18 and 80 years of age; and (3) patients who signed the informed consent forms.

2.4. Exclusion Criteria. The exclusion criteria are as follows: (1) patients with cardiac shock; (2) patients with valvular heart disease; (3) patients with cardiomyopathy; (4) patients who underwent coronary artery bypass grafting; (5) heart transplant recipients; (6) patients with contraindications to antiplatelet agents and anticoagulation; (7) patients with multiple organ failure; (8) patients with acute infection, autoimmune disorders, or advanced cancer; and (9) patients allergic to contrast agents. All patients were divided into the no-reflow group and normal reflow group according to TIMI flow grade during coronary angiography (as illustrated below).

2.5. Diagnosis of No-Reflow during Coronary Angiography. All patients were administered with oral aspirin (300 mg) and clopidogrel (300 mg) and intravenous unfractionated heparin (50–70 U/kg). PCI procedures were performed via the transradial and transfemoral approaches. Before PCI, each patient underwent left and right coronary angiography with at least two projections. The upfront intracoronary administration of glycoprotein IIb/IIIa receptor inhibitor (GPIIb/IIIa receptor inhibitor, Tirofiban) was left to the operator's discretion during PCI. After intervention, all patients were administered with clopidogrel (75 mg) and aspirin (100 mg) once daily for 12 months. Other treatments were provided according to the physician's clinical opinion.

At least two experienced cardiologists determined all parameters and strategies. No-reflow was defined as a post-PCI TIMI flow grade of ≤ 2 in the IRA in the absence of dissection, spasm, apparent thrombus, or flow-limiting residual stenosis $< 50\%$. TIMI flow grade 3 was considered as normal reflow [15–17].

2.6. Grouping. Based on the neutrophil count cut-off determined by the receiver operating characteristic (ROC) curve analysis, the patients were divided into two groups: group A (neutrophil count $< 9.14 \times 10^9$/L) and group B (neutrophil count $\geq 9.14 \times 10^9$/L), namely, group A (<9.4 G/L) and group B (≥9.4 G/L).

2.7. Laboratory Analysis. Blood samples were drawn from the antecubital vein on admission for laboratory analysis. Neutrophil count was determined from the whole blood using an automated haematology analyser. Samples were centrifuged within 30 min to separate plasma and to determine the serum creatinine (Scr), blood glucose (GLU), and blood lipid profiles (low-density lipoprotein, high-density lipoprotein cholesterol, total cholesterol, and triglycerides) using an automated biochemical analyser. Cardiac biomarkers and high-sensitivity C-reactive protein (Hs-CRP) levels were measured using standard methods.

2.8. Clinical Data Collection and Quality Control. Responsible physicians performed the physical examinations and the independent researchers recorded data related to smoking history and comorbidities (diabetes mellitus and hypertension) on admission. The Killip classification was used to assess the severity of heart failure. Primary PCI strategy, choice of stent, and medications administered during hospitalization were chosen by the individual interventional cardiologists or responsible physicians according to clinical symptoms and angiographic characteristics. Data used for statistical analysis were obtained and entered into a computerized database by the staff.

2.9. Statistical Analysis. Continuous variables were presented as means ± standard deviations. When the variables were normally distributed, Student's *t*-test was used to compare

two independent samples. The Mann–Whitney U test was used to compare nonnormally distributed data. Categorical variables were expressed as frequencies and percentages, and the chi-square test was used to compare the data. An ROC curve was used to determine neutrophil count cut-off level. The predictors of no-reflow were determined by univariate and multivariate logistic regression. In multivariate models, covariates included age ≥ 65, male, smoking history, hypertension, diabetes, Killip classification ≥ 3, the left anterior descending artery (LAD) as the IRA, neutrophil count ≥ 9.14×10^9/L, neutrophil/lymphocyte ratio, cardiac troponin I (cTNI), upfront intracoronary GPIIb/IIIa receptor inhibitor administration, aspiration thrombectomy, platelet counts, white blood cell (WBC) counts, hemoglobin (HGB), time from symptom onset to reperfusion (>6 hours), multivessel disease, and initial TIMI flow grade (0-1) (those with a P value < 0.1 in univariate analysis and those that were clinically relevant). Results were presented as adjusted odds ratios (ORs) with 95% confidence intervals (CIs). A two-sided P value < 0.05 was considered statistically significant. SPSS 17.0 software was used to analyse the data.

3. Results

3.1. Baseline Characteristics. ROC curve analysis revealed that neutrophil count predicted no-reflow. The area under the ROC curve was 0.604 (95% CI: 0.522–0.687, $P = 0.013$) (see Figure 1), and the neutrophil count cut-off value was 9.14×10^9/L, with 67.9% sensitivity and 57.7% specificity. Patients were divided into two groups according to the neutrophil count cut-off level: group A (<9.4 G/L) ($n = 194$) and group B (≥9.4 G/L) ($n = 167$).

The mean age in group A (<9.4 G/L) was greater than that in group B (≥9.4 G/L). Differences in gender, smoking history, hypertension, diabetes, and history of PCI between the two groups were not statistically significant. No statistically significant differences were found between group A (<9.4 G/L) and group B (≥9.4 G/L) in terms of blood pressure, time from onset of symptoms to reperfusion, and multivessel disease. There were 53 (17.3%) patients with Killip class I in group A (<9.4 G/L) compared to 35 (21.1%) in group B (≥9.4 G/L). There were 128 (66.0%) patients with Killip class II in group A (<9.4 G/L) compared to 119 (71.7%) in group B (≥9.4 G/L). There were 11 (5.7%) patients with Killip class III in group A (<9.4 G/L) compared to eight (4.8%) in group B (≥9.4 G/L). There were 2 (1.0%) patients with Killip class IV in group A (<9.4 G/L) compared to four (2.4%) in group B (≥9.4 G/L). There were more patients with multistent implantation in group A (<9.4 G/L) (68, 35.1%) than in group B (≥9.4 G/L) (41, 24.6%). Higher WBC counts, neutrophil/lymphocyte ratios, red blood cell counts, HGB, platelet counts, and cTNI were detected in group B (≥9.4 G/L), whereas the lymphocyte counts and proportion of lymphocytes were decreased correspondingly. The differences in other blood markers were not statistically significant (see Table 1).

3.2. The Incidence of No-Reflow in Group A (<9.4 G/L) and Group B (≥9.4 G/L). Eighteen (9.3%) patients in group A (<9.4 G/L) had no-reflow compared to 38 (22.8%) patients in group B (≥9.4 G/L). This difference was statistically significant (Table 2).

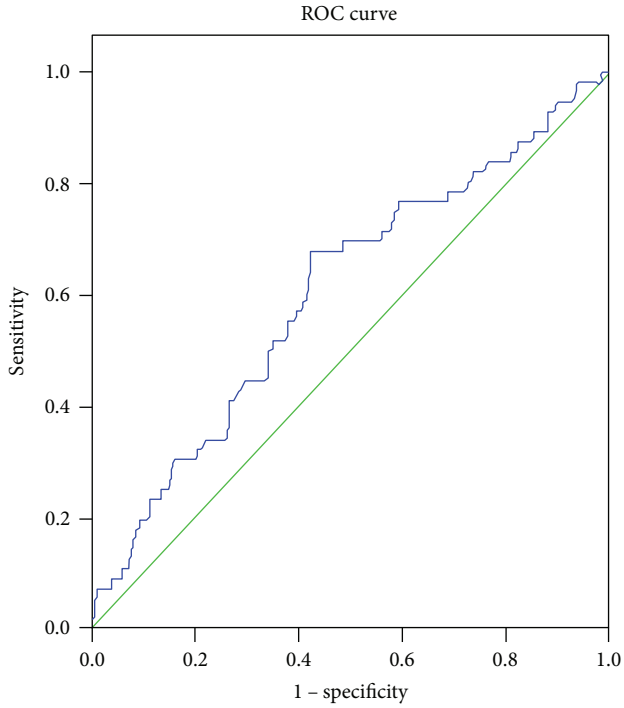

Figure 1: Receiver operating characteristic analysis of neutrophil count and no-reflow (area under the curve 0.604, 95% confidence interval: 0.522–0.687, $P = 0.013$).

3.3. Clinical Characteristics of Patients in the Normal Reflow Group and No-Reflow Group. No differences between patients in the no-reflow group and normal reflow group were detected in terms of age, gender, smoking history, comorbidities, history of PCI, and blood pressure. Higher values of WBC counts, neutrophil counts, neutrophil proportions, and neutrophil to lymphocyte ratio were detected in the no-reflow group compared to those in the normal reflow group. Lymphocyte counts and the proportion of lymphocytes were lower in the no-reflow group than those in the normal reflow group. Blood lipids, GLU, and other blood markers were not statistically significantly different between the two groups. The differences between the two groups with respect to left ventricular diastolic dysfunction and ejection fraction on admission were not statistically significant. Patients with no-reflow had significantly higher Killip classifications than those with normal reflow ($P < 0.05$) (Table 3).

3.4. Coronary Angiography Findings and Percutaneous Intervention Characteristics in the No-Reflow and Normal Reflow Groups. No-reflow, defined according to TIMI flow grade during coronary angiography, was more frequently observed among patients with the LAD as the IRA, while TIMI flow grade 3 was more frequently observed within the right coronary artery (RCA). There were more patients with anterior wall infarction in the no-reflow group than in the normal reflow group. The upfront intracoronary GPIIb/IIIa

Table 1: Baseline characteristics in group A and group B.

Parameters	Group A (<9.4 G/L) ($n = 194$)	Group B (≥9.4 G/L) ($n = 167$)	P value
Age (years, $\bar{X} \pm S$)	56.48 ± 10.85	53.57 ± 10.86	0.012*
Gender, male, n (%)	171 (88.1)	148 (88.6)	0.888
Smoker, n (%)	129 (66.5)	120 (71.9)	0.272
Hypertension, n (%)	105 (54.1)	90 (53.9)	0.965
Diabetes, n (%)	50 (25.8)	44 (26.3)	0.901
History of PCI, n (%)	12 (6.2)	7 (4.2)	0.398
SBP (mmHg, $\bar{X} \pm S$)	120.12 ± 17.93	116.60 ± 17.11	0.074
DBP (mmHg, $\bar{X} \pm S$)	74.93 ± 10.94	73.82 ± 11.40	0.579
Time from onset of symptoms to reperfusion (hour)	5.19 ± 2.72	5.25 ± 2.44	0.443
Killip classification, n (%)			0.395
1	53 (17.3)	35 (21.1)	
2	128 (66.0)	119 (71.7)	
3	11 (5.7)	8 (4.8)	
4	2 (1.0)	4 (2.4)	
WBC counts ($\times 10^9$/L)	8.83 ± 1.69	13.83 ± 2.62	0.0001**
Lymphocyte counts ($\times 10^9$/L)	1.65 ± 0.89	1.46 ± 0.81	0.014*
Proportion of lymphocytes (%)	18.84 ± 9.04	10.70 ± 5.25	0.0001**
Neutrophil/lymphocyte ratio	5.38 ± 4.12	9.89 ± 4.88	0.0001**
Red blood cell counts ($\times 10^{12}$/L)	4.56 ± 0.48	4.76 ± 0.57	0.0001**
HGB (g/L)	141.45 ± 14.33	145.72 ± 14.84	0.006**
Platelet counts ($\times 10^9$/L)	202.65 ± 55.48	221.62 ± 71.07	0.001**
PDW (%)	12.15 ± 1.85	12.46 ± 1.82	0.115
cTNI (ng/L)	65.64 ± 55.83	90.63 ± 71.91	0.0001**
Scr (μmol/L)	76.99 ± 18.63	79.37 ± 23.36	0.489
TG (mmol/L)	1.86 ± 1.50	1.84 ± 1.01	0.419
TCHO (mmol/L)	4.62 ± 0.89	4.65 ± 0.98	0.811
HDL-C (mmol/L)	1.02 ± 0.23	0.99 ± 0.23	0.246
LDL-C (mmol/L)	2.91 ± 0.78	3.02 ± 0.87	0.245
GLU (mmol/L)	7.88 ± 2.59	8.43 ± 3.07	0.129
Hs-CRP (mg/L)	9.54 ± 10.14	11.75 ± 10.47	0.051
FBG (g/L)	2.72 ± 0.56	2.72 ± 0.71	0.985
IRA, n (%)			0.802
LAD	95 (49.0)	79 (47.3)	
LCX	18 (9.3)	19 (11.4)	
RCA	81 (41.8)	69 (41.3)	
Multivessel disease, n (%)	48 (24.7)	42 (25.1)	0.929
Multistent, n (%)	68 (35.1)	41 (24.6)	0.030*

*$P < 0.05$; **$P < 0.01$. SBP: systolic blood pressure; DBP: diastolic blood pressure; WBC: white blood cell; HGB: hemoglobin; PDW: platelet distribution width; cTNI: cardiac troponin I; Scr: serum creatinine; TG: triglyceride; TCHO: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; GLU: glucose; Hs-CRP: high-sensitivity C-reactive protein; FBG: fibrinogen; IRA: infarct-related artery; LAD: left anterior descending; LCX: left circumflex artery; RCA: right coronary artery.

Table 2: The incidence of no-reflow in group A and group B.

Parameters	Group A (<9.4 G/L) ($n = 194$)	Group B (≥9.4 G/L) ($n = 167$)	P value
No-reflow, n (%)			0.0001**
Yes	18 (9.3)	38 (22.8)	
No	176 (90.7)	129 (77.2)	

**$P < 0.01$.

receptor inhibitor use rate was lower in the no-reflow group than the normal flow group. However, the incidence of no-reflow was not affected by multivessel disease, multistent implantation, and aspiration thrombectomy. Moreover, there were no significant differences between the no-reflow and normal reflow groups with respect to time from symptom onset to reperfusion and initial TIMI flow grade. Non-IRA intervention was not associated with no-reflow (Table 4).

Table 3: Clinical characteristics of patients with normal reflow and no-reflow.

Parameters	Normal reflow ($n = 305$)	No-reflow ($n = 56$)	P value
Age (years, $\bar{X} \pm S$)	54.96 ± 11.80	56.11 ± 11.66	0.470
Gender, male, n (%)	269 (88.2)	50 (89.3)	0.815
Smoker, n (%)	212 (69.5)	37 (66.1)	0.609
Hypertension, n (%)	165 (54.1)	30 (53.6)	0.942
Diabetes, n (%)	80 (26.2)	14 (25.0)	0.847
History of PCI, n (%)	18 (5.9)	1 (1.8)	0.330
SBP (mmHg, $\bar{X} \pm S$)	118.8 ± 17.6	116.9 ± 17.7	0.504
DBP (mmHg, $\bar{X} \pm S$)	74.4 ± 11.3	74.6 ± 10.6	0.983
Killip classification, n (%)			0.011*
1	82 (26.9)	7 (12.5)	
2	206 (67.5)	41 (73.2)	
3	12 (3.9)	7 (12.5)	
4	5 (1.6)	1 (1.8)	
WBC counts ($\times 10^9$/L)	10.99 ± 3.23	11.97 ± 3.61	0.042*
Proportion of neutrophils (%)	78.73 ± 11.11	82.73 ± 9.15	0.002**
Neutrophil counts ($\times 10^9$/L)	8.79 ± 3.21	10.01 ± 3.45	0.013*
Lymphocyte counts ($\times 10^9$/L)	1.61 ± 0.89	1.34 ± 0.45	0.034*
Proportion of lymphocytes (%)	15.59 ± 8.69	12.29 ± 7.19	0.002**
Neutrophil/lymphocyte ratio	7.17 ± 4.93	9.10 ± 5.19	0.001**
Red blood cell counts ($\times 10^{12}$/L)	4.66 ± 0.53	4.62 ± 0.56	0.649
HGB (g/L)	143.71 ± 14.55	141.88 ± 15.54	0.391
Platelet counts ($\times 10^9$/L)	212.57 ± 64.85	205.19 ± 57.79	0.467
PDW (%)	12.27 ± 1.87	12.44 ± 1.70	0.529
cTNI (ng/L)	74.09 ± 62.49	94.15 ± 75.05	0.078
Scr (μmol/L)	77.66 ± 21.19	80.45 ± 19.61	0.155
TG (mmol/L)	1.88 ± 1.37	1.70 ± 0.83	0.795
TCHO (mmol/L)	4.65 ± 0.92	4.53 ± 1.00	0.382
HDL-C (mmol/L)	1.00 ± 0.23	1.04 ± 0.25	0.310
LDL-C (mmol/L)	2.97 ± 0.81	2.92 ± 0.90	0.696
GLU (mmol/L)	8.17 ± 2.94	7.95 ± 2.19	0.717
Hs-CRP (mg/L)	10.62 ± 10.48	10.21 ± 9.49	0.869
FBG (g/L)	2.73 ± 0.64	2.64 ± 0.63	0.574
LVDD (mm)	49.70 ± 4.65	49.09 ± 7.81	0.728
EF (%)	54.30 ± 9.49	52.91 ± 10.28	0.355

*$P < 0.05$; **$P < 0.01$. LVDD: left ventricular diastolic diameter; EF: ejection fraction.

3.5. Univariate and Multivariate Logistic Regression. In univariate analysis, the Killip classification ≥ 3 (OR = 2.824, 95% CI: 1.155–6.904, $P = 0.023$), LAD as the IRA (OR = 1.821, 95% CI: 1.018–3.259, $P = 0.043$), neutrophil count ≥ 9.14×10^9/L (OR = 2.880, 95% CI: 1.573–5.275, $P = 0.001$), neutrophil/lymphocyte ratio (OR = 1.067, 95% CI: 1.015–1.121, $P = 0.011$), cTNI (OR = 1.004, 95% CI: 1.000–1.008, $P = 0.036$), and WBC count (OR = 1.086, 95% CI: 1.002–1.178, $P = 0.046$) were predictors for no-reflow. Upfront intracoronary GPIIb/IIIa receptor inhibitor use was negatively associated with no-reflow (OR = 0.303, 95% CI: 0.091–1.010, $P = 0.052$). In multivariate logistic regression analysis, neutrophil count ≥ 9.14×10^9/L was a predictor for no-reflow after adjusting for patients aged ≥ 65, male, smoking, hypertension, diabetes, Killip classification ≥ 3, LAD as the IRA, neutrophil/lymphocyte ratio, cTNI, upfront intracoronary GPIIb/IIIa receptor inhibitor use, aspiration thrombectomy, platelet counts, WBC counts, HGB, time from symptom onset to reperfusion (>6 hours), multivessel disease, and initial TIMI flow grade (0-1) (OR = 4.474, 95% CI: 1.610–12.433, $P = 0.004$) (Table 5).

4. Discussion

In the present study, there were 56 (15.5%) patients with angiographic no-reflow. Patients in the no-reflow group

Table 4: Percutaneous intervention findings of patients with normal reflow and no-reflow.

Parameters	Normal reflow ($n = 305$)	No-reflow ($n = 56$)	P value
Time from onset of symptoms to reperfusion (hour)	5.20 ± 2.70	5.29 ± 1.95	0.315
Time from onset of symptoms to reperfusion (>6 hours)	96 (31.5)	16 (28.6)	0.666
Multivessel disease, *n* (%)			
Yes	76 (24.9)	14 (25.0)	0.990
No	229 (75.1)	76 (24.9)	
Initial TIMI flow grade, *n* (%)			
0-1	192 (63)	38 (67.9)	0.483
≥2	113 (37.1)	18 (32.1)	
Multistent, *n* (%)			
Yes	93 (30.5)	16 (28.6)	0.774
No	212 (69.5)	40 (71.4)	
IRA, *n* (%)			
LAD	140 (45.9)	34 (60.7)	0.051
LCX	30 (9.8)	7 (12.5)	
RCA	135 (44.3)	15 (26.8)	
Infarct location, *n* (%)			0.149
Anterior wall	140 (45.9)	36 (64.3)	
Inferior wall	55 (18)	6 (10.7)	
Inferior and posterior wall	43 (14.1)	4 (7.1)	
Complicate by right ventricular	63 (20.7)	10 (17.9)	
Others	4 (1.3)	0 (0)	
Aspiration thrombectomy			
Yes	206 (67.5)	40 (71.4)	0.566
No	99 (32.5)	16 (28.6)	
Upfront intracoronary GPIIb/IIIa receptor inhibitor			
Yes	48 (15.7)	3 (5.4)	0.040*
No	257 (84.3)	53 (94.6)	
Non-IRA intervention, *n* (%)			
Yes	18 (5.9)	4 (7.1)	0.760
No	287 (94.1)	52 (92.9)	

*$P < 0.05$.

had significantly higher neutrophil counts than those in the normal reflow group. In univariate and multivariate logistic regression analyses, a neutrophil count above 9.14×10^9/L was independently associated with no-reflow after adjusting for age ≥ 65, male, smoking history, hypertension, diabetes, Killip classification ≥ 3, LAD as the IRA, neutrophil/lymphocyte ratio, cTNI, upfront intracoronary GPIIb/IIIa inhibitor administration, aspiration thrombectomy, platelet counts, WBC counts, HGB, time from symptom onset to reperfusion (>6 hours), multivessel disease, and initial TIMI flow grade (0-1).

The present result is supported by the retrospective study of Kosuge et al. [18] stating that a WBC count of 12,000 cells/mm^3 was an independent predictor of impaired myocardial reperfusion in patients with early recanalized anterior acute myocardial infarction (AMI). However, they only recruited the patients with anterior AMI. Furthermore, they did not analyse specific types of WBCs. Takahashi et al. [19] concluded that neutrophils over 10 G/L were associated with no reflow within 116 patients with a first anterior AMI. The present study recruited patients not limited to those with anterior AMI. The study of Wang et al. [20] which assessed the relationship between neutrophil counts and no-reflow included 217 patients in the author's center, age and blood cells counts were adjusted exclusively in the study, and the threshold of neutrophil count no-reflow was not analysed. In the present study, we obtained a cut-off value of 9.14×10^9/L (67.9% sensitivity and 57.7% specificity), with neutrophil counts above this threshold being associated with increased rates of no-reflow. There were studies on the prognostic value of neutrophil to lymphocyte ratio in predicting no-reflow [15, 16]; hence, the present study also included neutrophil/lymphocyte ratio in the multivariate analysis. Additionally, other factors that are clinically relevant to no-reflow including hypertension, blood pressure, Killip classification (≧3), cTNI, time from symptom onset to reperfusion (>6 hours), multivessel disease, and initial TIMI flow grade (0-1) were also included in the multivariate analysis in the

Table 5: Univariate and multivariate logistic analysis for no-reflow.

Parameters	Univariate logistic analysis			Multivariate logistic analysis		
	OR	95% CI	P	OR	95%CI	P
Age ≥ 65 years	1.116	0.567–2.199	0.751	1.068	0.467–2.442	0.877
Male	1.115	0.446–2.786	0.815	1.364	0.441–4.224	0.590
Smoking	1.171	0.640–2.143	0.610	1.148	0.549–2.400	0.714
Hypertension	1.021	0.577–1.809	0.942	0.979	0.519–1.845	0.947
Diabetes	0.938	0.486–1.807	0.847	0.821	0.395–1.710	0.599
Killip classification (≧3)	2.824	1.155–6.904	0.023*	4.072	1.391–11.916	0.010*
LAD as IRA	1.821	1.018–3.259	0.043*	2.457	1.226–4.925	0.011*
Neutrophil count ≥ 9.14×10^9/L	2.880	1.573–5.275	0.001**	4.474	1.610–12.433	0.004**
Neutrophil/lymphocyte ratio	1.067	1.015–1.121	0.011*	1.029	0.967–1.095	0.366
cTNI	1.004	1.000–1.008	0.036*	1.001	0.995–1.006	0.806
Upfront intracoronary GPIIb/IIIa receptor inhibitor	0.303	0.091–1.010	0.052	0.219	0.061–0.783	0.019*
Aspiration thrombectomy	1.201	0.642–2.250	0.566	1.253	0.469–3.347	0.652
Platelet counts	0.998	0.993–1.003	0.424	0.997	0.991–1.003	0.343
WBC counts ($\times 10^9$/L)	1.086	1.002–1.178	0.046*	0.940	0.798–1.107	0.459
HGB (g/L)	0.992	0.973–1.011	0.390	0.982	0.959–1.007	0.159
Time from symptoms onset to reperfusion (>6 hours)	0.871	0.465–1.632	0.666	0.932	0.469–1.851	0.840
Multivessel disease	1.004	0.520–1.940	0.990	0.987	0.470–2.075	0.973
Initial TIMI flow grade (0-1)	1.242	0.677–2.280	0.483	0.886	0.354–2.219	0.796

*$P < 0.05$; **$P < 0.01$. Age ≥ 65 years, male, smoking, hypertension, diabetes, Killip classification (≧3), LAD as IRA, neutrophil count ≥ 9.14×10^9/L, neutrophil/lymphocyte ratio, cTNI, upfront intracoronary GPIIb/IIIa receptor inhibitor, aspiration thrombectomy, platelet counts, WBC counts, HGB, time from symptoms onset to reperfusion (>6 hours), multivessel disease, and initial TIMI flow grade (0-1) were included in the multivariate analysis.

present study. The prognostic association of the neutrophil/lymphocyte ratio with no-reflow was lost after adjusting for neutrophil count ≥ 9.14×10^9/L. Furthermore, neutrophil/lymphocyte ratio was not associated with no-reflow in multivariate analysis without confounding for neutrophil counts (OR = 1.043, 95%CI: 0.984–1.106, $P = 0.152$). This could be explained by the elevated neutrophil/lymphocyte ratio in our study that mainly resulted from the increased neutrophil counts.

The underlying mechanism of the involvement of neutrophils in no-reflow is complex. Ischemic injury damages myocardiocytes, which presents as myocardial cell swelling and interstitial edema. The pathological changes in myocardial cells increase the compression of intramural vessels and induce neutrophil plugging and activation in the coronary microcirculation [4]. The oxygen-free radicals released by activated neutrophils contribute to endothelial injury and impaired reperfusion. At the time of reperfusion, there was a massive neutrophil adhesion to the endothelium due to the excessive generation of reactive oxygen species and subsequently activated NF-κB cascade. Structural luminal obstruction of the microvasculature resulted from microaggregates formed by neutrophils and platelets that aggravate the reperfusion injury [4, 21]. Moreover, intense and prolonged coronary microvascular vasoconstriction is attributable to vasoactive substances produced by neutrophils, platelets, and damaged endothelial cells [11]. In addition, the infiltration of neutrophils in the vulnerable myocardium as a result of increased vascular permeability enhances interstitial edema and extravascular mechanical compression, contributing to the pathological processes of no-reflow [4, 11].

ACS is a group of clinical syndromes characterized by rupture or erosion of coronary atherosclerotic plaques and subsequent complete or incomplete thrombotic occlusion [22]. Lipid-rich plaques are correlated with impaired reperfusion after restoration of the epicardial artery [23]. Noncalcified plaque burden is correlated with the neutrophil/lymphocyte ratio [24]. Neutrophils mediate apoptosis in endothelial cells and smooth muscle cells, contributing to plaque rupture [25–27]. Microvascular embolization and no-reflow occur when a mass of plaque fragments, leading to the release of cholesterol crystals and microthrombi into the bloodstream. Moreover, active neutrophils accelerate the formation of platelet-leukocyte aggregates, plugging the microvessel.

In the present study, we found that the Killip classification ≥ 3 was associated with no-reflow. Patients with no-reflow had advanced Killip classifications, coincident with the findings of Zhou et al. [17], despite that Killip classification 2 rate in the present was higher than that reported. There were 7.9% patients with Killip classifications 3 and 4, similar to the percentage in the study of Zhou et al. (8.9%) [17]. Killip classification of at least grade 3 on admission may be associated with larger infarctions and decreased coronary perfusion pressure [17]. The decreased coronary pressure accelerates plugging of neutrophils in the microvasculature, inducing no-reflow. Although

we observed the cTNI (associated with larger infarct area) level was higher in group B (<9.4 G/L) than in group A (≥9.4 G/L), there was no difference between the no-reflow group and normal reflow group. Furthermore, after adjusting for cTNI, neutrophil count was independently associated with no-reflow.

4.1. Limitations. There are some limitations to the present study. A relatively small sample size was used. Furthermore, only TIMI flow grade was used to identify no-reflow, and no other diagnostic methods were used because of limited data. This partially explains why the LAD as the IRA was a negative factor for no-reflow. However, this difference did not affect the outcomes of the study. A prospective study including a larger sample, and in which TIMI myocardial perfusion or myocardial blush grade is assessed, is needed in the future.

5. Conclusions

Inflammatory responses and the infiltration of neutrophils are associated with ischemia/reperfusion injury associated with no-reflow following PCI. A circulating neutrophil count $\geq 9.14 \times 10^9$/L is independently associated with no-reflow in patients with acute STEMI following successful primary PCI.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

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

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Research Article

Elevated Level of Troponin but Not N-Terminal Probrain Natriuretic Peptide Is Associated with Increased Risk of Sudden Cardiac Death in Hypertrophic Cardiomyopathy Calculated According to the ESC Guidelines 2014

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The aim of this study was to assess the relationship between biomarkers (high-sensitive troponin I [hs-TnI], N-Terminal probrain natriuretic peptide [NT-proBNP]) and calculated 5-year percentage risk score of sudden cardiac death (SCD) in hypertrophic cardiomyopathy (HCM). *Methods*. In 46 HCM patients (mean age 39 ± 7 years, 24 males and 22 females), echocardiographic examination, including the stimulating maneuvers to provoke maximized LVOT gradient, had been performed and next ECG Holter was immediately started. After 24 hours, the ECG Holter was finished and the hs-TnI and NT-proBNP have been measured. Patients were divided according to 1/value of both biomarkers (hs-TnI-positive and hs-TnI-negative subgroups) and 2/(NT-proBNP lower and higher subgroup divided by median). *Results*. In comparison between 19 patients (hs-TnI positive) versus 27 patients (hs-TnI negative), the calculated 5-year percentage risk of SCD in HCM was significantly greater ($6.38 \pm 4.17\%$ versus $3.81 \pm 3.23\%$, $P < 0.05$). In comparison between higher NT-proBNP versus lower NT-proBNP subgroups, the calculated 5-year percentage risk of SCD in HCM was not significantly greater ($5.18 \pm 3.63\%$ versus $4.14 \pm 4.18\%$, $P > 0.05$). *Conclusions*. Patients with HCM and positive hs-TnI test have a higher risk of SCD estimated according to SCD calculator recommended by the ESC Guidelines 2014 than patients with negative hs-TnI test.

1. Introduction

The risk factors of sudden cardiac death (SCD) for hypertrophic cardiomyopathy (HCM) in the ESC Guidelines [1] included echocardiogram, electrocardiographic (ECG) Holter monitoring, and clinical variables. The calculator for sudden cardiac death risk [1] has not included any biomarker. Recently, Kehl et al. [2] reviewed the available data regarding the usefulness of natriuretic peptides and troponins in HCM. Concentrations of natriuretic peptides, and to a lesser extent of troponins, correlate with left ventricular thickness, symptom status, and left ventricular hemodynamics by Doppler measurements (left ventricular filling pressure, left ventricular outflow tract gradient).

Neither ischemic biomarker nor signs and symptoms of myocardial ischemia are included in the calculator [1]. However, ischemic response to stress revealed by echocardiographic methods becomes important prognostic player [3, 4].

Currently used high-sensitivity troponin I (hs-TnI) is an super precise biomarker for the detection of myocardial ischemia. In previous HCM studies, measurements of hs-Tn were only at a resting (without stress in unnatural condition) echocardiography and not timely synchronized with maneuvers to provoke LVOTG by natural stimuli reflecting daily common physical activity for patients [5–8]. Moreover, measurements of hs-Tn were also not timely synchronized with the Holter monitoring. So far, we have used the following protocol: 24-hour cycle—8 a.m., echocardiography with

LVOTG provocation by natural stimuli (orthostatic test and Valsalva test [1, 9–13]; the observation was divided into 2 periods: day phase physical activity with probable episodes of provocable LVOTG (unmeasurable) and night phase period as a potential time for rise of troponin, in which the level has been measured after night at 8 a.m. in the next day). Between echocardiography and biomarker sampling, 24-hour Holter electrocardiography (ECG) was recorded and then the measurement of hs-TnI (the biomarker level has a close temporal relationship with findings on Holter ECG). This protocol seems to be reasonable because hs-TnI levels may be a potential cause of life-threatening ventricular arrhythmias occurring during the previous 24 hours.

The aim of this study was to assess the relationship between biomarker concentrations (hs-TnI, NT-proBNP) and calculated 5-year percentage risk score of SCD in HCM.

2. Methods

Consecutive patients with HCM were recruited to the study. Informed consent was obtained from each participant. All patients fulfilled conventional diagnostic criteria for HCM. The criteria for diagnosis of HCM, according to the ESC Guidelines, were the presence of left ventricular (LV) wall thickness of at least 15 mm without any other cause that could lead to ventricular hypertrophy [1, 13]. The exclusion criteria were as follows: sport activity more than recreational, prior myocardial infarction, current symptoms suggestive of coronary artery disease, concomitant neoplasm, infection, or renal failure. Subjects who had a history of alcohol septal ablation or septal myectomy were not included into the present study.

The final sample included 46 patients with HCM (mean ± SD age, 39 ± 7 years; 24 men and 22 women).

Patients on current pharmacotherapy were studied according to the abovementioned protocol. Patients have been asked to perform their common day physical activity and nocturnal resting. This protocol seems to be reasonable because hs-TnI levels may be related with labile, dynamic nature of LVOTG with fluctuating peaks in daytime (provoked LVOTG as a potential cause of myocardial ischemia).

2.1. First Model of Risk Calculation (Only the Current ECG Holter). For calculating the percentage value of HCM risk score SCD, we assessed the following parameters: episodes of nonsustained ventricular tachycardia (nsVT) in current Holter monitoring (defined as three or more consecutive ventricular beats > 120 beats per minute) and two-dimensional (2D) echocardiography with the assessment of the maximal left ventricular wall thickness in diastole (MWT), left atrial diameter (LAD), and maximal provocable left ventricular outflow tract (LVOT) gradient [1]. For the disease history, we check the following binary variables: syncope and family history of sudden death [1]. Finally, we include into the calculator the age of patients [1].

2.2. Second Model of Risk Calculation (All ECG Holter – Current + Previous ECG Holter). Every patient had at least 3 times 24-hour ECG Holter recordings during life. One ECG Holter is defined as current (simultaneous) with biomarker sampling, and the remaining 2 or more recordings took place in past history. Presence/absence of NsVT was assessed by summing the data of all Holter (previous and current). The remaining parameters used in calculation were identical as in the first model.

The study protocol was approved by a local institutional review board (Komisja Bioetyki Jagiellonian University).

2.3. Statistical Analysis. Normally distributed continuous variables were presented as mean ± SD. Differences between two groups were assessed using independent *t*-test. Categorical variables were assessed using the Fisher exact test and expressed as numbers (percentages). A P value of less than 0.05 was considered significant.

Table 1: Baseline characteristics of the patients.

NYHA class	2.3 ± 0.6
CCS class	1.5 ± 0.4
Syncope (*n*)	15
Sudden death in family history (*n*)	15
NSVT in current Holter (*n*)	11
Creatinine, μg/L	82.3 ± 11.6
Maximum LV thickness, mm	22.5 ± 4.2
Resting LVOT gradient, ≥30 mm Hg (*n*)	8
Provocable LVOT gradient, ≥30 mm Hg (*n*)	17
Left atrial diameter, mm, mean (SD)	4.83 ± 0.81
Drugs with negative chronotropic properties (*n*)	
β-Blocker	37
Verapamil	5
None	4

CCS: Canadian Cardiovascular Society; LVOT: left ventricular outflow tract; LV: left ventricular; NSVT: nonsustained ventricular tachycardia; NYHA: New York Heart Association.

3. Results

The baseline characteristics of HCM patients are displayed in Table 1.

Hs-TnI was detected in all HCM patients and patients with abnormal level > 19.5 ng/L were defined as positive troponin subgroup; nonelevated hs-TnI subgroup consisted of negative troponin subgroup. After NT-proBNP measurement, only 3 patients have a normal concentration < 125 pg/mL; thus, subgroup division has been created by a median.

In comparison between 19 patients (hs-TnI positive) versus 27 patients (hs-TnI negative), the calculated 5-year percentage risk of SCD in HCM was significantly greater, both in the first and in the second models (Table 2). In the second comparison between higher NT-proBNP versus lower NT-proBNP subgroups, the calculated 5-year percentage risk of SCD in HCM was not significantly greater in the first model as well as in the second model (Table 2).

4. Discussion

In the current study, patients with HCM and positive hs-TnI test have a higher risk of SCD estimated according to SCD

Table 2: Comparison between subgroups of hs-TnI positive versus negative and also between subgroup of lower versus higher NT-proBNT concentration (NS: nonsignificant).

First model—current Holter			
	Hs-TnI negative $n = 27$	Hs-TnI positive $n = 19$	
5-year SCD risk in HCM	$3.81 \pm 3.23\%$	$6.38 \pm 4.17\%$	$P < 0.05$
	Lower NT-proBNP $n = 23$	Higher NT-proBNP $n = 23$	
5-year SCD risk in HCM	$4.14 \pm 4.18\%$	$5.18 \pm 3.63\%$	NS
Second model—all Holter			
	Hs-TnI negative $n = 27$	Hs-TnI positive $n = 19$	
5-year SCD risk in HCM	$4.25 \pm 4.20\%$	$6.90 \pm 3.99\%$	$P < 0.05$
	Lower NT-proBNP $n = 23$	Higher NT-proBNP $n = 23$	
5-year SCD risk in HCM	$4.40 \pm 3.62\%$	$6.29 \pm 4.18\%$	NS

calculator recommended by the ESC Guidelines 2014 than patients with negative hs-TnI test. Level of NT-proBNP is not associated with the calculated 5-year risk of SCD (stratified by calculator).

4.1. Clinical Application of Biomarkers in HCM. In a recent review paper, authors ask the question: NT-proBNP versus troponin: is one better than the other [2]. Although both biomarkers correlate with indices of HCM disease progression, BNP may be a more sensitive indicator of left ventricular hypertrophy than troponin. It was documented that the wall thickness threshold was lower for BNP elevation than for TnI elevation [14]. Moreover, it has also stronger predictors of hemodynamic parameters and clinical symptoms than troponin. Although a correlation between elevated troponin and elevated BNP has been demonstrated [14, 15], it is not a consistent finding [8, 14].

Before our study, a strategy for application of these biomarkers to risk stratification has not yet been investigated. These biomarkers may be most useful when risk markers of SCD indicate intermediate or indeterminate risk.

The impact of stress echocardiography in HCM is limited by lack of standardization and outcome data. ECS guidelines recommend stress echo solely for evaluation of LVOT [3]. However, large-scale registry data show that stress echocardiography positivity for ischemic criteria (such as new wall motion abnormalities and coronary flow velocity reserve) rather than inducible gradients predicts adverse outcome in HCM [4]. In a large study [3], mortality was predicted using criteria for detecting ischemia on stress echocardiography. The investigators proposed that stress echocardiography has a signficant prognostic role in patients with HCM, with ischemic endpoints showing a greater predictive accuracy than hemodynamic endpoints [3].

In a recent review by McCarthy et al. [16], the utility of hs-Tn assessment in arrhythmic disease is only at the initial stage of investigations, but it has been postulated as a valuable screening marker for patients with HCM at high risk of SCD.

Regular training exercise (e.g., fitness activity) has recently been recommended for selected patients with HCM [17]. Based on current observation on the association between tachycardia and elevated troponin level in patients with HCM [18] and phenomenon of troponin release by exercise [19], we suggest that any exercise stress test in HCM patients (performed either for training or diagnostic purposes) should be controlled by troponin level measurements before exercise and 6 and 12 hours after the exercise. Especially predisposed to high LVOT gradient are HCM women > 50 years of age, due to smaller LV cavity size [20]. This subgroup of HCM patients may be particularly at risk to develop high gradient at peak/post exercise period resulting into increased calculated risk in calculator.

Our study has several limitations. First, our study group may appear too small to definitely rule out association between NT-proBNP and SCD HCM risk score. Secondly, the current pharmacological treatment was maintained, and particularly, β-blockers were not withdrawn. In our pilot study, we aimed to make the observation on the correlation between hs-TnI release and timely synchronized findings on ECG Holter and resting/stress echocardiography.

Our preliminary study showed that β-blocker withdrawal might not be safe in troponin-positive subgroup of patients. In future studies, we will attempt to increase the dose and use only one type of a β-blocker to decrease ischemia burden.

We decided to measure hs-TnI levels only once because our pilot study was conducted in an outpatient setting. The optimal protocol, that is, 48-hour profile of troponin measurement with the assessment of troponin with echocardiographic examination every 8 hours, and 48 hours Holter monitoring (recommended by the ESC Guidelines), would require the in-hospital setting for the study and would be more costly. Moreover, only an outpatient-based study provides an opportunity to assess the heart rate during

common daily physical activity of patients. At first look, the study by Kubo et al. [5] seems similar to our study; however, we have found essential differences from our investigation in many important points.

(i) Kubo et al. (in pre-era of the ESC Guidelines from 2014, authors did not use calculator risk factors for SCD) defined their study as analysis of combined cardiovascular events (with SCD episode [the most fatal end point] only in 4 patients—such number seems to be too small for proper statistical analysis). Our study was not follow-up designed, but focused on relationship between biomarker levels and calculated 5-year percentage risk score only for SCD in HCM. SDC is the most fatal, but an easy preventable complication of HCM (by ICD).

(ii) Morphologic/prognostic differences are also important (in Kubo et al., patients had less predisposition for SCD: a benign apical variant 28% versus 0% in our group). The benign morphologic pattern in Kubo et al. paper seems to correspond to the low number of SCD.

(iii) In Kubo et al., LVOT gradient was assessed in binary analysis < 30 mmHg or ≥ 30 mmHg and only in resting conditions. In contrast, we have assessed LVOTG more precisely as a continuous variable, measured both at rest and after provocation (the provocable LVOTG is absolutely needed to measure risk of SCD by ESC calculator).

(iv) Kubo et al. did not analyze nsVT in ECG-Holter (which is absolutely needed to measure the risk of SCD by calculator); moreover, nsVT assessment is needed also in the American Guideline from 2011 for risk stratification of SDC. Thus, in a paper by Kubo et al., the lack of ECG Holter analysis is a serious limitation.

(v) Kubo et al. did not describe the time period between blood sampling for biomarkers and measurement of echo parameters (nsVT in Holter was not studied). In our study, the time synchrony between echo/Holter and hs-TnI measurement was defined.

(vi) Kubo et al. did not analyze NT-proBNP, but only hs-Tn. Our study provides more information about two important biomarker sampling simultaneously with echocardiographic and ECG Holter measurements.

5. Conclusions

Patients with HCM and positive hs-TnI test have higher risk of SCD estimated according to SCD calculator recommended by the ESC Guidelines 2014 than patients with negative hs-TnI test.

5.1. Clinical Perspective. These findings suggest that hs-Tn may be useful as an additional biomarker for better risk stratification in HCM. Additionally, we have postulated to monitor also the biomarkers of endothelial dysfunction (impaired endothelium-dependent vasodilatation) [21].

Conflicts of Interest

The authors declare that there is no conflict of interest.

Authors' Contributions

Rafał Hładij contributed to the conception, contributed to acquisition and analysis, and gave final approval. Renata Rajtar-Salwa contributed to the conception and design; contributed to acquisition, analysis, and interpretation; critically revised the manuscript; and gave final approval. Paweł Petkow Dimitrow contributed to the conception and design; contributed to acquisition, analysis, and interpretation; drafted the manuscript; critically revised the manuscript; and gave final approval. Renata Rajtar-Salwa and Rafał Hładij contributed equally to this work.

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Research Article

Vitamin D Status, Disease Activity, and Endothelial Dysfunction in Early Rheumatoid Arthritis Patients

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Cardiovascular diseases represent important complications in rheumatoid arthritis (RA) patients, generated by an accelerated atherosclerosis. The aim of this study is represented by the assessment of the correlations between serum levels of vitamin D, disease activity, and endothelial dysfunction in patients with early RA. *Material and Methods*. The study was performed on a group of 35 patients with early RA and 35 healthy subjects matched for age and gender, as controls. In all studied subjects, the following were determined: inflammatory markers, insulin resistance, vitamin D levels, and endothelial dysfunction. Statistical analysis were performed using the Student's *t*-test and the Pearson's test. p values of less than 0.05 were considered statistically significant. *Results*. The group of patients with RA patients presented inflammation, low levels of vitamin D, elevated insulin resistance, and reduced flow-mediated vasodilation, statistically significant compared to the control group ($p < 0.00001$). Significant inverse correlations between the levels of 25(OH) vitamin D and DAS28, respective insulin resistance, and significant positive correlation between 25(OH) vitamin D and endothelial function were demonstrated. *Conclusion*. In early RA patients with moderate and high disease activity, low serum levels of vitamin D were associated with disease activity, increased insulin resistance, and endothelial dysfunction.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disorder, which typically involves small- and medium-sized joints. Rheumatoid synovitis generates cartilage breakdown, bony erosions, and loss of function of the involved joints [1]. But besides articular involvement, cardiovascular disease generated by accelerated, premature atherosclerosis represents a serious complication of RA. It is known that in RA patients, cardiovascular disease represents the cause of 40–50% of the deaths in this group of population [2, 3]. Atherosclerosis is a complex process, which develops over the course of many years, beginning in the early teenage years, and endothelial dysfunction represents the first step in its development [4, 5].

Lin et al. showed in their meta-analysis that the RA patients exhibit lower vitamin D levels than healthy controls, and, on the other hand, these levels present an inverse correlation with the disease activity [6]. Other studies demonstrated the role of vitamin D in RA activity [7–10]. Besides the role in bone mineral metabolism, vitamin D has anti-inflammatory and immunomodulatory roles. By antiproliferative, antiangiogenic, and antioxidant properties, vitamin D offers protective effects on the cardiovascular system [11, 12].

Table 1: Demographic data in RA patients and controls.

Parameter	Value (mean ± standard deviation)	
	RA patients	Controls
Sex [*n* (%)]	35	35
Males	12 (34.28%)	12 (34.28%)
Females	23 (65.72%)	23 (65.72%)
Mean age (years)	55.6 ± 9.74	54.14 ± 6.28
Mean length of RA evolution (months)	14.25 ± 5.27	—
The drugs used by the RA patients in the moment of investigation	Methotrexate (30 patients; 13.28 ± 2.25 mg/week) and leflunomide (5 patients; 20 mg/day)	—

The aim of this study is represented by the assessment of the associations between serum levels of vitamin D, disease activity, and endothelial dysfunction in patients with early rheumatoid arthritis.

2. Material and Methods

2.1. Patients. The study was performed on a group of 35 patients with early RA and 35 healthy subjects matched for age and gender, as controls. The diagnosis of RA was established based on the 2010 American College of Rheumatology/European League Against Rheumatism Classification Criteria for Rheumatoid Arthritis [13]. All patients had a disease duration of less than 2 years. Exclusion criteria were as follows: previous steroid therapy or drugs that alter insulin sensitivity, diabetes mellitus, uncontrolled arterial hypertension, dyslipidemia, chronic kidney disease, thyroid dysfunction, Cushing's syndrome, current smokers, patients with history of acute coronary syndrome during the last 6 months, pregnancy, and patients taking vitamin D replacement therapy. All patients gave their informed consent. The study was approved by the Ethics Committee of University of Medicine and Pharmacy "Victor Babeş" Timişoara, Romania.

2.2. Methods. In all patients, the following were determined: anti-citrullinated peptide antibodies (chemiluminescent microparticle immunoassay, serum), rheumatoid factor (turbidimetry, serum), erythrocyte sedimentation rate (ESR) (Electro Optical System Technologies), C-reactive protein (turbidimetry, serum), fibrinogen (coagulation, plasma citrate), TNF-α (chemiluminescence immunoassay, serum), IL-6 (electrochemiluminescence immunoassay, serum), fasting insulinemia (chemiluminescence immunoassay, serum) and glycemia (photometry, plasma NaF K2 oxalate), and vitamin D levels (25(OH) vitamin D) (chemiluminescence immunoassay, serum).

Rheumatoid arthritis activity was assessed using Disease Activity Score 28 (DAS28). DAS28 was calculated based on ESR, tender joint count (28 joints), swollen joint count (28 joints), and the patient's assessment of global well-being (100 mm visual analogue scale) (http://www.4s-dawn.com/DAS28/DAS28.html).

Insulin resistance was assessed by homeostasis model assessment of insulin resistance (HOMA-IR) index, using fasting insulin and glucose [14].

Endothelial dysfunction was assessed by means of flow-mediated vasodilation, on brachial artery, using B-mode ultrasonography (Siemens Acuson X300 Ultrasound System, with linear transducer of 10 MHz). Before the test, the patient was relaxed at a stable room temperature between 20–25°C; ingestion of caffeine, high-fat foods, and vitamin C was prohibited. The diameter of the brachial artery was measured incidentally with the *R* wave of the electrocardiograph trace (Di). Then, ischemia was induced by inflating the pneumatic cuff to a pressure 50 mmHg above systolic one, in order to obliterate the brachial artery and induce ischaemia. After 5 minutes, the cuff was deflated and the diameter was measured after 60-second postdeflation (Df). FMD was calculated with the formula: $\text{FMD} = [(\text{Df} - \text{Di})/\text{Di}] \times 100$ [15].

In controls, the following were determined: erythrocyte sedimentation rate (ESR), C-reactive protein, fibrinogen, TNF-α, IL-6, fasting insulinemia and glycemia, vitamin D levels, insulin resistance, and flow-mediated vasodilation, using the same methods.

2.3. Statistical Analysis. Data were expressed as mean ± standard deviation. Statistical analyses were performed using the Student's *t*-test and Pearson's correlation. Differences were considered statistically significant at the value of $p < 0.05$.

3. Results

The demographic data of patients and controls are presented in Table 1.

All patients were positive for rheumatoid factor and anti-citrullinated peptide antibodies.

The laboratory findings of RA patients and controls are presented in Table 2.

By analyzing these data, it can be observed that all RA patients presented inflammation, low levels of vitamin D, elevated insulin resistance, and reduced flow-mediated vasodilation. These parameters showed statistically significant differences between RA patients and controls, as presented in Table 2.

Table 2: Laboratory findings in RA patients and controls.

Parameter	Value (mean ± standard deviation)		p
	RA patients	Controls	
ESR (mm/h)	74.11 ± 18.47	8.45 ± 2.99	<0.00001
C-reactive protein (mg/l)	60.34 ± 27.8	2.88 ± 0.98	<0.00001
Fibrinogen (mg/dl)	693.52 ± 284.61	207.18 ± 95.24	<0.0001
DAS28	6.41 ± 0.94	—	—
TNF-α (pg/ml)	89.65 ± 21.41	3.76 ± 1.64	<0.00001
IL-6 (pg/ml)	90.15 ± 20.79	4.41 ± 1.78	<0.00001
FMD (%)	7.94 ± 0.81	13.78 ± 1.43	<0.00001
HOMA-IR	5.97 ± 1.89	1.23 ± 0.19	<0.00001
25(OH) vitamin D	14.90 ± 2.81	36. 39 ± 7.78	<0.00001

Table 3: Differences of FMD, HOMA-IR, and 25(OH) vitamin D between moderate and high disease activity RA patients.

Parameter	Disease activity		p
	Moderate	High	
Number of patients	4	31	
DAS28	4.63 ± 0.26	6.64 ± 0.73	<0.0001
FMD (%)	8.88 ± 0.88	7.23 ± 0.82	<0.05
HOMA-IR	4.27 ± 1.23	6.13 ± 1.92	<0.05
25(OH) vitamin D	19.15 ± 0.72	14.35 ± 2.49	<0.0001

Among the RA patients, only one had vitamin D insufficiency, while another 34 patients had vitamin D deficiency.

Based on DAS28 value, the RA patients were divided into two subgroups: one with moderate disease activity and the other with high disease activity. The RA patients with high disease activity presented lower values of FMD and 25(OH) vitamin D and higher values of HOMA-IR, compared with the RA patients with moderate disease activity (Table 3).

The results of this study showed that the entire group of patients with early active RA presented low levels of 25(OH) vitamin D, high insulin resistance, and endothelial dysfunction.

There were demonstrated significant inverse correlations between the levels of 25(OH) vitamin D and DAS28 ($p = 0.0011$), and respective insulin resistance ($p = 0.0389$), and significant positive correlation between 25(OH) vitamin D and endothelial function, expressed as FMD ($p = 0.0010$). The same correlations were identified in the two subgroups of patients with moderate disease activity and respective high disease activity (Table 4, Figures 1, 2, and 3).

Studying the correlations between proinflammatory cytokines and the levels of 25(OH) vitamin D, significant inverse correlations were identified between 25(OH) vitamin D and TNF-α ($r = -0.4269$, $p = 0.0105$), respective IL-6 ($r = -0.3627$, $p = 0.0322$).

On the other hand, high disease activity of RA, expressed as DAS28, has been positively correlated with insulin resistance ($r = 0.3692$, $p = 0.0029$) and negatively correlated with FMD ($r = -0.3912$, $p = 0.0020$).

4. Discussion

Rheumatoid arthritis affects up to 1% of adults worldwide, representing a serious public health problem, because of articular and extra-articular involvement. Morbidity and mortality due to atherosclerotic cardiovascular diseases are increased in RA patients [16, 17]. Based on the studies of Meune et al. and Avina-Zubieta et al., RA is associated with a 1.48-fold increase in atherosclerotic cardiovascular diseases and a 1.6-fold increase in cardiovascular disease-related death, compared to the general population [18, 19]. Atherosclerotic cardiovascular disease in RA patients has more severe presentation and worse outcomes compared to the general population [20]. Traditional cardiovascular risk factors and RA-related risk factors contribute to this excess of cardiovascular morbidity and mortality of these patients. Among RA-related risk factors, proinflammatory cytokines (TNF-α, IL-6), oxidative stress, an increase of leptin and resistin (proatherogenic hormones) and the decrease of adiponectin (antiatherogenic hormone), and insulin resistance play the most important role in accelerated atherogenesis. The first step in the atherosclerosis process is represented by the endothelial dysfunction [4, 19, 21].

In the last couple of years, it has been emphasized the role of vitamin D in health and disease. Besides the well-known effects on bone metabolism, vitamin D has effects on the immune and cardiovascular systems [22–24].

Normal levels of vitamin D are required to maintain the physiological innate and adaptive immune responses and the immune tolerance of self-antigens. Vitamin D deficiency is associated with the loss of immune tolerance and the appearance of autoimmunity processes, including rheumatoid arthritis [16, 22, 25–27]. The protective effects of vitamin D on the cardiovascular system are represented by the increase of anti-inflammatory cytokine expression (such as IL-10) and by the decrease of proinflammatory molecule expression (such as TNF-α and IL-6) [28].

The connections between low levels of vitamin D, inflammation, insulin resistance, and endothelial dysfunction in rheumatoid arthritis are very complex.

Accelerated atherosclerosis associated with RA is related to systemic inflammation, which characterized this disease [29]. Systemic inflammation contributes to the initiation

Table 4: Correlations between 25(OH) vitamin D and disease activity, insulin resistance, and endothelial function in RA patients.

Correlation	RA patients		
	Whole group	Moderate disease activity group	High disease activity group
25(OH) vitamin D-DAS28	$r = -0.5253$	$r = -0.9072$	$r = -0.3198$
	$p = 0.0011$	$p = 0.046$	$p = 0.0397$
25(OH) vitamin D-HOMA-IR	$r = -0.3506$	$r = -0.9813$	$r = -0.3200$
	$p = 0.0389$	$p = 0.009$	$p = 0.0396$
25(OH) vitamin D-FMD	$r = 0.5287$	$r = 0.9001$	$r = 0.5144$
	$p = 0.0010$	$p = 0.049$	$p = 0.001$

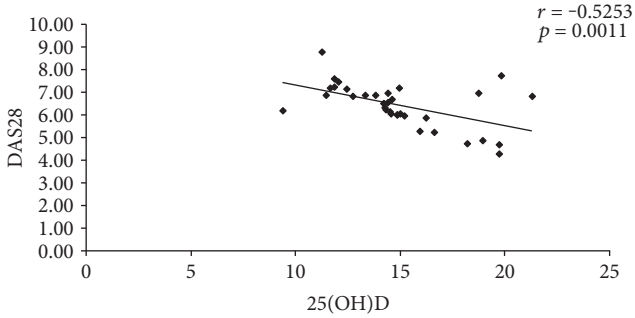

Figure 1: Correlation between 25(OH)D and DAS28.

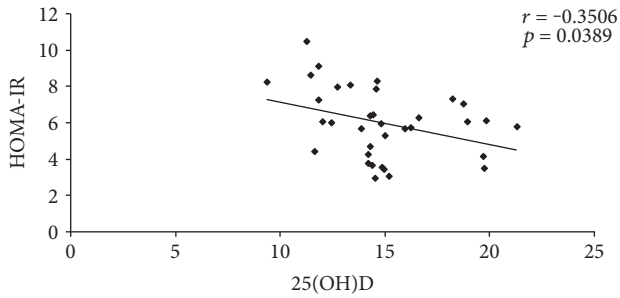

Figure 2: Correlation between 25(OH)D and HOMA-IR.

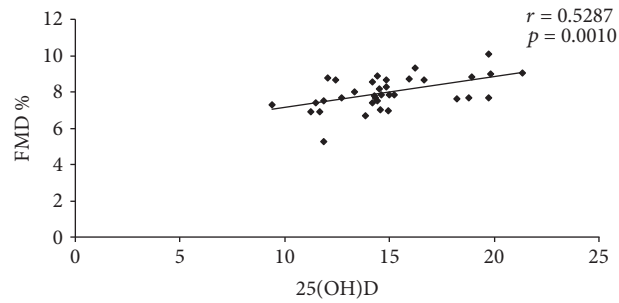

Figure 3: Correlation between 25(OH)D and FMD%.

and development of accelerated atherosclerosis, since inflammatory processes in the rheumatoid synovium and atherosclerotic plaques are remarkably similar. Inflammatory response acts in endothelial dysfunction appearance by means of proinflammatory cytokines, such as TNF-α, IL-6, and IL-1 [30]. In RA, low levels of vitamin D are noted to be common and are even more prevalent than in the general population. These low levels are described to be associated with some cardiovascular risk factors [2, 12, 31]. But vitamin D deficiency is associated with an exacerbation of Th1 immune response, resulting in the upregulation of the expression and production of several proinflammatory cytokines including TNF-α, IL-1β, IL-6, and IL-8, too [32–34]. Inflammatory responses play a crucial role in the pathogenesis and development of IR [35], and the low levels of 25(OH) vitamin D are associated with IR [36]. Insulin resistance induces vasoconstriction and vascular smooth muscle cell proliferation, generating endothelial dysfunction [37].

In the present study, the levels of 25(OH) vitamin D were decreased in all studied patients, compared with controls ($p < 0.00001$), as it has been shown in other studies [2, 12, 31]. Significant inverse correlations ($p = 0.0105$ for TNF-α, respective $p = 0.0322$ for IL-6) have been highlighted between the levels of proinflammatory cytokines (TNF-α and IL-6) and the levels of 25(OH) vitamin D. Welsh et al. showed that the degree of systemic inflammation is inversely associated with the circulating levels of vitamin D [31].

Vitamin D may also have a role in modulating RA disease activity [7]. The results of the present study showed that the low levels of 25(OH) vitamin D were correlated in an inversely manner with the RA activity, expressed as DAS28 ($p = 0.0011$). The same inverse correlations were identified in RA patients with moderate disease activity ($p = 0.046$) and respective high disease activity ($p = 0.0397$). Rossini et al., studying 1191 RA patients and 1019 healthy controls, identified a significant inverse correlation between the logarithm of the 25(OH) vitamin D levels and the RA activity ($p = 0.002$) [38]. Hong et al. demonstrated on the 130 patients with RA and 80 healthy controls that the RA patients had lower levels of 25(OH) vitamin D and these levels correlated significantly with the RA activity ($r = -0.43$) [16]. El-Barbary et al. included in their study forty early RA patients and forty healthy controls. The authors demonstrated the significant negative correlation between 25(OH) vitamin D levels and DAS28, respective IL-6 ($p < 0.001$) [39]. Other studies obtained the same relationship between the levels of serum 25(OH) vitamin D and disease activity in RA patients, reporting the correlation

coefficient as $r = -0.57$ [40], $r = -0.431$ [41], $r = -0.42$ [42], and $r = -0.604$ [43].

Hypovitaminosis D increases the insulin resistance and may trigger endothelial dysfunction [44]. In our study, it was identified a significant inverse correlation between low levels of serum 25(OH) vitamin D and insulin resistance, expressed as HOMA-IR ($p = 0.0389$). Strong inverse correlation was obtained in both subgroups of rheumatoid patients with moderate disease activity ($p = 0.009$) and respective high disease activity ($p = 0.0396$). Hirschler et al. reported the same results in their study ($r = -0.29$, $p = 0.002$) [45]. The role of vitamin D in modulating insulin sensitivity is emphasized in the study published by Miñambres et al. [46].

Previous studies have shown that insulin resistance is associated with endothelial dysfunction, an early marker of atherosclerosis [21, 47, 48].

Borges et al. demonstrated that vitamin D improved the endothelial function, suggesting the relationship between hypovitaminosis D and endothelial dysfunction [49]. The present study showed the positive correlation between the serum levels of 25(OH) vitamin D and endothelial function expressed as FMD ($p = 0.0010$). In the subgroup of RA patients with moderate disease activity, it was shown a positive correlation between the serum levels of 25(OH) vitamin D and endothelial function ($p = 0.049$), while in a subgroup of patients with high disease activity, the correlation was positive, too, but more significant ($p = 0.001$). The explanation of this finding is based on the fact that high disease activity is associated with lower levels of 25(OH) vitamin D, higher inflammatory responses and insulin resistance, and significant endothelial dysfunction [35–37]. In their study, Jablonski et al. found endothelial dysfunction in adults with hypovitaminosis D, as opposed to patients with normal levels of this vitamin [50]. In RA patients with vitamin D deficiency, Ranganathan et al. revealed a significant correlation between serum 25(OH) vitamin D and endothelial function ($p = 0.04$) [51].

Our study revealed that low levels of 25(OH) vitamin D were associated with high disease activity, high insulin resistance, and endothelial dysfunction in early RA patients. But disease activity in itself contributes to the development of atherosclerosis through insulin resistance [52–54]. Between DAS28, as a marker of RA activity and FMD, it was observed as an inverse correlation ($r = -0.3912$, $p = 0.020$), and, on the other hand, between DAS28 and insulin resistance it was identified as a positive significant correlation ($r = 0.3692$, $p = 0.029$).

The present study had some limitations. The first limitation was the small sample size, being investigated only 35 patients. Second, only the serum 25(OH)D was determined. The free bioavailable vitamin D and vitamin D-binding protein were not measured.

5. Conclusion

In early RA patients with moderate and high disease activity, low serum levels of vitamin D were associated with disease activity, increased insulin resistance, and endothelial dysfunction.

Conflicts of Interest

The authors declare no conflicts of interest.

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Research Article

Detection of Pathological Changes in the Aorta during Thoracic Aortic Aneurysm Progression on Molecular Level

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The progression of thoracic aortic aneurysm depends on regulation of aortic wall homeostasis and on changes in the structural components of the extracellular matrix, which are affected by multiple molecular signalling pathways. We decided to correlate the diameter of ascending thoracic aneurysm with gene expression of inflammation markers (IL-6, CRP), cytokine receptors (IL-6R, TNFR1, and TNFR2), and extracellular matrix components (Emilin-1, MMP9, and TIMP) for detection of the degree of pathological process of TAA formation. The experimental group was divided into three groups according to the diameter of the aortic aneurysm. Whole blood and tissue samples were properly collected and used for nucleic acid, chromatin, and protein isolation. The mRNA levels were detected by qRT-PCR. For the detection of protein levels a Cytokine Array IV assay kit was used in combination with a biochip analyzer. In aortic tissue, significant positive correlations were found between increased mRNA levels of inflammatory cytokines (CRP and IL-6) on both mRNA levels in tissue and protein from the blood with maximum in stage 3. Changes of gene expression of selected genes can be used for the experimental study of the inflammatory receptor inhibitors during trials targeted on slowing down the progress of aortic wall aneurysm.

1. Introduction

Thoracic aorta diseases are among the most common causes of death in the US and other developed countries. The incidence of thoracic aortic aneurysm has been estimated at 6 cases per 100,000 persons per year [1], and its prevalence has been estimated at 3-4% in patients over age 65 years old. According to possible endovascular therapy (EVT) or its combination with surgical therapy, it seems most practical to divide thoracic aorta diseases into acute aortic syndromes (AAS) and chronic aneurysmal transformation (thoracic aortic aneurysm—TAA) [2]. The AAS group includes penetrating aortic ulcer (PAU), bordered aortic intramural hematoma (IMH), and classical acute aortic dissection, while chronic aneurysmal transformation includes right degenerative aneurysm of the ascending or descending aorta, false TAA (pseudoaneurysm), and posttraumatic TAA [3].

The growth rate of TAA varies by lesion location, with ascending aneurysms growing at a rate of 0.07 cm per year. The rate of growth has also been demonstrated to increase with aneurysm size [4]. When the diameter of the thoracic aorta is more than 3.5 cm, or twice the normal diameter, the thoracic aorta is considered aneurysmal. The risk of aneurysm rupture is proportionate to its size [5]. The decision of whether to surgically treat a patient is based on aneurysm size and patient operative risk. Annual risk of rupture, dissection, or death is 14.1% in patients with

Table 1: Demographic and clinical characteristics of subjects.

	Control	Stage 1	Stage 2	Stage 3
Number of patients	35	12	34	14
Gender (female %)	20/35 (57%)	2/12 (17%)	5/34 (14.7%)	2/14 (14.3%)
Age (years)	45 ± 9.6	49.9 ± 16.6	55.1 ± 14.7	54.5 ± 14.1
Aorta diameter (mm)	—	43 ± 2.3	51 ± 2.8	59.5 ± 3.7
AR/AS	—	6/6 (50/50%)	19/15 (56/44%)	8/6 (57/4%)
BAV/TAV	—	8/4 (67%)	21/13 (62%)	8/6 (57%)
Hypertension	5/35 (14%)	8/12 (67%)	18/34 (52.9%)	12/14 (85.7%)
Diabetes mellitus	0	1/12 (8%)	3/34 (8.8%)	2/14 (14.3%)
Current smokers	9/35 (26%)	7/12 (58.3%)	22/34 (64.7%)	11/14 (78.6%)
BMI	26.4 ± 4.2	29.4 ± 6.4	29.6 ± 4.9	26.99 ± 4.9
Weight (kg)	76.9 ± 15.2	89.2 ± 16.9	89.3 ± 13.7	87.4 ± 16.3

aneurysms larger than 6 cm, compared with 6.5% for aneurysms between 5 and 6 cm [6]. Additionally, repair is suggested in patients with documented aneurysm growth of >1 cm per year [7]. The progression of aortic aneurysm probably results from a combination of chronic inflammation, hemodynamic stress, aortic mechanical injury, and epidemiologic risk factors. Aortic functions as well as regulation of aortic wall homeostasis depend on changes in the structural components of the extracellular matrix (ECM), which are affected by multiple molecular signalling pathways.

Microfibrils, as the main structural component of aortic wall ECM, provide a scaffold for the lysyl oxidase protein family to cross-link tropoelastin monomers to form mature elastic fibres [8]. Microfibrils are composed mainly from fibrillin and several microfibril-associated proteins (elastin microfibril interface-located protein 1 (Emilin-1), microfibril-associated glycoproteins (MAGP-1,2), and fibulins) [9]. Emilin-1 is known to be a binding precursor of TGF-β, called pro-TGF-β, and inhibits its maturation by furin convertases [10]. Defects in Emilin-1 expression affect the formation and function of elastic lamellae, increasing the degree of inflammation.

Inflammation participates in the pathogenesis of aortic aneurysm formation by the migration of T-lymphocytes and macrophages diffusely throughout the adventitia to the tunica media [11]. Several cytokines and chemokines that are produced during their transfer (TNF-α, interferon-γ, IL-1, IL-2, IL-6, and IL-8) are upregulated and promote the recruitment of other inflammatory cells to the aortic wall [12]. The main protein of the acute phase of inflammation—serum C-reactive protein (CRP)—has been reported in patients with stenotic atherosclerotic disease and is associated with an increased risk of developing cardiovascular events [13]. However, only a few articles have been published describing serum CRP and IL-6 in patients with TAA. Inflammatory changes consequently induce the secretion of matrix metalloproteinases (MMP), elastase, and collagenase from macrophages and neutrophils that are capable of directly degrading the ECM and may also contribute to the detachment of smooth muscle cells from the ECM, leading to cell death [14]. The important regulators of local MMP activity are tissue inhibitors of metalloproteinases (TIMPs) [15]. Animal data support a role for MMP and TIMP in the development of aortic aneurysm, especially in Marfan syndrome [16]. Most of the data on MMPs and aortic aneurysm are from studies of abdominal aortic aneurysms, while data is lacking on MMP in TAA. Studies of TAA have focused on changes in genetic expression, which does not necessarily translate into the changes in protein expression that determine the effect on the aorta [17]. The proteolytic theory of TAA development envisions increased concentrations of MMP and reduced concentrations of TIMP in the aorta acting in concert to increase ECM degradation, leading to an aorta that is more likely to expand in response to the hemodynamic load [18]. In this study, we decided to correlate the diameter of ascending thoracic aneurysm with gene expression of inflammation markers (IL-6, CRP), cytokine receptors (IL-6R, TNFR1, and TNFR2), and components of ECM (Emilin-1, MMP9, and TIMP) for detection of the degree of pathological process of TAA formation. The obtained results could help surgeons decide if the progression of aortic aneurysm is too fast and give them a chance to improve the lifetime and healthcare of patients suffering with progressive TAA.

2. Materials and Methods

2.1. Experimental Groups and Sample Collection. The experimental group ($n = 60$) consisted of patients suffering from thoracic aortic aneurysm, regurgitation, and aortic valve stenosis, who were divided into three groups according to the diameter of the aortic aneurysm (stage 1: 43 ± 2.3 mm, stage 2: 51 ± 2.8 mm, and stage 3: 59.5 ± 3.7 mm). Each group was characterized by age, gender, BMI, presence of aortic regurgitation or aortic stenosis (AR/AS), bicuspid or tricuspid aortic valve (BAV/TAV), hypertension, diabetes mellitus, current smoking, and family predispositions for cardiovascular diseases (Table 1). Results of preoperative medical tests of individual biochemical and haematology biomarkers were within acceptable physiology intervals according to the patient's clinical status.

Whole blood was collected from all patients of the experimental group during the standard preoperative examination done in cooperation with the Eastern Slovak Institute of

Cardiovascular Diseases (VUSCH). Results of preoperative medical tests of individual biochemical and hematology biomarkers were within acceptable physiology intervals according to the patient's clinical status. The tissue samples of patients in the experimental group were collected during the following surgical procedures: replacement of the ascending aorta, valve-sparing procedure (aortic root remodeling), and replacement of the aortic valve (AVR) with a mechanical or biological prosthetic valve. Other concomitant procedures were replacement of the mitral valve (MVR) and off-pump coronary artery revascularization (OPCAB). The operations were performed through a median sternotomy with the use of cardiopulmonary bypass, mild hypothermia, and cardioplegic arrest. Cardio anesthesia was performed according to the standard protocol.

2.2. Control Group and Sample Collection. Control group ($n = 35$) blood samples were taken from blood donors in cooperation with the UNLP Department of Haematology and Transfusiology. The control group was composed of people with negative results from biochemical and haematological screening medical tests. Donors declared themselves free of any symptoms of cardiovascular diseases, and their clinical imaging methods, ultrasonography and computed tomography angiography, were also negative. Control materials of the ascending part of thoracic aortic tissues ($n = 10$) were obtained from the Department of Forensic Medicine of LF UPJŠ and UNLP. These necroptic samples were collected immediately after death. Cause of deaths was not related to pathology, such as thoracic aortic aneurysm, regurgitation or stenosis of the aortic valve, or any pathology associated with myocardial damage. Histopathology detection of the myocardial tissue samples did not show any noticeable changes.

All clinical investigations using human samples have been carried out in accordance with the code of ethics of the world medical association (Declaration of Helsinki). Healthy subjects in the control group and patients in the experimental group answered a medical sheet and questionnaire. Patients were informed by their doctor about the use of their blood and tissue for experimental diagnostic purposes. Informed consents were signed. Ethical consent for this study was given by the institutional committee on human research, was approved by the ethical committee of the Eastern Slovak Institute of Cardiovascular diseases (VUSCH), and was compliant with ethical standards on human experimentation and with the Declaration of Helsinki.

2.3. RNA, DNA, and Chromatin Isolation. All tissue samples were frozen immediately after harvesting at −196°C and stored in a freezer at −80°C. Whole blood samples, collected into Paxgene test tubes, were stored in a fridge for 2 hours and then used for isolation of DNA and RNA. For isolation of total RNA from 40 mg of tissue or 2 ml of whole blood, the isolation protocol RNeasy Mini Kit (Qiagene, Hilden, Germany) was used following the manufacturer's protocols. Chromatin isolation starts from 40 mg of tissue from the ascending part of the thoracic aorta. The tissue was washed using ice cold 1× PBS. After centrifugation at 1300 rpm/5 min/4°C, the supernatant was carefully removed and the pellet was resuspended thoroughly in 2 ml of 1× PBS containing of 1% formaldehyde for cross-linking the cells/for 8 min/37°C. Cross-linking was stopped by the addition of 125 mM of glycine. After centrifugation at 1300 rpm/5 min/4°C, the pellet was washed 5× with ice cold 1× PBS. The pellet was lysated with lysis buffer (LB consists of 155 mM NH4Cl, 10 mM KHCO3, and 0.1 mM EDTA pH 7.4) on ice for 10 minutes with gentle mixing. After centrifugation at 1500 rpm/5 min/4°C, the cell lysates were sonicated with a Bioruptor (Diagenode, Denville, USA) at high intensity for 5 min, with 30 s on/off intervals. For preclearing of chromatin, Protein G agarose beads (Merck Millipore, Prague, Czech Republic) were used for 1 hour/4°C. Chromatin fragments with lengths of 200 bp were visualized on agarose gel. Isolated cells were snap-frozen and stored at −80°C. A Nanodrop LC 3000 (Thermo Scientific, Bratislava, Slovak Republic) was used for measuring the concentration and purity of isolated chromatin, DNA, and RNA.

2.4. qRT-PCR. For detection of changes in the mRNA expression levels of specific genes *IL-6*, *hsCRP*, *TIMP*, *Emilin-1*, and *MMP9*, a Rotor-Gene Q-PCR thermocycler (Qiagene, Hilden, Germany) was used. RNA isolated from blood was transcribed into cDNA by using specific reverse primers individually for each gene and an M-MLV reverse transcriptase kit (Sigma-Aldrich). We also isolated chromatin from tissue and detected transcription activity of RNA Pol II, Emilin-1, and MMP9 using qRT-PCR methods after chromatin immunoprecipitation. In all, triplicated analyses were performed for each gene. Selected experimental genes (*IL-6*, *hsCRP*, *TIMP*, *Emilin-1*, and *MMP9)* and control housekeeping genes (*HPRT*, *ETNK*, *and GAPDH)* were amplificated by using 34 cycles (95°C/5 min, 95°C/15 s, 58–62°C/20 s, and 72°C/25 s) using appropriate specific primer sequences (Table 2). More detailed primer sequences are in Supplementary Table 5 available online at https://doi.org/10.1155/2017/9185934. Numerical quantification of changes in the expression of mRNA levels was evaluated by the comparative quantification and Ct value Q Rotor gene Software. The determination starts when, for each sample, difference between ΔCt of studied gene and control gene was calculated, then subtracted between ΔCt of sample with unknown concentration and ΔCt of the calibrator. The final result was a multiple of the calibrator concentration.

2.5. Copy Number Variation Analysis. Analysis of gene copies was performed after the isolation of DNA, using specific primers for all exon-specific gene domains of *IL-6*, *hsCRP*, *TIMP*, *Emilin-1*, and *MMP9* in comparison with HPRT and GAPDH. Amplification of specific genes was run for 33 cycles (95°C 5 min, 95°C for 15 seconds, 58°C–60°C for 20 seconds, and 72°C for 25 seconds) using the appropriate primer sequences with the Rotor-Gene Q-PCR (Qiagene, Hilden, Germany) thermocycler.

2.6. ChIP qRT-PCR. Precleared chromatin (125 ng) was incubated with selected antibodies (anti-RNAPII CTD YSPTSPS, anti-Emilin-1, and anti-MMP9, ab817, ab185953, ab 38898

Table 2: Localization of the chromosome of specific genes (http://www.genome.ucsc.edu).

Name of gene	Chromosomal localization	Size of gene in bp including UTR side	Analysis place of gene Ex-exon
IL-6	7p15.3	22, 766, 766–22, 770, 157	Prom, Ex1, Ex4
CRP	1q23.2	159, 682, 079–159, 684, 379	Ex1, Ex2
TIMP	Xp11.23	47, 441, 712–47, 446, 188	Ex1, Ex3
Emilin-1	2p23.3	27, 301, 435–27, 309, 265	Prom, Ex1, Ex3, Ex4, Ex6
MMP9	20q13.12	44, 635, 634–44, 647, 114	Prom, Ex1, Ex5, Ex9, Ex13
GAPDH	12p13.1	6, 643, 585–6, 647, 537	Ex2, Ex3, EX4
HPRT	Xq26.2-q26.3	133, 594, 175–133, 634, 698	Ex3, Ex6
ETNK	12p12.1	22, 778, 076–22, 843, 608	Ex1, Ex3, Ex7

(Abcam, Cambridge, UK) at 4°C overnight. The RNase treatment was done 30 minutes before incubation using a mix of RNase A/T (Roche Slovakia, Bratislava, Slovak Republic). Protein IgG agarose beads (Merck Millipore, Prague, Czech Republic) were used for bonding with immunocomplexes and cross-linking them. The next procedure used the downward line of cleaning buffer solutions in the order buffers I and II (500 mM NaCl, 50 mM HEPES (pH 7.5), 1% Triton-X-100, 0.1% sodium deoxycholate, and 1 mM EDTA (pH 7.5)), buffer III (10 mM Tris-Cl, 250 mM LiCl, 0.5% NP-40 (pH 8.0), 0.5% sodium deoxycholate, and 1 mM EDTA (pH 7.5)), and buffer IV (1 mM EDTA, 10 mM Tris-HCl). Immunoprecipitated DNA was eluted from the beads in TE Tris-EDTA buffer with 1% SDS. For reverse cross-linking of the samples, a solution was used containing 5 mol/l NaCl, 5 g/ml of enzyme RNaseA (Roche Slovakia, Bratislava, Slovak Republic), 1 M Tris-HCL (Sigma-Aldrich, Bratislava, Slovak Republic), and 20 g of proteinase K (Roche Slovakia, Bratislava, Slovak Republic), which was incubated at 65°C overnight and purified using a Qiagen PCR purification columns kit (28104, Qiagene, Hilden, Germany). DNA was eluted twice with 30 μl of RNAase/DNAase free water (Qiagene, Hilden, Germany). An aliquot of 2 μl of each sample was used for qRT-PCR using SensiMix (Bioline, Luckenwalde, Germany). Amplification was performed on a Qiagene Rotor-Gene Q-PCR thermocycler using the protocol: 30 cycles (95°C for 5 min, 95°C for 15 s, 60°C for 20 s, and 72°C for 25 s). All primer pairs (Sigma-Aldrich, Bratislava, Slovak Republic) used for ChIP analysis were designed using the Internet databases (http://www.genome.ucsc.edu/ and http://www.bioinformatics.org/sms/rev_comp.html). The primer pairs are listed in Table 2.

2.7. Protein Analysis by Randox Biochip. For the detection of protein levels in the serum of both the experimental and control groups, a Cytokine Array IV assay kit was used in combination with a biochip analyzer (Evidence Investigator, Randox Laboratories Ltd., London, UK). Detection of proteins IL-6, IL-6R, hsCRP, MMP9, TNFR1, and TNFR2 started with the incubation of a sample with 200 μl of assay buffer for 1 hour/37°C/370 rpm of 100 μl. After incubation, the procedure continued by decantation of the liquid and the washing of each well 2 times. The second incubation using the same conditions continued after adding conjugation buffer. After the second incubation, another decantation of liquid and the washing of each well 4 times were done. A mixture of luminol-EV-70l together with hydrogen peroxide was added to each well and incubated for 2 minutes. Visualization and calculation of the proteins levels (ng/ml) of each biomarker were performed using the Evidence Investigation biochip software version 4.

2.8. Statistical Analysis. All values are expressed as means ± SD for normally distributed data. Differences in proportions of categorical variables were analysed using Pearson chi-squared test, and continuous variables with normally distributed values were analysed using the Student *t*-test, whereas nonnormally distributed continuous data were analysed with Mann–Whitney *U*-test for two independent samples and Kruskal-Wallis test for more than two independent samples. Possible associations between aneurysm diameter and selected markers were evaluated by Pearson correlation test. Relationship between progression of aneurysm and selected markers was evaluated by linear regression. The level of statistical significance was set at $p < 0.05$. All analyses were performed using IBM SPSS 22.00 statistical software package.

3. Results

During the analysis of demographic data impact on the formation and progression of the aortic aneurysm, there were no any significant correlations found. We found that there is no difference in mean age between groups divided by aortic diameter. Differences are not statistically significant ($rs = 0.293$, $p = 0.084$). Due to the low number of women, there is no statistically significant difference in the male/female ratio in the individual aortic diameter categories ($rs = 0.240$, $p = 0.070$). Other demographic data (obesity, smoking, hypertension, and diabetes) also showed no significant correlation with aortic diameter with $p > 0.05$.

3.1. Changes in the Levels of Cytokines and Their Receptors. The real-time PCR for IL-6 mRNA (Figure 1) in aortic tissue showed increased expression rising from stage 1 to the maximum in stage 3 (650% higher, $p < 0.001$) versus aortic controls.

Immunochemical evaluation (Figure 2) of the blood serum showed a similar increase in the levels of final protein IL-6.

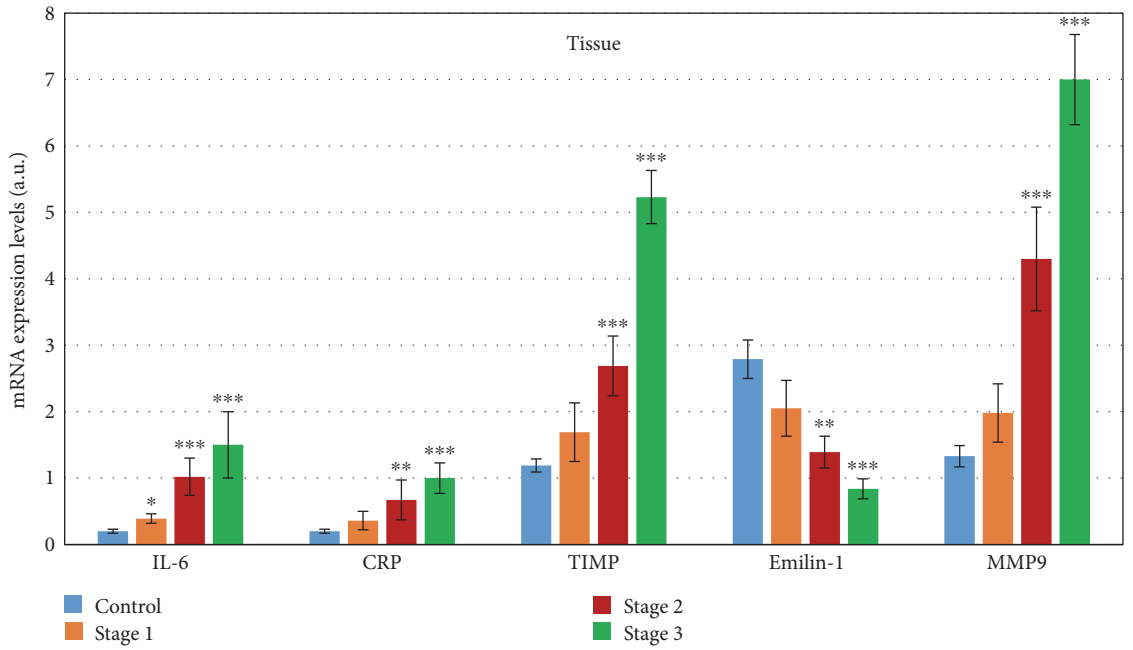

Figure 1: Expression of marker genes on mRNA levels in tissue of the patients with different stages of TAA. The mRNA levels of all detected genes were compared to controls (C, $n = 10$). All data are presented as average ± SD: 1—mean aortic diameter 43 ± 2.3 mm ($n = 10$), 2—mean aortic diameter 51 ± 2.8 mm ($n = 34$), and 3—mean aortic diameter 59.5 ± 3.7 ($n = 14$). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ mean statistical significance. Maximal levels reached values about 400% higher than controls ($p < 0.001$).

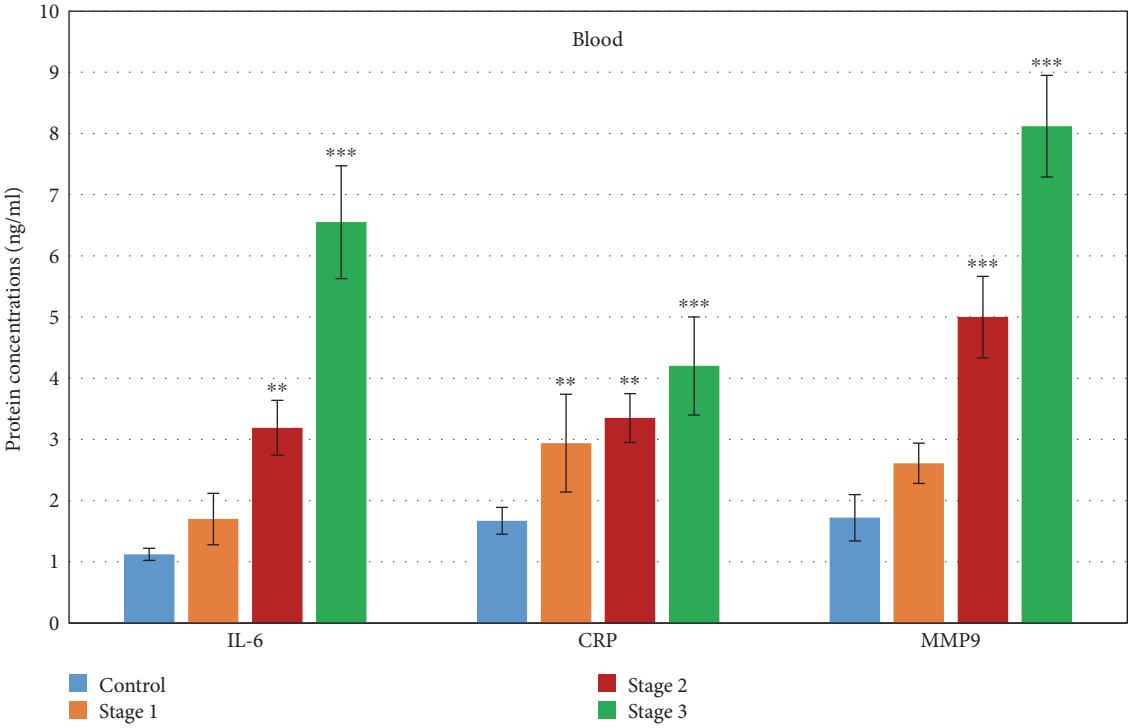

Figure 2: Expression of marker genes on protein levels in the blood of patients with different stages of TAA. The protein levels of all detected genes were compared to controls (C, $n = 10$). All data are presented as average ± SD: 1—mean aortic diameter 43 ± 2.3 mm ($n = 10$), 2—mean aortic diameter 51 ± 2.8 mm ($n = 34$), 3—mean aortic diameter 59.5 ± 3.7 ($n = 14$)., $^{**}p < 0.01$ and $^{***}p < 0.001$ mean statistical significance.

Table 3: Spearman correlations and linear regression between the mRNA, protein levels, and aortic diameter for selected cytokines and their receptors.

	mRNA in aortic tissue		Protein levels in blood				
	CRP	IL-6	CRP	IL-6	IL-6R	TNFR1	TNFR2
Correlation coefficient	0.489**	0.554**	0.025	0.320	−0.37*	0.272	0.268
p value	0.015	0.017	0.642	0.524	0.024	0.058	0.063
Unstandardized coefficient	0.105	0.710***	0.027	−0.006	0.007	0.966	0.771
p value	0.197	0.000	0.051	0.503	0.004	0.177	0.120

Statistical significance: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

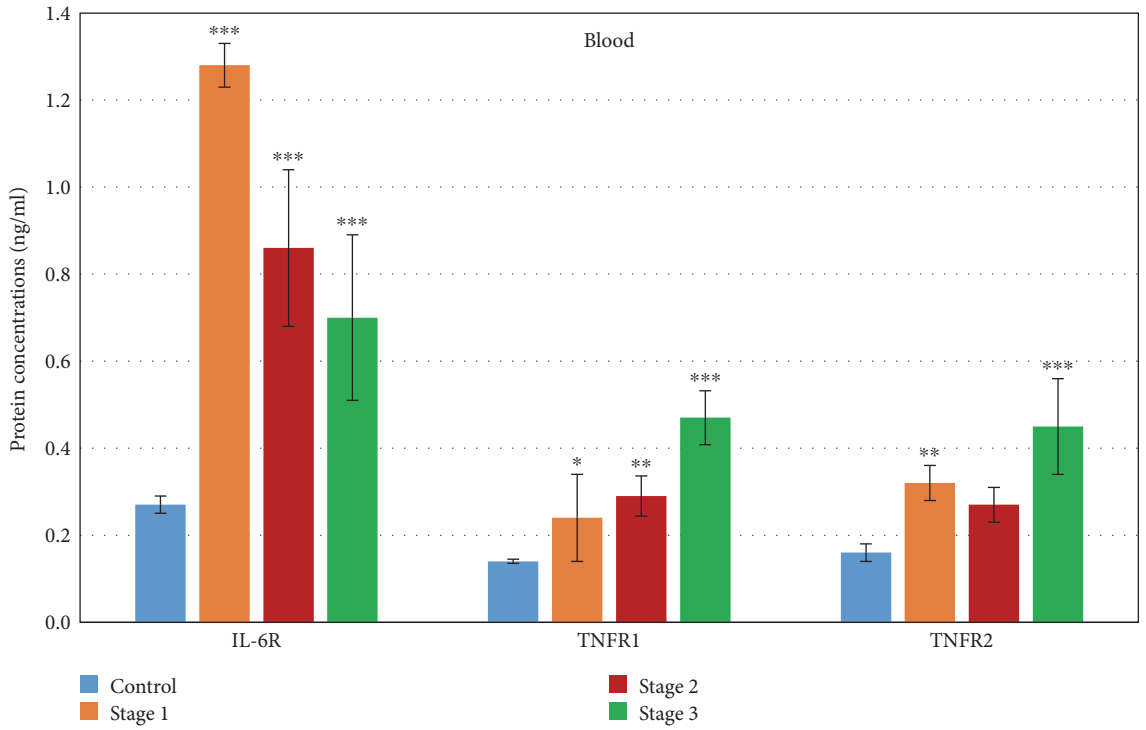

Figure 3: Expression of cytokine receptors on protein levels in blood of patients with different stages of TAA. The protein levels of all detected genes were compared to controls (C, $n = 10$). All data are presented as average ± SD: 1—mean aortic diameter 43 ± 2.3 mm ($n = 10$), 2—mean aortic diameter 51 ± 2.8 mm ($n = 34$), 3—mean aortic diameter 59.5 ± 3.7 ($n = 14$). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ mean statistical significance.

According to the Spearman correlation coefficients (nonparametric correlation), a strong correlation exists between the rising expression of IL-6 mRNA and increased aorta diameter (Table 3). Linear regression showed using categorical variable aorta diameter as a dependent variable that if the aorta diameter increases about one then an expression of IL-6 mRNA in tissue will be elevated about 0.710 ($p < 0.001$). Serum protein IL-6 did not correlate with the aorta diameter. The detection of mRNA levels of CRP in the aortic tissue (Figure 1) showed almost exponential growth from stage 1 to stage 3 against the control tissues.

Protein levels of CRP in blood serum (Figure 2) did not increase so dramatically. We found a maximal increase in stage 3 (about 151% times higher, $p < 0.001$) in comparison with the control serum.

We also demonstrated that the mRNA of CRP is produced in aneurysmal tissue, and its rising concentrations are associated with aneurysmal size, proved by Spearman correlation (Table 3).

Serum protein CRP was not correlated with the aorta diameter, because its levels could be affected by multiple factors related to nonspecific inflammation or infection and not only by aortic tissue damage. The most significant differences in protein levels of the cytokine receptors in blood were detected using antibodies for IL-6 receptor, where we found decreasing levels of protein from stage 1 with 374% higher levels than the control ($p < 0.001$) to almost 159% higher against the control in stage 3 ($p < 0.001$) (Figure 3).

In contrast to these data, the protein levels of both TNFR receptors (TNFR1 and 2) were significantly elevated to the

Table 4: Spearman correlations and linear regression between the mRNA, protein levels, and aortic diameter for a selected member of ECM.

	mRNA in aortic tissue			Protein levels in blood
	MMP9	Emilin-1	TIMP	MMP9
Correlation coefficient	0.386*	−0.496**	0.470**	0.320*
p value	0.048	0.015	0.025	0.045
Unstandardized coefficient	0.193***	−0.533***	0.280***	0.007
p value	0.000	0.000	0.000	0.004

*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ mean statistical significance.

maximum in stage 3 (Figure 3), with values about 230% and 181% higher than controls (both with $p < 0.001$).

During the statistical evaluation of Spearman correlation data, we found medium negative correlation between the aortic aneurysm diameter and protein levels of IL-6R ($rs = -0.37$, $p < 0.024$). The protein levels of TNFR1 positively correlated nonsignificantly with the progress of TAA ($rs = 0.272$, $p < 0.058$). All data are shown in Table 3.

3.2. Changes in the Expression of Components of ECM. The expression of Emilin-1 in aortic tissue on mRNA levels revealed a nonsignificant decrease from 28% less than control in stage 1 to a 70% smaller value against controls ($p < 0.001$) in stage 3. Linear regression showed using categorical variable aorta diameter as a dependent variable that if the aorta diameter increases about one then an expression of Emilin-1 mRNA in tissue will be decreased about 0.533 ($p < 0.001$). The pathological changes in ECM were confirmed by the detection of mRNA for MMP9 and TIMP (Figure 1). We found that the increase in mRNA levels of MMP9 (with the maximum in stage 3 about 490% higher than controls, $p < 0.001$) also affected the expression of TIMP; the mRNA levels of which were also elevated. Linear regression showed using categorical variable aorta diameter as a dependent variable that if the aorta diameter increases about one then an expression of MMP9 mRNA in tissue will be elevated about 0.193 ($p < 0.001$). Maximal levels of TIMP mRNA were detected in stage 3, with the value about 340% higher than in controls ($p < 0.001$). Linear regression showed using categorical variable aorta diameter as a dependent variable that if the aorta diameter increases about one then an expression of TIMP mRNA in tissue will be elevated about 0.280 ($p < 0.001$). The MMP9 protein levels in the blood were measured in the time-course of the growing diameter of the thoracic aorta aneurysm. We found that protein levels of MMP9 were nonsignificantly elevated from the initial stage of TAA (52% higher than controls) to highly significant levels about 372% higher in stage 3 ($p < 0.001$).

Spearman correlation of aneurysm diameter revealed a weak positive correlation in the mRNA of MMP9 ($rs = 0.386$, $p < 0.048$), which was also confirmed by the weak positive correlation of TAA progress with the blood level of protein MMP9 ($rs = 0.320$, $p < 0.045$). In agreement with this data, we found a medium positive correlation in mRNA levels of MMP9 and inhibitor TIMP ($rs = 0.470$, $p < 0.025$). The other marker of ECM degradation progress, Emilin-1, showed medium negative correlation with an aneurysm diameter on the mRNA level ($rs = -0.496$, $p < 0,015$). All data are shown in Table 4.

3.3. Results of Spearman Correlation Analysis. The mRNA levels of both IL-6 and CRP had a similar rising ratio in the aortic tissue in all stages, which was also confirmed in the levels of both proteins in blood serum, where the IL-6 concentrations were about 160% higher than the levels of CRP ($p < 0.001$). During the statistical analysis of obtained results, we found a highly significant positive correlation between the expressions of mRNA of IL-6 in aortic tissue and protein levels of IL-6 in the blood ($rs = 0.449$, $p < 0.01$). A similar statistically significant positive correlation (Figure 4) was found between the mRNA expression of CRP in aortic tissue and protein levels of CRP in the blood ($rs = 0.394$, $p < 0.01$).

All of the results showed a strong positive correlation between mRNA levels of MMP9 and its protein MMP9 in blood ($rs = 0.989$, $p < 0.001$), which suggests that the progressive pathological changes of the TAA tissue cause the release of MMP9 into the blood of patients. This fact is also supported by the strong positive correlation between mRNA levels of TIMP and MMP9 in the tissue ($rs = 0.934$, $p < 0.01$, Figure 4). Linear regression revealed that if the level of MMP9 mRNA in tissue will be elevated about one then the mRNA of TIMP will be increased about 1.188 ($p < 0.001$). Because of the inhibitory activity of TIMP on MMP9, the imbalance between these genes is considered to be important in the degenerative process. Linear regression showed using categorical variable MMP9 mRNA as a dependent variable and protein MMP9 as an independent variable that if concentration of mRNA in tissue increases about one then the level of protein MMP9 in blood will be elevated about 0.013 ($p < 0.051$). Similarly to that also linear regression showed using IL-6 mRNA as a dependent variable and protein IL-6 as an independent variable that if concentration of mRNA in tissue increases about one then the level of protein IL-6 in blood will be elevated about 0.021 ($p < 0.022$).

4. Discussion

The occurrence and expansion of an aneurysm probably depend on local hemodynamic factors and intrinsic factors of the affected arterial segment. The medial layer of the aorta wall is responsible for its tensile elasticity and strength. During the formation of thoracic aorta aneurysm, elastin content in the ECM of the ascending aorta is progressively degrading. The activity and gene expression of specific enzymes (TGF, MMP) are increased, and this leads to the

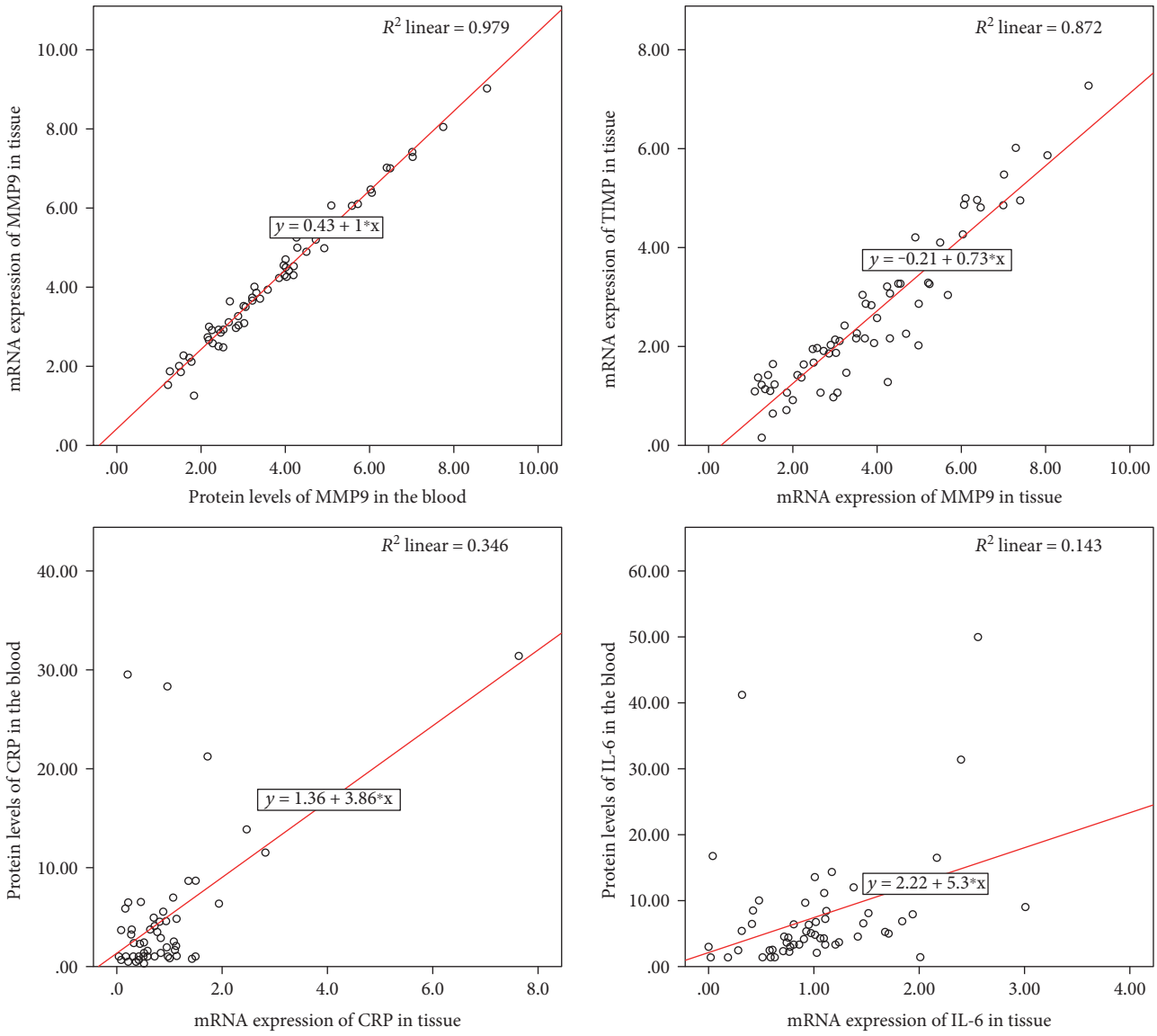

Figure 4: Graphical expression of the Spearman correlation of selected cytokines and markers of ECM damage.

degradation of the structural proteins (elastin, fibullin, and collagen). Elastic fibre fragmentation and loss together with degeneration of the media result in a weakening of the aortic wall, loss of elasticity, and consequent dilation [19].

One of the main members of ECM is Emilin-1 [20]. Emilin-1 has multiple roles, like inhibiting elastin deposition by smooth muscle cells (SMC) [21] as well as regulation of the bioavailability of TGF-β by inhibiting proteolysis of the proTGF-β precursor to LAP/TGF-β, a complex from which the growth factor can be subsequently released for receptor binding [22]. The absence of Emilin-1 causes a remarkable increase of active TGF-β, which consequently through the SMAD cascade upregulates genes involved in ECM destruction (MMP9) or decreases SMC proliferation (p27) [23]. The importance of Emilin-1 expression was also confirmed by the experiment of Pivetta et al. [10], which showed that mice deficient in *Emilin-1* had increased TGF-β activity; however, these mice had a low incidence of aneurysms and no dissection. Another study, by Lee et al. [24], however, showed that embryonic mutation of the type II TGF-β receptor gene (*Tgfbr2*) impaired elastogenesis and resulted in aneurysm formation and inflammation. We found that the increasing diameter of aortic aneurysm significantly correlates negatively with decreasing levels of Emilin-1 mRNA in the affected tissue, which confirmed the direct involvement of Emilin-1 in the regulation of degradation processes of ECM in the aortic wall.

Another impairment in the maintaining of physiological conditions of ECM in aortic wall is upregulation of the expression of MMPs, which are defined as proteases produced by leukocytes and smooth muscle cells (SMCs) within the aortic wall and acting on a variety of extracellular protein substrates [25]. Specific MMP9 degrades type IV collagen, elastin, and various basement membrane proteins of SMCs. Its expression increases within 3 hours of the onset of dissection [11]. Ruddy et al. [26] observed that the activity

of protein MMP9 in the aortic tissue of patients with Marfan syndrome, detected by zymography, was increased in comparison to controls. They also found that the concentration of protein MMP9 in the blood was significantly elevated as compared to controls. Gene expression analyses in animal models made by Trollope et al. [27] demonstrated the upregulation of the mRNA encoding MMP9 corresponding to increased extracellular matrix degradation. The results of Swedenborg et al. [28] have determined that the main producers of MMP9 in normal human aorta are mast cells. They found a higher amount of MMP9 in comparison to their expression in atherosclerosis, which demonstrates their involvement in extracellular matrix degradation, smooth muscle cell apoptosis, renin-angiotensin system activity, and neovascularization. In agreement with those results, we detected significantly elevated levels of both mRNA in aortic tissue (about 490% higher than controls in stage 3) and protein levels (with a maximum in the same stage of about 340% higher than controls). According to the statistically strong correlation between mRNA, protein levels of MMP9, and aortic diameter, we suggest that MMP9 expression was shown to be directly dependent on aneurysm diameter. For more precise confirmation, we measured the expression of mRNA for TIMP.

TIMP1 inhibits the activities of all MMPs and plays a role in regulating ECM in different physiological processes [26]. Structure function studies have separated the MMP inhibitory activity of TIMP1 from its growth promoting effect [29]. These TIMPs can express MMP-dependent and MMP-independent actions in the regulation of cell death, cell proliferation, and angiogenesis, involving specific signal transduction pathways. Several studies exist describing the ratio of MMP9 to TIMP1 expression in TAA. The study of Mi et al. [30] revealed that the ratio of MMP9 to TIMP1 in TAA tissue was 3.7-fold higher in TAA compared to controls. Another study of Ikonomidis et al. [31] confirmed the ratio of MMP9/TIMP1 was over 3.5-fold greater than controls. We found elevated mRNA levels of TIMP1 in all stages of TAA (with a maximum in stage 3, with levels about 340% higher than controls). We also correlated the mRNA ratio of MMP9 and TIMP and confirmed 1.7-fold higher values in aortic tissue against controls. Therefore, we suggest that an imbalance between MMP and TIMP expression is responsible for the shift toward a proteolytic state of ECM.

SMC apoptosis and ECM destruction in the aortic wall are accompanied by an increased degree of inflammation [11]. Several cytokines and chemokines that promote the recruitment of inflammatory cells to the aortic wall, such as tumour necrosis factor α, interferon (IFN)-γ, and interleukins IL-2 and IL-6 are upregulated [12]. These findings indicate that damage to the ECM, resulting in elastic fibre fragmentation, can trigger an inflammatory process by recruiting, activating, and inducing the differentiation of immune cells.

A classical plasma protein marker of acute phase of inflammation, infection, and tissue damage is CRP [32]. CRP is mainly expressed by hepatocytes, and its synthesis is regulated at the posttranscriptional level by cytokines, mainly by IL-6 with a synergic effect of IL-1 [13]. CRP can also be produced locally in atherosclerotic lesions [33]. A study by De Haro et al. [34] showed that patients with symptomatic and ruptured aneurysms had elevated serum CRP compared with patients with asymptomatic AAA. CRP directly influences several phases of atherosclerosis via complement activation, apoptosis, vascular cell activation, monocyte recruitment, lipid accumulation, and thrombosis [35]. CRP was normal and increased significantly since day 2 in the impaired oxygenation group [36]. Increased admission CRP correlated with high mortality irrespective of management policy [37]. However, Sakakura et al. [38] proposed that it may take 3–6 days to reach peak CRP; thus, initial CRP levels might not reflect the whole severity of aortic dissection. Our findings showed a rising concentration of both CRP mRNA in the tissue and also protein CRP in the blood of patients with a growing size of aortic diameter. Therefore, we supported the increasing inflammation in the TAA tissue and its spreading into the bloodstream, which was confirmed by elevated levels of IL-6 protein and mRNA in the blood and tissue.

IL-6 is involved in acute and chronic inflammation associated with aneurysm formation [39]. Both thoracic and abdominal aortic aneurysms are positively correlated to high circulating levels of IL-6 [13]. We found that soluble IL-6 in the samples with the highest aortic diameter had levels elevated by about 84% in comparison to controls. Our results are also confirmed by the study of Dawson et al. [40]. They demonstrated that TAA is a source of IL-6 in circulation, which was also demonstrated in the study of Golledge et al. [41], which showed that IL-6 values in AAA patients increased in a stepwise fashion among groups of aortic size and peaked in patients with aortic dilatation. This result confirmed that the aneurysm tissue is the source of the soluble IL-6, which is probably one of the key factors required for promoting Th17 cell differentiation; thus, one of the possible mechanisms of IL-6 action could be the regulation of Th17 cells in progression of TAA.

Another marker of increased inflammation enhanced by aortic dilatation is IL-6 receptor, which forms a dimer with the ubiquitously expressed signal transducer glycoprotein-130 (gp-130). Attachment of IL-6 to its receptor leads to the activation of the intracellular receptor-associated kinases and downstream effects via the transcription factor STAT3. The membrane-bound IL-6R (mIL-6R) is expressed in hepatocytes and cells of the innate immune system. In transsignalling, IL-6 binds to the circulating soluble IL-6R (sIL-6R), and this complex is capable of binding to gp130 in a wide range of cell types [42]. It has previously been shown that the expression of IL-6 and downstream mediators of IL-6 signalling, such as STAT3, are greater in AAA than in nonaneurysmal aortic tissue [32]. Till now, no data exists comparing the soluble protein of IL-6R concentrations and the diameter of TAA. We found a medium negative correlation of IL-6R with the diameter of aorta aneurysm.

We also studied the effect of the inflammatory cytokine TNF-α according to the expression of its receptors in the blood. TNF-α initiates its biological actions by binding to a 55-KDa receptor (TNFR1) or a 75-KDa receptor (TNFR2) [43]. TNFR1 is constitutively expressed in most tissues, binding primarily to the soluble form of TNF-α, and is the key mediator of TNF-α signalling in many cell types. TNFR2 is

typically expressed in endothelial- and immune-related cells and is activated by membrane-bound TNF-α. The major difference between the two receptors is the death domain (DD) of TNFR1 that is absent in TNFR2. Meng et al. [44] suggest that during early stages of aneurysm formation, TNFR2 signalling is activated by membrane-bound TNF-α and when sufficient TNF-α is secreted, it activates TNFR1 signalling, resulting in inflammation and apoptosis [45]. We found that both TNFR receptors (TNFR1 and TNFR2) had protein levels significantly elevated to the maximum in the group of patients with the higher aneurysm diameter. This confirms the nonsignificantly positive correlation of TNFR receptors with the progress of TAA.

5. Conclusion

The asymptomatic progress of TAA predetermines the detection of early pathological changes in aortic tissue as one of the most important goals of current cardiovascular treatment. Recently used detection techniques (ultrasonography and MRI) have very high specificity and efficiency. However, the possibility of repeating measurements during the control examination is limited regardless of the time occupancy of individual scanning equipment. Therefore, the aims of this paper were focused on the detection of basic inflammatory markers as well as markers of ECM degradation in both the aortic wall and blood. From the obtained results, a positive correlation of parameters like IL-6, CRP, TNFR1, and TNFR2 together with MMP9 and TIMP between the growing diameter of aneurysm and mRNA from tissue or protein levels from blood is obvious, although we also found significant negative correlation of Emilin-1 mRNA in the tissue and protein soluble receptor of IL-6 in the blood, which suggests that the release of inflammatory mediators dramatically increases the degradation of ECM in the aortic wall. Changes of gene expression of selected genes can be used for more purposes, like the detection of progressive pathological changes of aortic wall, for the experimental study of the inflammatory receptor inhibitors or effect of the gene polymorphism on the receptor functions during trials targeted on slowing down the progress of aortic wall aneurysm or for a decision about the consequential surgical options of wall recovery.

Conflicts of Interest

The authors state that there are no conflicts of interest regarding the publication of this article.

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Review Article

The Role of Hematological Indices in Patients with Acute Coronary Syndrome

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An increased systemic and local inflammation plays a key role in the pathophysiology of acute coronary syndrome (ACS). This review will discuss the role of hematological indices: white blood cells (WBC), neutrophil to lymphocyte ratio (NLR), red cell distribution width (RDW), and platelet indices, that is, platelet to lymphocyte ratio (PLR), mean platelet volume (MPV), and platelet distribution width (PDW) in the case of ACS. In recent years, a strong interest has been drawn to these indices, given that they may provide independent information on pathophysiology, risk stratification, and optimal management. Their low-cost and consequent wide and easy availability in daily clinical practice have made them very popular in the laboratory testing. Furthermore, many studies have pointed at their effective prognostic value in all-cause mortality, major cardiovascular events, stent thrombosis, arrhythmias, and myocardial perfusion disorders in terms of acute myocardial infarction and unstable angina. The most recent research also emphasizes their significant value in the combined analysis with other markers, such as troponin, or with GRACE, SYNTAX, and TIMI scores, which improve risk stratification and diagnosis in ACS patients.

1. Introduction

Coronary heart disease (CHD), most commonly caused by atherosclerosis, is the leading cause of death worldwide. Atherosclerosis is a systemic, lipid-driven immune inflammatory disease [1]. Inflammation, one of the factors leading to coronary artery disease (CAD), can be not only local but also systemic. Research carried out by Dutta et al. [2] proved that myocardial infarction is linked to an increased myeloid activity. Interestingly, it has also been shown that in the case of mice with an induced myocardial infarction, the sympathetic nervous system (SNS) becomes activated. This, in turn, induces the release of hematopoietic stem cells (HSPCs) from bone marrow niches, which consequently causes the further systemic stimulation of atherosclerotic plaques.

The chronic low-grade inflammation plays a key role in the initiation and development of the atherosclerotic plaque, which subsequently leads to the plaque's instability with a thrombus formation. Inflammation is also considered to be one of the main causes of diabetes, hyperlipidemia, metabolic syndrome, and endothelial dysfunction [3]. The inflammation leading to ACS encourages research into the clinical usage of new inflammatory biomarkers.

In this review, we shall describe the main hematological indices and their prognostic and diagnostic value in patients with ACS. In recent years, strong interest has arisen in these indices, given that they may provide independent information on pathophysiology, risk stratification, and optimal management.

The main advantage of hematological indices is that they are relatively inexpensive and thus widely and easily available in daily clinical practice. They have also proven their diagnostic and prognostic value in many cardiovascular diseases including CAD, atrial fibrillation following

the coronary artery bypass graft (CABG) procedure, acute and chronic cardiac insufficiency, cardiac arrhythmias, and pulmonary hypertension.

2. White Blood Cell Count (WBC)

Leukocytes play a key role in the pathophysiology of ACS, given their effect on the instability of atherosclerotic plaques. In the initial stage, leukocytes permeate endothelial cells and become activated when reaching the tunica intima. They induce the formation of microvascularity there and, as a result, make plaques more susceptible to rupture [4]. Many studies have indicated that leukocytosis is related to an increased cardiovascular mortality rate. What is more, leukocytosis also proved to be of prognostic value when assessing adverse clinical outcomes [5–7].

In the study of Sabatine et al., the elevated WBC count was found to be a relevant death risk factor during the first 30 days and 6 months following the myocardial infarction among patients with ACS (UA, NSTEMI). Furthermore, the elevated level of WBC was also related to a more advanced CAD as well as epicardial and myocardial perfusion disorders [8]. In another study, the WBC > 10,000 pointed to increased mortality among AMI and UA patients. [9]

Many prospective studies have shown that the increased concentration of leukocytes on admission was connected not only to the development of worse microvascular injury, congestive heart failure, and shock but also to the elevated mortality rate in patients with ACS [10].

3. Neutrophil to Lymphocyte Ratio (NLR)

NLR is easily measured by dividing neutrophil count by lymphocyte count in a differential white blood cells (WBC) sample. It is one of the best-assessed hematological biomarkers, which provides prognostic and diagnostic information in ACS. Its role in cardiovascular diseases has been studied extensively in the past few years [11, 12].

The study of Sezer et al. proved that the increased number of neutrophils and MPV in patients with a front wall myocardial infarction is strongly and independently connected to the development of microvascular reperfusion injury after recanalisation of infarct-related artery [13]. In another study, activated neutrophils called polymorphonuclear cells (PMN) were found in coronary thrombi in patients with myocardial infarction who were undergoing primary percutaneous coronary intervention (PCI). PMN release neutrophil extracellular traps (NETs) at the culprit lesion site. NETs are highly proinflammatory and prothrombotic fibers which can entrap leucocytes and propagate thrombosis. NETs proved to be correlated negatively with ST-segment resolution (STR) and positively with infarct size [14]. By contrast, lymphocytes, especially B2 and T helper, as the elements of the adaptive immune system, could mute and limit inflammation. The lower lymphocyte counts were associated with atherosclerosis progression and adverse clinical outcomes in patients with heart failure and ACS [15–17].

The combination of neutrophil and lymphocyte parameters has a better prognostic value than each parameter separately [18]. Kalay et al. demonstrated that NLR is related to the progression of coronary atherosclerosis, the process which is a strong and independent predictor of future coronary events [19]. In Wang et al. meta-analysis, NLR was a predictor of all-cause mortality and cardiovascular events in patients undergoing angiography or cardiac revascularization [20].

In the study of Tamhane et al., the admission NLR was described as a predictor of in-hospital and 6-month mortality in patients who undergo PCI. In the same study, it was proved that higher NLR was associated with diabetes and heart failure [21].

In recent years, numerous papers have been published regarding the value of NLR in predicting short- and long-term mortality in patients with ST-segment elevation (STEMI) [22–25] and with non-NSTEMI [26]. Preprocedural elevated NLR was also linked to an increased risk of significant ventricular arrhythmias during PCI [27].

NRL enables a clinician to predict stent thrombosis and the high mortality rate among patients with STEMI. NLR > 4.9 had 70% accuracy and 65% specificity in predicting in-hospital mortality. In a multidimensional analysis, NLR was strongly linked to stent thrombosis [28].

Furthermore, NRL itself is referred to the complexity and severity of ACS assessed by SYNTAX score, GRACE scale, and TIMI score [29–31].

4. Red Cell Distribution Width (RDW)

RDW which is a part of a standard complete blood count (CBC) is a measure of variations in the volume of red blood cells. An elevation in RDW is known as anisocytosis. An increased level of RDW has been found in patients with vitamin B12, iron, and folate deficiency. RDW has also been observed after blood transfusion and hemolysis [32].

In the study of Patel et al., the RDW values above 14.0% were significantly related to a decreased red blood cell deformability, which can impair the blood flow through microcirculation. The resultant diminution of oxygen supply at the tissue level may help to explain the increased risk of adverse cardiovascular events associated with elevated RDW [33]. In 2007, Felker was one of the first authors who proved that the elevated RDW is a useful biomarker of morbidity and mortality among patients with heart failure [34]. In the study of Arbel et al., the RDW level of 12% and above is associated with an increased risk of cardiovascular morbidity and all-cause mortality in both anemic and nonanemic patients [35].

Many studies have highlighted that the increased RDW has also been linked to peripheral artery disease (PAD) [36], chronic obstructive pulmonary disease (COPD) [37], renal failure [38], sepsis and shock sepsis [39], cerebral atherosclerosis [40], stroke [41], and pulmonary hypertension [42]. Tonelli et al. indicated a relationship between higher levels of RDW and the risk of death and adverse cardiovascular outcomes in people with prior myocardial infarction but without symptomatic heart failure [43]. Moreover, it was shown that the elevated RDW was connected to a higher

mortality rate in patients with a myocardial infarction (with or without anemia) [44–47].

In their study, Lippi et al. showed that the combined measure of RDW and troponin T (cTnT) increased diagnostic sensitivity to 99%, which meant that the combined measure was more effective in diagnosing ACS than the measure of cTnT alone [48]. Moreover, it was proved that RDW is an essential predictor of CAD severity among patients with acute myocardial infarction (AMI) [49].

5. Platelet Indices: PLR, PDW, and MPV

Regardless of their role in the general (systemic) inflammatory response, platelets have been closely related to the activation and coordination of endothelium. It has recently been observed that there is a close relation between cardiovascular mortality and the number of platelets or their ability to aggregate. Platelets play a key role in the pathophysiology of ACS. Compounded with fibrin, platelets form coronary thrombus [1]. The CADILLAC study has shown that the level of platelets (which does not affect the effectiveness of percutaneous interventions) is significantly correlated with the incidence of restenosis and stent thrombosis [50], given the function of platelets in the local as well as general inflammatory response and their aspirin resistance [51, 52].

Platelets participate in the creation of blood clots and deliver mediators which develop and sustain a local inflammatory response [53]. MPV and PDW are important and simple markers which significantly increase during platelet activation [54]. Furthermore, these indices are helpful in the evaluation of thromboembolic diseases.

6. Platelet to Lymphocyte Ratio (PLR)

It turns out that the platelet to lymphocyte ratio is a useful parameter describing the systemic inflammatory response. Thus, it has become an important prognostic factor in numerous diseases. It has been shown that PLR correlates with the prognosis in esophageal, ovarian, rectal, and hepatocellular carcinoma as well as glioma multiform [55].

The roles of PLR and other complex markers of systemic inflammatory response have been primarily described in relation to the prognosis of ACS. It has been shown that PLR correlates with a greater overall mortality in patients with NSTEMI [56]. In the recently published (prospective) study involving 5886 patients, the same relation for STEMI has been presented [57]. The same study also showed that high PLR correlates with the recurrence of myocardial infarction, stroke, and subsequent heart failure. It seems that PLR is also helpful in predicting long-term results of percutaneous interventions and it can help select patients with a higher risk of no-reflow syndrome after pPCI [58, 59].

7. Platelet Distribution Width (PDW)

Platelet distribution width (PDW) indicates a varied size of platelets. The number of large immature platelets in patients with ACS is caused by an increased bone marrow activity during the process known as thrombocytopoiesis.

PDW measured on admission is a cheap and generally available biomarker which allows for predicting the development of heart failure in patients with ACS after PCI [60]. Bekler et al. showed that an increased level of PDW (>17%) was related to the severity of CAD in patients with ACS. In the same study, an elevated PDW, diabetes mellitus, and myocardial infarction (MI) were positively correlated with a high Gensini score [61]. In a different study, PDW was greater in patients with STEMI than in those with stable CAD [62]. PDW also serves as a useful prognostic factor for long-term mortality in patients after AMI [63, 64].

8. Mean Platelet Volume (MPV)

MPV is a useful, indirect, and easily marked biomarker of platelet activity. Numerous studies support the association of MPV with adverse cardiac outcomes in patients with ACS.

MPV was a strong and independent predictor of impaired reperfusion and 6-month mortality in STEMI patients who underwent PCI [65, 66]. A similar correlation was found in NSTEMI patients [67, 68]. Moreover, Chu et al. showed that in patients who underwent PCI, the elevated MPV occurred in patients who developed restenosis [69]. Similarly, Huczek et al. proved that MPV was significantly higher in patients with ACS who developed an early stent thrombosis. It correlated with a poor dual antiplatelet responsiveness [70]. In another study of 462 patients with CAD who underwent PCI, higher MPV levels were independently associated with high residual platelet reactivity after both aspirin and clopidogrel treatments [71]. This is due to the fact that larger platelets are more often reticulated than smaller platelets containing more prothrombotic material (thromboxane A2, platelet factor 4, alpha-granules, P-selectin, and platelet-derived growth factor), which is an independent predictor of a poor response to dual antiplatelet therapy [72].

MPV turned to be independently responsible for the slow coronary flow (SCF) occurrence and its extent [73]. In the recent years, the correlation of WBC to MPV has been named as the WBC/MPV ratio (WMR). The relationship between WMR and major adverse cardiovascular events (MACE) in patients with NSTEMI [74] and STEMI [75] was more prominent than with WBC and MPV, respectively.

9. Conclusions

There is a high demand for a reliable, accessible, noninvasive, and hematological prognostic marker in ACS, which would identify patients of high cardiovascular risk in secondary prevention and tailor the therapy to their needs. Many of the indices presented here reflect the complex pathophysiology of ACS. The inflammatory processes play a key role in the development of atherosclerosis, destabilisation of atherosclerotic plaques and formation of clots on the plaque surface [76]. The significance of NLR, PLR, PDW, MPV, and RDW in the prognosis of ACS has been indicated in many studies as it has been shown above. The most crucial studies concerning hematological indices have been summarized in Table 1.

Table 1: Summary of some studies investigating diagnostic and prognostic role of the most important hematological indices.

Study type	Study	Sample size	Main findings	References
WBC				
Retrospective	Cannon et al. (2001)	7651 patients with ACS	WBC count of >10,000 was associated with increased 30-day and 10-month mortality.	[9]
Retrospective	Barron et al. (2000)	975 patients with MI	Elevation in WBC count was associated with reduced epicardial blood flow and myocardial perfusion, thromboresistance, and a higher incidence of new congestive heart failure and death.	[10]
Prospective	Sabatine et al. (2002)	2220 patients with UA/NSTEMI	Higher baseline WBC count was associated with impaired epicardial and myocardial perfusion, more extensive CAD, and higher six-month mortality rates.	[8]
Retrospective	Gurm et al. (2003)	4450 patients	A low or an elevated preprocedural WBC count in patients undergoing PCI is associated with an increased risk of long-term death.	[11]
Prospective	Chia et al. (2009)	363 patients with STEMI	Elevated leucocyte and neutrophils are predictors of adverse cardiac events.	[12]
NLR				
Prospective	Duffy et al. (2006)	1046 patients who underwent PCI	The NLR was an independent significant predictor of long-term mortality in patients who have undergone coronary angiography.	[18]
Prospective	Tamhane et al. (2008)	2833 patients with ACS	NLR was a predictor of in-hospital and 6-month mortality in patients who undergo PCI.	[21]
Prospective	Núñez et al. (2008)	515 patients with STEMI	NLR was a useful marker to predict subsequent mortality in patients admitted for STEMI, with a superior discriminative ability than total WBC.	[22]
Prospective	Azab et al. (2010)	1345 patients with NSTEMI	NLR is an independent predictor of short-term and long-term mortalities in patients with NSTEMI.	[26]
Retrospective	Chatterjee et al. (2011)	30,798 records who have undergone coronary angiography	A preprocedural NLR, elevated WBC count, and neutrophils were predictors of significant ventricular arrhythmias in patients undergoing PCI.	[27]
Prospective	Akpek et al. (2012)	418 patients with STEMI who underwent PCI	The NLR was independently associated with the development of no-reflow and in-hospital MACEs in patients with ST-segment elevation myocardial infarction undergoing primary PCI.	[23]
Prospective	Sahin et al. (2013)	840 patients with STEMI who underwent PCI	NLR was the independent predictor for SYNTAX score in patients with STEMI.	[24]
Retrospective	Sawant et al. (2014)	250 consecutive STEMI patients	NLR based on an optimal cut-off value of 7.4 was an excellent predictor of short- and long-term survival in patients with revascularized STEMI.	[31]
Retrospective	Ayça et al. (2015)	102 patients with stent tshrombosis and 450 patients with STEMI	In patients with STEMI, preprocedural high NLR was associated with both stent thrombosis and higher mortality rates.	[28]
Prospective	Yaylak et al. (2016)	A total of 213 subjects with inferior STEMI	NLR was an independent predictor of RVD in patients with inferior STEMI undergoing primary PCI.	[25]
RDW				
Retrospective	Nabais et al. (2009)	1796 patients with ACS	There is a graded independent association between higher RDW values and adverse outcomes in patients with ACS.	[45]

Table 1: Continued.

Study type	Study	Sample size	Main findings	References
Prospective	Lippi et al. (2009)	456 patients with ACS	RDW at admission might be considered with other conventional cardiac markers for the risk stratification of ACS patients admitted to emergency departments.	[48]
Prospective	Dabbah et al. (2010)	1709 patients with AMI	RDW is a predictor of mortality after AMI. Moreover, an increase in RDW during hospitalization also portends adverse clinical outcome.	[44]
Retrospective	Uyarel et al. (2011)	2506 STEMI patients	RDW at admission was a predictor of in-hospital and long-term cardiovascular mortality.	[46]
Prospective	Isik et al. (2012)	135 patients with STEMI	RDW is a marker indicating long-term prognosis.	[47]
Prospective	Timóteo et al. (2015)	787 patients with ACS	Combination of RDW with GRACE score improves the predictive value for all-cause mortality.	[80]
PLR				
Observational study	Azab et al. (2012)	619 patients with NSTEMI	PLR is a significant independent predictor of long-term mortality after NSTEMI.	[56]
Prospective	Kurtul et al. (2014)	1016 patients with ACS	PLR at admission is significantly associated with the severity and complexity of coronary atherosclerosis in patients with ACS. Increased PLR is an independent predictor of higher SYNTAX score in patients with ACS who undergo urgent CA.	[82]
Retrospective	Acet et al. (2016)	800 patients with STEMI	PLR, RDW and monocyte were associated with GRACE score in patients with STEMI.	[81]
Retrospective	Yildiz et al. (2015)	287 patients with STEMI	High preprocedural PLR and NLR levels are significant and independent predictors of no-reflow in patients undergoing primary PCI.	[58]
Prospective	Sun et al. (2017)	5886 patients with STEMI	Higher PLR was associated with recurrent myocardial infarction, heart failure, ischemic stroke, and all-cause mortality in patients with STEMI.	[57]
Prospective	Vakili et al. (2017)	215 patients with STEMI	PLR and NLR were associated with no-reflow phenomenon in patients with STEMI treated with pPCI.	[59]
PDW				
Prospective	De Luca et al. (2010)	1882 patients undergoing coronary angiography + IMT in 359 patients	PDW is not related to the extent of CAD and carotid IMT. PDW positively correlated with age, weight, waist circumference, and prevalence of diabetes.	[78]
Prospective	Rechciński et al. (2013)	538 patients who underwent primary PCI in acute MI	PDW and P-LCR are prognostic predictors after MI.	[63]
Retrospective	Celik et al. (2015)	306 patients with STEMI	Baseline PDW and MPV are independent correlates of no-reflow and in-hospital MACEs among patients with STEMI undergoing pPCI.	[64]
Retrospective	Bekler et al. (2015)	502 patients with ACS were enrolled.	The group with PDW > 17% had significantly higher Gensini score.	[61]
MPV				
Prospective	Huczek et al. (2005)	398 patients with STEMI	MPV is a predictor of impaired reperfusion and mortality in STEMI treated with pPCI.	[65]

Table 1: Continued.

Study type	Study	Sample size	Main findings	References
Case-control study	Huczek et al. (2010)	36 consecutive ST cases and 72 matched controls	Baseline platelet size is increased in patients with ACS developing early stent thrombosis and correlates with future residual platelet reactivity.	[70]
Systematic review + meta-analisis	Chu et al. 2010	Pooled results from 16 cross-sectional studies involving 2809 patients with CAD	Elevated MPV is associated with AMI, mortality following myocardial infarction, and restenosis following coronary angioplasty.	[69]
Retrospective	Isik et al. (2012)	2467 who underwent coronarography with CAD	Diabetes, smoking, hemoglobin, and MPV were found to be the independent correlates of SCF presence. Moreover, only MPV was identified as an independent correlate of extent of SCF.	[73]
Prospective	Wan et al. (2014)	297 ACS patients	Both MPV and the GRACE score were significant and independent predictors for CVD events. Combination of MPV with the scoring system improved the predictive value.	[83]
Prospective	Niu et al. (2015)	506 ACS patients	Elevated MPV was an independent predictor of 6-month mortality or MI in patients with ACS. The addition of MPV to the GRACE model improved its predictive value.	[84]

ACS: acute coronary syndrome; AMI: acute myocardial infarction; NSTEMI: non-ST elevation myocardial infarction; MI: myocardial infarction; MPV: mean platelet volume; STEMI: ST elevation myocardial infarction; CAD: coronary artery disease; TIMI: thrombolysis in myocardial infarction; WBC: white blood cell count; RDW: red blood cell distribution width; PDW: platelet distribution width; PLR: platelet lymphocyte ratio; P-LCR: platelet large cell ratio; CVD: cardiovascular disease; HF: heart failure; PCI: percutaneous coronary intervention; RVD: right ventricular dysfunction; MACEs: major adverse cardiac events; GRACE: global registry of acute coronary events; SCF: slow coronary flow; IMT: intima media thickness.

Dutta et al. showed that ACS increases inflammation in the atherosclerotic plaques within months [2]. Therefore, the questions to be asked are at which stage of ACS would it be best to test biomarkers and would repeated tests improve their prognostic value? It is worth emphasizing that EDTA as an anticoagulant can cause platelet swelling thereby affecting the value of PDW and MPV [77]. Moreover, it is worth paying attention to the possible influence of medication on the value of hematological biomarkers; for example, statins had an effect on the higher value of PDW [78]. In a different study, statins considerably diminished MPV [79]. It is also important to mention that the value of hematological biomarkers in ACS patients is affected by other health disorders such as chronic renal failure, anemia, thrombocytopenia, thyroid dysfunction, dyslipidemia, diabetes, and hypertension. What is more, these disorders anticipate worse prognosis for patients with ACS, given their increased chance of heightened inflammation, oxidative stress, and apoptosis in bone marrow, which all negatively affect the function of erythropoiesis (inflammatory cytokines suppress the maturation of erythrocytes). Interestingly, biomarkers have an additional prognostic value for ACS patients if they are analysed with other inflammatory markers (such as CRP and fibrinogen) or with GRACE, SYNTAX, and TIMI risk scores [80–84].

Conflicts of Interest

The authors declare no conflict of interests.

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Research Article

Association between Cullin-3 Single-Nucleotide Polymorphism rs17479770 and Essential Hypertension in the Male Chinese Han Population

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Background. Hypertension, including essential and secondary hypertension, is a multifactorial disease, affecting more than one billion people worldwide. Secondary hypertension can result from mutations of cullin-3 (*CUL3*); however, whether polymorphisms of *CUL3* are associated with essential hypertension (EH) has not been reported. Here, we investigated the association between *CUL3* SNPs rs17479770 and rs3738952 and EH in the Chinese Han population. *Methods*. This case-control study investigated 520 representatives, including 259 patients with EH and 261 normotensive controls matched for age, gender, BMI, TG, TC, and HbA1c for the distribution of functional rs17479770 and rs3738952 within the *CUL3* gene by using PCR and RFLP. *Results*. Our results showed that there was no significant difference in allele and genotype distribution of rs3738952 and haplotype distribution of rs17479770 and rs3738952 between the EH group and normotensive group, whereas the rs17479770 TT genotype in male and the full data set were significantly associated with the decreased risk of EH ($P = 0.050$, $P = 0.042$), and rs17479770 allele T in male was shown to have the correlation tendency of the decreased risk of EH ($P = 0.064$). *Conclusion*. Our data suggest that the *CUL3* rs17479770 variant could be a protective factor in the pathogenesis of EH.

1. Introduction

Cullin-3 (CUL3) is the core component of multiple cullin-RING-based BCR (BTB-CUL3-RBX1) E3 ubiquitin-protein ligase complexes which mediate the ubiquitination and subsequent proteasomal degradation of target proteins [1]. The CUL3-RING E3 ubiquitin ligase (CRL) complex controls the ubiquitination of with-no-lysine kinase (WNK) and enhances the levels of WNK isoforms, whose function is similar to that of a serine-threonine protein kinase critical in controlling potassium, sodium, and pH homeostasis. The CRL complex also plays a major role in regulating blood pressure by increased activity of ion cotransporters in the kidney [2, 3]. Through stabilizing WNK isoforms, the mutation of *CUL3* has been linked to Pseudohypoaldosteronism type II (PHAII), a rare Mendelian syndrome featuring hypertension [4]. Vascular actions of CUL3 may contribute to hypertension, because McCormick and colleagues proposed that CUL3 regulates vascular tone via RhoA/Rho kinase signaling [5].

Hypertension is considered one of the most important diseases with a great burden on health care systems around the world. Hypertension is highly prevalent in the Asia-Pacific [6]. About 2 million 430 thousand people died of hypertension, accounting for about 24.6% of all deaths in China in 2010. In 2013, China's total health expenditure is

31869 billion yuan, of which the direct economic burden of hypertension accounted for 6.61% [7]. Essential hypertension (EH), which accounts for 90% of hypertensive cases, is a polygenic and multifactorial disease caused by the interaction of genetic determinants and environmental factors [8, 9]. The pathogenesis and etiology of EH include a multifactorial imbalance which results from complicated gene-gene and gene-environment interaction [10].

Single-nucleotide polymorphisms (SNPs) are the variation in the genomes which can be used to associate genotypic variation with the phenotype [11]. SNPs underlie differences in our susceptibility to disease. Associations of some SNPs in *WNK1* and *WNK4* with EH have been observed in the general population [12–14], and one study had identified that *rs3738952* of *CUL3* was significantly associated with head and neck squamous cell carcinoma (HNSCC) risk [15], but very few reports investigated the relationship between SNPs of *CUL3* and EH [16]. Genome linkage studies have identified numerous gene variants that associated with EH, and a few genetic loci and candidate genes (variants) have been identified by genome-wide association studies (GWAS) [17]. Through GWAS, a variety of common genetic variants are analyzed and identified for disease association, which have potential association with blood pressure and the development of EH [18, 19], whereas some gene variants have been shown to have contribution to EH according to ethnicity or gender [20]. The development of genetic studies has revealed that some SNPs within genes, such as *ATP1B1* [21], *CD36* [22], *CYP2J2* [23], *CYP4A11* [24, 25], *CYP4F2* [26], *CYP17A1* [27], and *TPRC6* [28], are closely related to the progression of EH.

However, it remains unknown whether there are other new gene variants which can influence the progression of EH. Therefore, we selected rs17479770 and rs3738952 SNPs in the *CUL3* gene after the haploview analysis in the Han population of China and examined the possible relationship between the SNPs and EH in this study. Haploview software was used to conduct linkage disequilibrium and haplotype block analyses, using the Hapmap phase IV genotype data for chromosomal region 2: 225043534–225157486 (CHB database, Hapmap release 24 (2008, November)). The criterion for r^2 was set at 0.8. The Han population is the largest ethnic group in China, and the association of *CUL3* with EH in the Chinese Han population has not yet been reported. Therefore, our results could provide new insights into the pathogenesis of EH by studying *CUL3* SNPs.

2. Patients and Methods

2.1. Ethics. The present study was performed with the approval of the ethics committee of Guangzhou General Hospital of Guangzhou Military Command and is in compliance with the Helsinki Declaration. Informed consents were collected from all the candidate subjects.

2.2. Subjects. Patients diagnosed with EH were recruited from Guangzhou General Hospital of Guangzhou Military Command from 2012 to 2015. In total, 259 patients in the EH patient group and 261 control subjects in the normotensive group, matched for age, sex, BMI, TG, TC, and HbA1c, were enrolled in this study (Table 1). All of the EH patients received antihypertensive drug treatment, so it does not need to match the blood pressure between the two groups. All participants were unrelated and belonged to the Chinese Han population. EH patients were diagnosed according to JNC 7 [29] and JNC 8 [30]: systolic blood pressure (SBP) > 140 mmHg and diastolic blood pressure (DBP) > 90 mmHg, without any antihypertensive medication or confirmed diagnosis of EH by a cardiovascular specialist. The subjects who had secondary hypertension caused by another disease or are in the acute phase with cardiovascular, lung, liver, kidney, and other somatic diseases or with malignant tumor were excluded from the EH patient group. The normotensive group was selected based on the following criteria: SBP < 129 mmHg and DBP < 85 mmHg and without any antihypertensive medication. Subjects who had been currently diagnosed with malignant tumor and diabetes or in the acute phase with cardiovascular, lung, liver, kidney, and other somatic diseases were excluded from the normotensive group. One subject with diabetes in the EH patient group was excluded, and one subject with impaired glucose tolerance in the normotensive group was also excluded from analysis.

Table 1: Baseline of the study population.

Parameters	EH group ($n = 259$)	Normotensive group ($n = 261$)	P
Age, y	77.81 ± 7.640	76.78 ± 9.095	0.163*
Gender, male, %	50.2%	50.9%	0.930
BMI	23.654 ± 3.871	23.305 ± 3.754	0.297*
HbA1c, %	5.3286 ± 0.901	5.258 ± 0.871	0.364*
TC, mmol/L	4.448 ± 0.893	4.327 ± 0.836	0.111*
TG, mmol/L	1.436 ± 0.926	1.346 ± 1.141	0.324*

*Analyzed by one-way ANOVA.

2.3. DNA Extraction. Genomic DNA was extracted from circulating leukocytes using commercial DNA isolation kits (Tiangen Biotech, Beijing, China). Briefly, the red blood cells, as well as the nuclei of leukocytes, were lysed. Subsequently, proteins were precipitated, followed by the precipitation of DNA using isopropanol. The DNA pellet was washed with ethanol. Finally, DNA was rehydrated with the DNA Rehydration Solution and preserved in liquid nitrogen.

2.4. Genotyping. Genomic DNA was isolated from peripheral blood leukocytes, according to the standard procedures by using commercial DNA isolation kits (Tiangen Biotech, Beijing, China). We performed genotyping for *CUL3* rs17479770 and rs3738952 polymorphisms by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) by using the Sequenom Mass ARRAY system (Sequenom Inc., CA, USA). Primers used for genotyping were designed manually: rs17479770 forward 5′-TGCCA TTTCCTGCTAGCAACCT-3′ and reverse 5′-

TCTTGGAAGGAAAGCTGTTGCATA-3′ and rs3738952 forward 5′-CCCAGGTCAACATAAATCACACATCA-3′ and reverse 5′-TTCTGCAGATCTCAATG CCACAT-3′. The concentration of rs17479770 primers was 1 μM, and that of rs3738952 primers was 2 μM in the PCR reaction system. PCR was performed in a reaction mixture volume of 20 μL, which included 1× HotStarTaq buffer, 3.0 mM Mg^{2+}, 0.3 mM dNTP, 1 U HotStarTaq polymerase (Qiagen Inc., MD, USA), and 1 μL DNA template. Amplification was performed under the following conditions: initial denaturation of 2 min at 95°C followed by 11 cycles of denaturation at 94°C for 20 s, annealing at 59.5°C for 40 s, and extension at 72°C for 1 min and 30 s and 24 cycles of denaturation at 94°C for 20 s, annealing at 59°C for 30 s, and extension at 72°C for 1 min and 30 s followed by a final extension at 72°C for 2 min. PCR products were stored at 4°C. Restriction DNA fragments were separated by electrophoresis on 1% agarose gel and stained with ethidium bromide.

Linking primers used for coupled reaction for genotyping were designed manually: rs17479770RC, TTCCGCGTTCG GACTGATATTCAGCAAAATTAGAAGTCATTTCTAGT CCTGAG; rs17479770RP2, AGCAGAARTAATTAGAAA TGTTAACATTTAAGTGCTTTTTTTTTTT; rs17479770 RT, TACGGTTATTCGGGCTCCTGTTCAGCAAAATT AGAAGTCATTTCTAGTCCTGAA; rs3738952RC, TCT CTCGGGTCAATTCGTCCTTTCTCAATGCCACATTTT ATGGACAAG; rs3738952RP, TTAAAAAGGTAAATAT TGATAGTTTGAACGTATTAAGTAATTTTT; and rs373 8952RT, TGTTCGTGGGCCGGATTAGTTCTCAATGC CACATTTTATGGACGAA. The reaction system of coupled reaction was performed in a reaction mixture system including 1 μL 10× connection buffer, 0.25 μL ligase, and 0.4 μL 5′ primer mixture (1 connection M) and 0.4 μL 3′ primer mixture (2 connection M), after purification of 2 μL multiple PCR products and 6 μL ddH_2O mixture. Coupled reaction was performed under the following conditions: 38 cycles of denaturation at 94°C for 1 min and annealing at 56°C for 4 min. Reaction products were stored at 4°C. Allelic discrimination was measured automatically on the ABI3730XL (Applied Biosystems) using the GeneMapper 4.1 software (95% confidence intervals).

2.5. Statistical Analysis. Statistical analysis was performed using the SPSS 20.0 software (SPSS Inc., Chicago, IL, USA).

The haplotype of rs17479770 and rs3738952 was analyzed by PHASE 2.0 (University of Manchester, Manchester, UK). One-way ANOVA was used to match the values of BMI, HbA1c, TC, and TG between the EH patient group and normotensive group. The chi-squared test was used to examine whether the genotype distributions differed from the expected Hardy-Weinberg equilibrium (HWE) and the distribution of rs17479770 and rs3738952 genotypes and alleles between the EH patient group and normotensive group. Odds ratios (OR) and its corresponding 95% confidence intervals (CI) were estimated to compare the distribution of genotypes and alleles between the patients and control subjects. Analyses used two-tailed estimation of significance. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Baseline Characteristics of the Study Population. The Han population consisted of 259 patients in the EH patient group (130 males and 129 females with an average age of 77.81 ± 7.640 years old) and 261 control subjects in the normotensive group (133 males and 128 females with an average age of 76.78 ± 9.095 years old), and there was no significant difference in age between the two groups ($P = 0.163$). The gender ratio of the two groups was insignificant ($P = 0.930$). The clinical representative characteristics including ID, age, gender, height, weight, TG, TC, and HbA1c were collected. There were no significant differences between the two groups including BMI, HbA1c, TC, and TG (P values were 0.297, 0.364, 0.111, and 0.324, resp.) (Table 1). The systolic blood pressure (SBP) and diastolic blood pressure (DBP) values were not included because all EH patients received antihypertensive medication.

3.2. Allele and Genotype Frequencies of CUL3 rs17479770 and rs3738952 in the EH Patient Group and Normotensive Group. The allele and genotype frequencies of rs17479770 and rs3738952 SNPs are shown in Tables 2 and 3. Genotype distribution in the EH patient group and normotensive group did not deviate from HWE ($P > 0.05$). Comparison of allele frequencies between the patient group and normotensive group by the chi-squared test revealed that the allele and genotype frequencies of rs3738952 had no statistically significant difference between the EH patient group and the normotensive group (Table 3), whereas the rs17479770 TT genotype in males and the full data set had a significantly protective effects on EH ($P = 0.050$; OR = 0.578, 95% CI = 0.344–0.970; $P = 0.042$; OR = 0.674, 95% CI = 0.468–0.971) (Table 2) and the T allele in males and the CT genotype in the full data set show some protective trend of EH ($P = 0.064$; $P = 0.066$). When the data further stratified the haplotype frequency distributions of *CUL3* rs17479770 and rs3738952 SNPs, there is no statistically significant difference between the EH patient group and normotensive group (Table 4).

Overall, these results suggest that the TT genotype of rs17479770 in males and all representatives indicate a significantly protective effect on EH and the T allele shows some protective trend to the male representatives and the same as the CT genotype to all representatives.

4. Discussion

We investigated rs17479770 and rs3738952 SNPs in the *CUL3* gene as genetic risk factors for EH in a case-control study of a well-characterized Southern Chinese Han population. Our results demonstrate that the frequencies of the rs17479770 TT genotype in male EH patients and in all representatives were significantly decreased compared to those in the normotensive group, whereas there was no statistically significant difference in allele and genotype frequencies of *CUL3* in males between the EH patient group and the normotensive group. Moreover, there was no significant difference of haplotype frequency distributions of the two SNPs

Table 2: Distribution frequency of *CUL3* rs17479770 polymorphism in the EH and control groups.

	CUL3	rs17479770 (C/T)	EH group *n*	Normotensive group *n*	*P*	OR (95% CI)
Full data set	Allele	C	229	207		
		T	289	317	0.132	1.213 (0.948–1.553)
	Genotype	CC	47	46	0.909	1.041 (0.665–1.630)
		CT	135	115	0.066	1.392 (0.986–1.965)
		TT	77	101	0.042	0.674 (0.468–0.971)
M	Allele	C	121	102		
		T	139	164	0.064	1.400 (0.989–1.980)
	Genotype	CC	27	22	0.430	1.323 (0.709–2.467)
		CT	67	58	0.218	1.375 (0.846–2.235)
		TT	36	53	0.050	0.578 (0.344–0.970)
F	Allele	C	108	107		
		T	150	151	1.000	1.016 (0.716–1.442)
	Genotype	CC	20	25	0.512	0.763 (0.400–1.457)
		CT	68	57	0.213	1.408 (0.863–2.299)
		TT	41	47	0.512	0.813 (0.485–1.361)

Table 3: Distribution frequency of *CUL3* rs3738952 polymorphism in the EH and control groups.

	CUL3	rs3738952 (C/T)	EH group *n*	Normotensive group *n*	*P*	OR (95% CI)
Full data set	Allele	C	387	399		
		T	131	123	0.564	0.911 (0.686–1.209)
	Genotype	CC	145	151	0.723	0.927 (0.655–1.311)
		CT	97	97	1.000	1.012 (0.710–1.444)
		TT	17	13	0.459	1.340 (0.637–2.819)
M	Allele	C	201	204	0.368	1.202 (0.810–1.786)
		T	59	72		
	Genotype	CC	76	79	0.901	0.962 (0.589–1.572)
		CT	49	46	0.610	1.144 (0.692–1.893)
		TT	5	8	0.572	0.625 (0.199–1.963)
F	Allele	C	186	195	0.315	0.808 (0.544–1.201)
		T	72	61		
	Genotype	CC	69	72	0.707	0.894 (0.547–1.462)
		CT	48	51	0.702	0.895 (0.541–1.479)
		TT	12	5	0.130	2.523 (0.862–7.382)

of *CUL3* in the EH patient group and normotensive group. The results of statistical analysis suggest that all *CUL3* rs17479770 TT genotypes were associated with the protection of the Chinese Han population especially male subjects from EH.

RhoA activation contributes to vascular constriction and hypertension, and CUL3Δ9-associated ubiquitin ligase activity toward RhoA is impaired, suggesting that CUL3Δ9 mutations may act dominantly by sequestering substrate adaptors and disrupting CUL3WT complexes [16]. CUL3 and KLHL3 are expressed in the distal nephron of the kidney, suggesting a mechanistic link between KLHL3 and CUL3 mutations, increased Na^+-Cl^- reabsorption, and disease pathogenesis [31]. CUL3 provides a scaffold that binds to the BTB domain of *KLHL3* through its N-terminus region [32]. *WNK1* and *WKN4* regulate sodium and potassium flux through regulation of the thiazide-sensitive Na^+/Cl^- cotransporter (NCC) and the renal outer medullary potassium channel (ROMK) in the distal nephron [33, 34]. The *WNK1* and *WNK4* isoforms, through directing two homologous kinases, SPS1-related proline/alanine-rich kinase (SPAK, also known as serine threonine kinase 39, STK39) and oxidative stress-responsive kinase1 (OSR1) which phosphorylates and activates NCC and $Na^+/K^+/2Cl^-$ cotransporters (NKCC) 1 and

Table 4: Haplotype frequency distributions of the 2 SNPs of *CUL3* in the EH and normotensive groups.

CUL3	Haplotype	EH group ratios	Normotensive group ratios	χ^2	*P* value	OR (95% CI)
Full data set	CC	229 : 259	207 : 261	0.700	0.436	1.115 (0.864–1.438)
	TC	158 : 259	192 : 261	1.824	0.189	0.829 (0.632–1.088)
	TT	131 : 259	123 : 261	0.213	0.647	1.073 (0.795–1.449)
Male	CC	121 : 130	102 : 133	1.128	0.317	1.214 (0.849–1.735)
	TC	80 : 130	102 : 133	1.293	0.288	0.802 (0.549–1.173)
	TT	59 : 130	62 : 133	0.015	0.913	0.974 (0.633–1.498)
Female	CC	108 : 129	105 : 128	0.012	0.926	1.021 (0.710–1.468)
	TC	78 : 129	90 : 128	0.577	0.488	0.860 (0.583–1.270)
	TT	72 : 129	61 : 128	0.545	0.521	1.171 (0.770–1.782)

2, thereby play important roles in controlling blood pressure [35–38]. The kidney plays a central role in the pathophysiology of EH, and the NCC is physiologically relevant to the development of EH. Based on the previous studies, we inferred a mechanism that the mutations in *CUL3* might influence susceptibility to EH. In this study, we analyzed the association of *CUL3* rs17479770 and rs3738952 polymorphisms with EH in the Southern Chinese Han population, but several limitations of this study should be mentioned. The main limitation is the relatively small sample size: only 520 participants, including 259 EH patients and 261 normotensive people, were recruited, which is insufficient for an SNP association study of a rarer mutation site. The other limitation is that only two SNPs within the *CUL3* gene were analyzed. Additional in-depth studies are needed to confirm the functional importance of *CUL3* rs17479770 polymorphism in EH and to elucidate its precise role in the pathogenesis of EH.

5. Conclusion

This study demonstrates that *CUL3* rs17479770 is a candidate SNP that could be further examined as a possible protective genetic factor for EH progression, especially in male population. However, no significant association was detected between *CUL3* rs3738952 polymorphism and EH in the Chinese Han population, and the study of haplotype frequency distributions of *CUL3* rs17479770 and rs3738952 in the EH and normotensive groups had no significant association. Our results revealed that the *CUL3* rs17479770 TT genotype was associated with protection against EH in male and all representatives. This study offers a new direction to understand the mechanisms underlying EH and suggest novel therapeutic targets for the disease treatment. Further population-based genetic studies will be required to confirm our results and consolidate the role of CUL3 on EH in populations living in different environments or regions.

Abbreviations

ANOVA: Analysis of variance
BMI: Body mass index
BP: Blood pressure
BTB: Bric-a-brac tramtrack broad complex
CI: Confidence intervals
CRL: Cullin-RING E3 ubiquitin ligase
CUL3: Cullin-3
CYP: Cytochrome P450
DBP: Diastolic blood pressure
EH: Essential hypertension
GWAS: Genome-wide association studies
HbA1c: Hemoglobin A1c
HNSCC: Head and neck squamous cell carcinoma
HWE: Hardy-Weinberg equilibrium
JNC 7: Seventh report of the Joint National Committee on prevention, election, evaluation, and treatment of high blood pressure
JNC 8: 2014 evidence-based guideline for the management of high blood pressure in adults: report from the panel members appointed to the Eighth Joint National Committee
KLHL3: Kelch-like 3
MALDI-TOF MS: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
NCC: Na^+/Cl^- cotransporter
NKCC: $Na^+/K^+/2Cl^-$ cotransporter
OR: Odds ratios
PCR: Polymerase chain reaction
PHAII: Pseudohypoaldosteronism type II
RFLP: Restriction fragment length polymorphism
ROMK: Renal outer medullary potassium channel
SBP: Systolic blood pressure
SNPs: Single-nucleotide polymorphisms
TC: Total cholesterol
TG: Triglyceride
WNK: With-no-lysine kinase.

Additional Points

Highlights. The *CUL3* rs17479770 TT genotype was associated with protection from EH in male and in the full data set. *CUL3* rs17479770 is a possible protective genetic factor for EH progression.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Jin Li and Jing Hu have contributed equally to this work.

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Research Article

Serum Gamma-Glutamyl Transferase and Ferritin Synergistically Associated with the Rate of Chronic Kidney Disease

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The present study investigated the effects of GGT and SF on the risk of CKD. 1024 participants (436 men and 588 women) were divided into three groups according to GGT and SF levels: group 1 (both GGT and SF not in the fourth quartile), group 2 (only GGT or SF in the fourth quartile), and group 3 (both GGT and SF in the fourth quartile). The risks of CKD in different levels of GGT and SF and in groups 2-3 compared with group 1 were analyzed by multiple logistic regression. GGT or SF in the highest quartile was associated with increased risk of CKD. Such associations attenuated after adjustment for confounding factors. The incidences of CKD, especially albuminuria, increased across the three groups. Correspondingly, malondialdehyde (MDA) levels gradually increased from group 1 to group 3. The risks of CKD were higher in groups 2 and 3 than that in group 1. In group 3, the increased rate was independent of age, BMI, alcohol drinking, diabetes mellitus, hypertension, hypertriglyceridemia, and metabolic syndrome (odds ratios from 1.887 to 2.293, $P < 0.05$). In summary, this study suggested that GGT and SF synergistically influence the rate of CKD.

1. Introduction

The incidence of chronic kidney disease (CKD) is increasing in recent decades in China and in other parts of the world [1, 2]. CKD is one of the leading causes for kidney failure and contributes to systemic pathophysiological processes and, thus, is becoming a great burden to public health [2, 3].

Unfortunately, the etiology of CKD is still undetermined. Risk factors of CKD included hypertension, hyperglycemia, hyperuricemia, and metabolic syndrome [1, 4, 5]. However, the exact link between CKD and these factors is unclear. A speculation is that both CKD and the aforementioned risk factors were caused by the same underlying mechanism [2, 6].

Oxidative stress is recognized as common soil of many noninfectious chronic diseases including CKD [7, 8]. Serum gamma-glutamyl transferase (GGT) and serum ferritin (SF) are two frequently used biomarkers in clinical practice. GGT cleavages glutathione, one of the most important extracellular antioxidative agents. This process will lead to increase production of reactive oxygen species [9]. SF is an index of body iron storage. Iron in the form of Fe(III) and Fe(II) is necessary for GGT's action on the catabolism glutathione [9]. Therefore, GGT and SF could have synergetic effect on oxidative stress and further affect the risk of CKD. Our previous study showed that serum GGT was correlated with SF, and they could synergistically affect the risk of type 2 diabetes [10]. Several but not all studies showed that GGT was associated with increased risk of CKD [11–13]. Few studies analyzed the association between SF and CKD, especially the interaction between GGT and SF on CKD. To clarify these issues, the present study employed the data from a recent survey in a Chinese minority and further investigated the potential effects of GGT and SF.

2. Methods

2.1. Study Design. This was a cross-sectional study initially designed to investigate the prevalence of type 2 diabetes in

Sichuan province, including Xichang area where Yi nationality population inhabits, an old minor nationality in West South of China.

2.2. Participants. All participants included in this study were from Yi nationality. Details of this population and study design were described in a previously published article [10]. In brief, a total of 1288 individuals of Yi nationality, 20–74 years of old, participated in a nationwide survey. 1024 subjects (84.0%) with integral data were enrolled. Patients were excluded if they were previously diagnosed with type 2 diabetes (T2D), type 1 diabetes, or other special type diabetes, previously diagnosed liver-biliary disease. This study was a part of a national survey of the prevalence of type 2 diabetes and metabolic syndrome in China, which was approved by China-Japan Friendship Hospital's Drugs/Medical Apparatus & Instruments Ethics Committee (07020470055), and all subjects gave their informed consent. This study was registered on the website of Chinese clinical trial registry (TR-CCH-ChiCTR-CCH-00000361).

2.3. Measurements. At the site of survey, a fasting morning spot urine sample was collected, stored at −20°C for less than 2 months, and then was transported in package conditioned by dry ice to China-Japan Friendship Hospital in Beijing for measurement. Serum GGT, albuminuria, and urinary creatinine were measured according to standard laboratory procedures (with immunoturbidimetric tests and Jaffe's kinetic method, resp.). The urinary albumin to creatinine ratio (ACR; mg/g creatinine) was calculated. SF level was measured with a radioimmunoassay kit (Beijing North Institute of Biological Technology). Serum creatinine level was measured at the survey site by Hitachi automatic biochemical analyzer. Estimated glomerular filtration rate (eGFR) was calculated according to a formula described in previous studies [1, 5]. Malondialdehyde (MDA) as a marker of oxidative stress was measured by MDA detection kit (Nanjing Jiancheng Bioengineering Institute).

2.4. Definition. Patients with an ACR more than 30 mg/g were defined as having albuminuria (no patients in this study had an ACR more than 300 mg/g). Chronic kidney disease is defined by microalbuminuria or eGFR less than 60 mL/min per 1.73 m^2 as described in the previous studies [1, 5].

3. Statistical Analysis

Serum levels of GGT and SF were firstly divided into quartiles with values specific to each gender from each geological location (urban or rural). Then, all subjects were reconstituted into three groups: group 1 (both GGT and SF were not in the fourth quartile), group 2 (only GGT or SF was in the fourth quartile), and group 3 (both GGT and SF were in the fourth quartile). The odds ratios (OR) and 95% confidential interval (95% CI) of CKD in different levels of GGT and SF, and in groups 2 and 3 compared with group 1, were assessed by multiple logistic regressions. The differences in other categorical variables were analyzed with chi-square tests, and those in continuous variables were compared by Student's *t*-tests or one-way ANOVA. All *P* values were two tailed, and those $\alpha < 0.05$ were considered statistically significant.

4. Results

4.1. Demographic and Clinical Characteristics of the Participants in the Study. Overall, participants from low to high quartiles of GGT or SF tended to be older, obese, with high levels of triglyceride, MDA, urinary albumin, and high incidences of diabetes mellitus, hypertension, and metabolic syndrome (Tables 1 and 2).

As shown in Table 3, participants were older and with higher BMI from group 1 to group 3. There were more drinkers and heavy drinkers in group 3 than in group 1 and group 2. The prevalence of diabetes mellitus, hypertension, and metabolic syndrome and the levels of triglyceride and MDA gradually increased from group 1 to group 3.

4.2. The Relationship between GGT and SF. As shown in our previous study, GGT was positively associated with SF ($r = 0.237$ to 0.303, all $P < 0.05$), which was independent of BMI [10].

4.3. CKD Incidences in Different Levels of GGT or SF. CKD rates were higher in the third and fourth quartiles of GGT when compared with the lowest quartile (OR 1.422, 95% CI 1.106, 1.830; OR 1.364, 95% CI 1.156, 1.610). Such associations were attenuated when adjusted for age and BMI plus diabetes mellitus, hypertension, or metabolic syndrome (Table 4).

CKD rates were higher in the fourth quartile of SF when compared with the lowest quartile (OR 1.373, 95% CI 1.174, 1.606). Such an association was attenuated but still existed when adjusted the above confounding factors (Table 5).

4.4. CKD Incidence and MDA Levels in Different Groups of GGT and SF Combination. ACR levels were significantly higher in groups 2 and 3 than in group 1 and was higher in group 3 than in group 2 but with no significant difference. Similarly, eGFR levels were significantly lower in group 2 and group 3 compared with group 1, and no significant difference was observed between group 2 and group 3 (Table 3). Moreover, the rates of microalbuminuria (14.2%, 21.4%, and 27.4% in groups 1 to 3, resp., $P < 0.01$) and CKD (14.8%, 23.0%, and 30.2% in groups 1 to 3, resp., $P < 0.01$) were significantly increased from group 1 to group 3, while that of reduced eGFR (less than 60 mL/min per 1.73 m^2) was not different across groups (Figure 1).

4.5. Synergetic Effects of GGT and SF on the Rates of CKD. The results of logistic regression showed that CKD rates increased in groups 2 and 3 compared with group 1 (OR 1.743; 95% CI 1.228, 2.474; and OR 2.413; 95% CI 1.504, 3.871). The ORs were independent of age and BMI but attenuated after adjustment for heavy drinking, diabetes mellitus, hypertension, hypertriglyceridemia, and metabolic syndrome, respectively, in group 2, and slightly attenuated but still be significant in group 3 (OR from 1.887 to 2.293, $P < 0.05$; Table 6). This synergy appears to be a novel underlining mechanism for CKD.

Table 1: General characteristics of individuals stratified by different levels of GGT.

	Men				Women			
	Quartile 1	Quartile 2	Quartile 3	Quartile 4	Quartile 1	Quartile 2	Quartile 3	Quartile 4
n	94	122	109	111	133	144	162	149
Age, years (S. E.)	43.2 (1.9)	44.6 (1.4)	48.6 (1.3)†	43.7 (1.1)	42.6 (1.4)	42.6 (1.1)	45.8 (1.1)†‡	45.7 (1.0)‡
BMI, kg/m^2	20.7 (0.3)	21.4 (0.3)	22.4 (0.3)†‡	24.0 (0.3)†‡§	20.6 (0.2)	21.7 (0.3)†	22.4 (0.3)†‡	24.5 (0.3)†‡§
Drinking, % (*n*)	46.8 (44)	59.0 (72)	61.5 (67)	71.2 (79)*	12.0 (16)	15.3 (22)	13.6 (22)	21.5 (32)
Heavy drinking[b], % (*n*)	40.4 (38)	47.5 (58)	50.5 (55)	64.0 (71)*	8.3 (11)	6.3 (9)	3.7 (6)	13.4 (20)*
Diabetes mellitus[c], % (*n*)	7.4 (7)	6.6 (8)	8.3 (9)	12.6 (14)	1.5 (2)	4.2 (6)	4.3 (7)	10.1 (15)*
Hypertension[d], % (*n*)	7.4 (7)	18.0 (22)	28.4 (31)	28.8 (32)*	11.3 (15)	11.8 (17)	19.1 (31)	25.5 (38)*
TG, mmol/L	1.3 (0.1)	1.2 (0.1)	1.6 (0.1)	2.6 (0.3)†‡§	1.3 (0.1)	1.3 (0.1)	1.5 (0.1)	2.2 (0.1)†‡§
Metabolic syndrome[e], % (*n*)	4.3 (4)	5.7 (7)	14.7 (16)	23.4 (26)*	8.3 (11)	11.8 (17)	21.0 (34)	42.3 (63)*
SF, μmol/L	130.2 (70.9, 166.3)	158.0† (124.3, 253.8)	193.5†‡ (127.6, 288.4)	270.6†‡§ (180.6, 332.1)	43.4 (27.9, 96.8)	49.7 (23.7, 101.6)	81.8†‡ (35.6, 156.3)	104.3†‡§ (56.2, 157.4)
GGT, U/L	13 (10, 14)	21† (17, 29)	36†‡ (29, 48)	105.5†‡§ (65.8, 172.0)	9 (8, 11)	14† (12, 18)	21†‡ (17, 27.5)	50†‡§ (38, 82)
MDA, mmol/L	4.4 (0.2)	4.3 (0.1)	4.4 (0.1)	4.5 (0.1)	3.7 (0.1)	3.7 (0.1)	4.0 (0.1)†‡	4.1 (0.1)†‡
eGFR, mL/min per 1.73 m^2	96.7 (1.8)	91.4 (2.1)	91.5 (2.6)	89.6 (1.5)†	90.0 (1.2)	90.4 (1.3)	86.0 (1.3)†‡	86.2 (1.3)†‡
ACR, mg/g creatinine	10.1 (4.9, 19.7)	10.1 (3.3, 19.8)	11.0 (4.5, 20.7)	9.3 (4.2, 29.3)	10.1 (5.3, 15.5)	10.4 (3.6, 22.5)	11.2 (5.7, 23.4)	14.4†‡ (5.8, 28.3)

$^{\dagger}P < 0.05$ compared with quartile 1; $^{\ddagger}P < 0.05$ compared with quartile 2; $^{\S}P < 0.05$ compared with quartile 3.$^{*}P < 0.05$ compared across all quartiles. [b]Drinking more than 60 mL of alcohol per day. [c]Diabetes mellitus was defined according to the criteria of the American Diabetes Association (1999). [d]Hypertension was defined as systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg. [e]Metabolic syndrome was defined according to the criteria of the IDF (2005).

Table 2: General characteristics of individuals stratified by different levels of SF.

	Men				Women			
	Quartile 1	Quartile 2	Quartile 3	Quartile 4	Quartile 1	Quartile 2	Quartile 3	Quartile 4
n	108	109	110	109	146	147	148	147
Age, years (S. E.)	42.9 (1.6)	45.0 (1.5)	44.6 (1.3)	47.8 (1.2)†	35.1 (0.8)	40.0 (1.0)†	45.8 (1.0)†‡	56.4 (0.9)†‡§
BMI, kg/m^2	20.9 (0.3)	21.7 (0.3)	22.4 (0.3)†	23.6 (0.3)†‡§	22.2 (0.3)	21.6 (0.3)	23.0 (0.3)‡	22.7 (0.4)‡
Drinking, % (*n*)	51.9 (56)	62.4 (68)	53.6 (59)	72.5 (79)*	15.1 (22)	16.3 (24)	15.5 (23)	15.6 (23)
Heavy drinking[b], % (*n*)	40.7 (44)	55.0 (60)	46.4 (51)	61.5 (67)*	6.2 (9)	7.5 (11)	8.8 (13)	8.8 (13)
Diabetes mellitus[c], % (*n*)	7.4 (8)	11.0 (12)	6.4 (7)	10.1 (11)	2.7 (4)	2.7 (4)	5.4 (8)	9.5 (14)*
Hypertension[d], % (*n*)	13.9 (15)	22.9 (25)	19.1 (21)	28.4 (31)	11.6 (17)	8.2 (12)	20.3 (30)	28.6 (42)*
TG, mmol/L	1.3 (0.1)	1.4 (0.1)	1.6 (0.2)	2.5 (0.3)†‡§	1.2 (0.1)	1.4 (0.1)†	1.6 (0.1)†	2.1 (0.1)†‡§
Metabolic syndrome[e], % (*n*)	4.6 (5)	11.0 (12)	11.8 (13)	21.1 (23)*	13.0 (19)	10.9 (16)	23.6 (35)	37.4 (55)*
SF, μmol/L	71.7 (54.0, 95.2)	143.1† (128.2, 174.1)	210.0†‡ (172.5, 274.8)	340.4†‡§ (306.1, 379.7)	18.5 (10.3, 27.3)	44.8† (37.4, 56.9)	99.9†‡ (80.6, 117.3)	202.0†‡§ (152.0, 262.1)
GGT, U/L	19 (13, 31.5)	26† (16, 40.5)	34†‡ (20, 65.8)	53.5†‡§ (29.2, 121.8)	14 (11, 22)	16 (11, 28.5)	19†‡ (12, 32)	25†‡§ (14, 47)
MDA, mmol/L	4.2 (0.1)	4.1 (0.1)	4.4 (0.1)	4.9 (0.1)†‡§	3.6 (0.1)	3.8 (0.1)	4.0 (0.1)†	4.1 (0.1)†‡
eGFR, mL/min per 1.73 m^2	95.8 (1.8)	91.7 (2.3)	91.9 (2.7)	88.9 (1.3)†	94.1 (1.2)	90.5 (5.2)†	86.2 (1.2)†‡	81.4 (1.4)†‡§
ACR, mg/g creatinine	8.6 (3.3, 19.4)	12.2 (4.1, 20.3)	10.4 (4.4, 16.9)	11.4† (5.1, 34.5)	9.8 (3.6, 15.5)	10.3 (5.0, 26.0)	11.9 (5.6, 23.0)	14.6† (6.6, 28.0)

$^{\dagger}P < 0.05$ compared with quartile; $^{\ddagger}P < 0.05$ compared with quartile 2; $^{\S}P < 0.05$ compared with quartile 3; $^{*}P < 0.05$ compared across all quartiles. [b]Drinking more than 60 mL of alcohol per day. [c]Diabetes mellitus was defined according to the criteria of the American Diabetes Association (1999). [d]Hypertension was defined as systolic blood pressure $\geq$ 140 mmHg and/or diastolic blood pressure $\geq$ 90 mmHg. [e]Metabolic syndrome was defined according to the criteria of the IDF (2005).

Table 3: General characteristics of individuals in groups stratified by different combinations of GGT and SF levels[a].

	Group 1	Group 2	Group 3
n	614	304	106
Age, years (S. E.)	41.9 (0.6)	48.4 (0.7)†	49.3 (1.2)†
Male, % (*n*)	56.4 (346)	38.2 (116)	49.1 (52)
BMI, kg/m^2	21.4 (0.1)	23.3 (0.2)†	24.3 (0.4)†‡
Drinking, % (*n*)	31.3 (192)	36.5 (111)	48.1 (51)*
Heavy drinking[b], % (*n*)	23.1 (142)	26.6 (81)	42.5 (45)*
Diabetes mellitus[c], % (*n*)	4.9 (30)	7.2 (22)	15.1 (16)*
Hypertension[d], % (*n*)	14.2 (87)	22.7 (69)	34.9 (37)*
TG, mmol/L	1.26 (0.0)	1.98 (0.1)†	2.81 (0.3)†‡
Metabolic syndrome[e], % (*n*)	8.1 (50)	29.3 (89)	36.8 (39)*
SF, μmol/L	72.5 (35.9, 131.0)	164.6 (96.2, 269.3)†	300.8 (211.2, 359.2)†‡
GGT, U/L	16 (12, 23.3)	34 (20, 63.5)†	75 (49.8, 127.3)†‡
MDA, mmol/L	4.0 (0.1)	4.2 (0.1)†	4.5 (0.1)†
eGFR, mL/min per 1.73 m^2	92.2 (0.8)	86.1 (0.9)†	86.1 (1.7)†
ACR, mg/g creatinine	10.3 (4.0, 19.2)	12.2 (5.8, 24.2)†	14.4 (5.4, 35.0)†

$^{\dagger}P < 0.05$ compared with group 1; $^{\ddagger}P < 0.05$ compared with group 2; $^{*}P < 0.05$ compared across groups. [a]Group 1: both GGT and SF were not in the fourth quartile; group 2: only GGT or SF was in the fourth quartile; group 3: both GGT and SF were in the fourth quartile. [b]Drinking more than 60 mL of alcohol per day. [c]Diabetes mellitus was defined according to the criteria of the American Diabetes Association (1999). [d]Hypertension was defined as systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg. [e]Metabolic syndrome was defined according to the criteria of the IDF (2005).

Table 4: The risks of CKD in groups with different levels of GGT.

	Quartile 1	Quartile 2	Quartile 3	Quartile 4
Model 0	1	1.457 (0.861, 2.464)	1.422 (1.106, 1.830)	1.364 (1.156, 1.610)
Model 1	1	—	1.360 (1.003, 1.699)	1.220 (1.012, 1.471)
Model 2	1	—	1.297 (0.997, 1.689)	1.215 (1.007, 1.466)
Model 3	1	—	1.303 (1.001, 1.697)	1.163 (0.960, 1.409)
Model 4	1	—	1.293 (0.992, 1.686)	1.178 (0.973, 1.427)
Model 5	1	—	1.302 (1.001, 1.695)	1.216 (1.007, 1.467)
Model 6	1	—	1.302 (1.000, 1.695)	1.200 (0.993, 1.451)
Model 7	1	—	1.308 (1.005, 1.703)	1.217 (1.009, 1.468)

Model 0, no confounding factors were adjusted; model 1, adjusted for age and BMI; model 2, model 1 plus drinking; model 3, model 1 plus T2D; model 4, model 1 plus hypertension; model 5, model 1 plus hypertriglyceridemia; model 6, model 1 plus metabolic syndrome; model 7, model 1 plus MDA.

5. Discussion

The present study found that subjects with high level of GGT or SF had increase prevalence of CKD compared with those with relatively low levels of GGT and SF. When GGT and SF were both at high levels, the CKD rates were the highest which were independent of age, BMI, drinking status, diabetes mellitus, hypertension, hypertriglyceridemia, and metabolic syndrome. The increased rates of CKD from group 1 to group 3 were accompanied by gradual increased levels of MDA.

Several published studies had investigated the association between GGT and CKD. Some studies showed that serum GGT within the physiologic range independently predicted albuminuria among patients with or without hypertension or diabetes [11, 12], while another study showed that there was no association between increased levels of serum GGT and CKD [13]. The discrepancy among these studies was unclear. What is clear is that most of these studies were based on the same speculation that the effect of GGT is related to oxidative stress. GGT plays important roles in GSH metabolism, and the latter is the most important extracellular antioxidative agent. In the process of GGT-mediated cleavage of glutathione, iron exerts important effect as an electron transporter [9]. Theoretically, the circulatory iron could interact with GGT, lead to enhanced oxidative stress, and increase the risk of CKD. Unfortunately, few study investigated the association between SF and CKD. In the present study, SF in the highest quartile was associated with increased risk of CKD, but such an association was dependent on confounding factors such as drinking status, diabetes mellitus, hypertension, hypertriglyceridemia, or metabolic syndrome. However, when GGT and SF were analyzed in combination, it can be seen that the incidence of CKD was the highest in group 3 where GGT and SF were both at the highest quartile and which was accompanied by the highest MDA level.

Table 5: The risks of CKD in groups with different levels of SF.

	Quartile 1	Quartile 2	Quartile 3	Quartile 4
Model 0	1	1.551 (0.945, 2.544)	1.278 (1.000, 1.174)	1.373 (1.174, 1.606)
Model 1	1	—	1.103 (0.850, 1.431)	1.259 (1.051, 1.510)
Model 2	1	—	—	1.265 (1.053, 1.519)
Model 3	1	—	—	1.246 (1.036, 1.499)
Model 4	1	—	—	1.248 (1.040, 1.498)
Model 5	1	—	—	1.250 (1.038, 1.504)
Model 6	1	—	—	1.242 (1.034, 1.492)
Model 7	1	—	—	1.253 (1.038, 1.512)

Model 0, no confounding factors were adjusted; model 1, adjusted for age and BMI; model 2, model 1 plus drinking; model 3, model 1 plus T2D; model 4, model 1 plus hypertension; model 5, model 1 plus hypertriglyceridemia; model 6, model 1 plus metabolic syndrome; model 7, model 1 plus MDA.

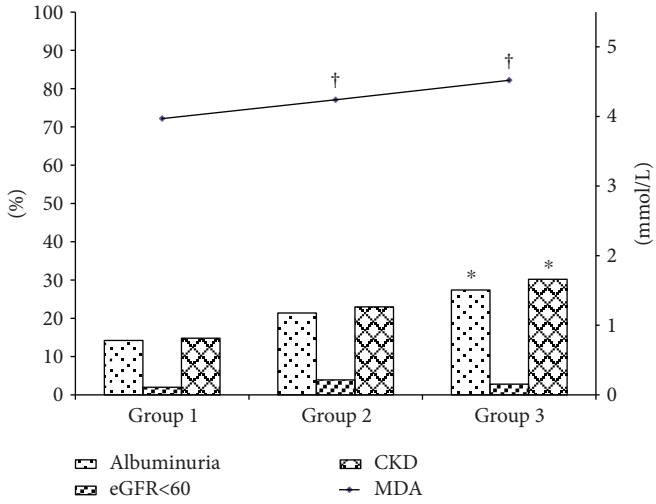

Figure 1: The prevalence of CKD in the different groups according to different combination of GGT and SF quartiles. Group 1: both GGT and SF were not in the fourth quartile; group 2: only GGT or SF was in the fourth quartile; group 3: both GGT and SF were in the fourth quartile. $^{\dagger}P < 0.05$ compared with group 1; $^{*}P < 0.05$ compared across groups.

Table 6: The risks of CKD in groups with the highest quartile of serum GGT and/or SF compared with group with other quartiles of serum GGT and SF levels[a].

	Group 1	Group 2	Group 3
Model 0	1	1.743 (1.228, 2.474)	2.413 (1.504, 3.871)
Model 1	1	1.440 (1.000, 2.075)	2.227 (1.355, 3.662)
Model 2	1	1.438 (0.998, 2.072)	2.293 (1.382, 3.805)
Model 3	1	1.429 (0.990, 2.062)	2.051 (1.233, 3.413)
Model 4	1	1.376 (0.951, 1.991)	1.996 (1.201, 3.317)
Model 5	1	1.286 (0.878, 1.882)	1.887 (1.104, 3.226)
Model 6	1	1.267 (0.867, 1.852)	2.079 (1.241, 3.482)
Model 7	1	1.461 (1.012, 2.109)	2.215 (1.342, 3.658)

[a]Group 1: both GGT and SF were not in the fourth quartile; group 2: only GGT or SF was in the fourth quartile; group 3: both GGT and SF were in the fourth quartile. Model 0, no confounding factors were adjusted; model 1, adjusted for age and BMI; model 2, model 1 plus drinking; model 3, model 1 plus T2D; model 4, model 1 plus hypertension; model 5, model 1 plus hypertriglyceridemia; model 6, model 1 plus metabolic syndrome; model 7, model 1 plus MDA.

These data highly suggested that GGT and SF worked together to promote increased oxidative stress and independently led to higher rate of CKD.

A subgroup analysis from the Nurses' Health Study showed that western diet (featured by higher portion of red and processed meats, saturated fats, and sweets) is associated with a significantly increased odds of microalbuminuria and rapid kidney function decrease [14]. High intake of red meat was also associated with elevated GGT and SF levels [15, 16]. So, it could be proposed that excessive red meat intake could lead to increased serum concentration of GGT and SF and thus increased the risk of CKD.

The major limitation of this study is its cross-sectional design, which could only suggest potential association between GGT, SF, oxidative stress, and CKD. Another limitation is that the sample size of the population was not large enough when both GGT and SF were taken into account, which hampers us to further analyze according to different gender and location; for example, GGT and SF synergy might be more obvious in men (Supplementary Tables 1 and 2 available online at https://doi.org/10.1155/2017/9765259). Third, due to insufficient blood sample, only MDA was measured as a marker of oxidative stress. This prevented us from analyzing the reason that the CKD risks in groups 2 and 3 were not dramatically weakened after adjustment for MDA level. Fourth, a large portion of participants were alcohol drinkers. Alcohol drink could increase serum levels of GGT and SF. Although the association between GGT, SF, and CKD still existed after adjustment for alcohol drinking or heavy drinking, potential interference might still exist.

6. Conclusions

In conclusion, this study found that GGT and SF could act together to increase the rate of CKD. The potential mechanism might be related to enhanced oxidative stress. Prospective studies or delicate designed animal studies are warranted to further investigate the potential values of GGT and SF in clinical practice with CKD.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Tao Chen and Yan Ren contributed equally to this paper. Tao Chen conceived, designed, and performed the experiments, analyzed the data, and wrote the paper. Yan Ren conceived and designed the experiments. Yun Gao collected the data. Haoming Tian conceived and designed the experiments.

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Research Article

Associations between *Interleukin-31* Gene Polymorphisms and Dilated Cardiomyopathy in a Chinese Population

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To explore the role of *Interkeulin-31 (IL-31)* in dilated cardiomyopathy (DCM), in our study, two SNPs of *IL-31*, rs4758680 (C/A) and rs7977932 (C/G), were analyzed in 331 DCM patients and 493 controls in a Chinese Han population. The frequencies of C allele and CC genotype of rs4758680 were significantly increased in DCM patients ($P = 0.005$, $P = 0.001$, resp.). Compared to CC genotype of rs4758680, the A carriers (CA/AA genotypes) were the protect factors in DCM susceptibility while the frequencies of CA/AA genotypes were decreased in the dominant model for DCM group ($P < 0.001$, OR = 0.56, 95%CI = 0.39–0.79). Moreover, IL-31 mRNA expression level of white blood cells was increased in DCM patients (0.072 (0.044–0.144) versus 0.036 (0.020–0.052), $P < 0.001$). In survival analysis of 159 DCM patients, Kaplan-Meier curve revealed the correlation between CC homozygote of rs4758680 and worse prognosis for DCM group ($P = 0.005$). Compared to CC genotype, the CA/AA genotypes were the independent factors in both univariate (HR = 0.530, 95%CI = 0.337–0.834, $P = 0.006$) and multivariate analyses after age, gender, left ventricular end-diastolic diameter, and left ventricular ejection fraction adjusted (HR = 0.548, 95%CI = 0.345–0.869, $P = 0.011$). Thus, we concluded that *IL-31* gene polymorphisms were tightly associated with DCM susceptibility and contributed to worse prognosis in DCM patients.

1. Introduction

Dilated cardiomyopathy (DCM) as a primary myocardial disease is marked by dilation of the left ventricle as well as systolic dysfunction that is with progressive functional and structural changes [1, 2]. It affects ~1/2500 adults and more common in men than in women [3, 4]. DCM is one of the pivotal causes of sudden cardiac death and congestive heart failure concurrent with the main indication for heart transplantation [5, 6]. Over the last years, substantial studies have focused on the etiology and development of DCM, whereas the exact cause of DCM was still not understood. Increasing evidence supports several cytokines implicated in the inflammatory, and immune responses are participating in the pathological process of DCM even congestive heart failure [7, 8]. It has been delineated that the gene polymorphisms of proinflammatory cytokines such as interleukin- (IL-) 6 and tumor necrosis factor-α (TNF-α) are associated with the susceptibility and prognosis of DCM or heart failure [7–9].

Interleukin-31 (IL-31) is a novelly detected proinflammatory cytokine belonging to gp130/IL-6 cytokine family which includes IL-6, IL-11, IL-27, oncostatin M, cardiotrophin-1, cardiotrophin-like cytokine, and leukemia inhibitory factor [10, 11]. It is produced principally by the activated $CD4^+$ T cells, especially when cells are skewed toward a Th2 phenotype [10, 12]. Distinguished with gp130 family, it acts through a heterodimeric receptor consisting of IL-31RA which is gp130-like receptor and oncostatin receptor (OSMR) [10]. As previously reported, IL-31 significantly

Table 1: Information about PCR-RFLP in DCM and control groups.

SNP ID	Primer sequence	Major/minor gene	Product (bp)	Annealing temperature (°C)	Restriction enzyme	Allele (bp)
rs4758680	F:5′-GATCACCCGGACTCAAAACGTG-3’	C/A	263	60	MboII	A (263)
	R: 5′-TTGTGCAAACCACACCTCTTCG-3’	—	—	—	—	C (210 + 53)
rs7977932	F:5′-GGTCAGTGTTGGGTTTGCAATG-3’	C/G	121	60	ScrFI	G (74 + 57)
	R:5′-TTGGTGATGGCACAGCCTCATA-3’	—	—	—	—	C (131)

stimulated the secretion of proinflammatory cytokines, such as IL-6 from monocytes and macrophages [13] and human colonic subepithelial myofibroblasts [14]. IL-6 could induce a hypertrophic response in myocytes [15], and TNF-α could trigger the left ventricular dilation [16]. In addition, the available data exhibited that IL-31 contributed to atopic dermatitis [17, 18], nonatopic eczema [19], systemic lupus erythematosus (SLE) [20], asthma [21], inflammatory bowel disease (IBD) [14], familial primary cutaneous amyloidosis [22], Kawasaki disease [23], hepatitis B virus liver failure [24], and allergic rhinitis [25]. These observations imply that IL-31 may contribute to the pathogenesis of DCM via cytokine modulation of immune response.

However, thus far, no study on the correlation between *IL-31* and DCM was reported. Therefore, we conducted the pilot study to clarify the role of *IL-31* in DCM patients in a Chinese population.

2. Materials and Methods

2.1. Study Subjects. The case group contained 331 subjects (male/female: 214/117, mean age: 50.16 ± 14.01 years) diagnosed as DCM recruited from the West China Hospital from June 2002 to October 2015. Since the median of the left ventricular ejection fraction (LVEF) among DCM patients was 30%, DCM patients were divided into two groups (LVEF < 30% versus LVEF ≥ 30%) in SNP-stratified analysis. The diagnosis of DCM was made in consistent with the criteria established by the World Health Organization/International Society and Federation of Cardiology Task Force on the Classification of Cardiomyopathies in 1995 (before 2006) and the scientific statement on the definitions and classification of cardiomyopathies proposed by the American Heart Association in 2006 (after 2006) [2, 26]. Meanwhile, for comparison, we recruited the control group from a routine health survey, and finally, 493 healthy unrelated individuals (male/female: 312/181, mean age: 49.15 ± 8.82 years) were consecutively enrolled. The patients with hypertension, coronary heart disease, cardiac valve disease, tachyarrhythmia, acute viral myocarditis, heavy alcohol intake, skeletal myopathies, systemic diseases of putative autoimmune origin, diabetes, and obesity or insulin resistance were excluded from the study. Written informed consents were obtained from all included subjects, sequentially, and 10 mL of peripheral venous blood was drawn from each of the DCM patient and control subjects. The present study was approved by the hospital ethics committee.

2.2. Extraction of DNA and Genotyping. Genomic DNA was extracted from 200 μl EDTA-anticoagulated peripheral blood sample with a DNA isolation kit (BioTeke, Peking, China) as the manufacturer's direction. DNA was stably stored at −20°C until assayed. Genotyping of the *IL-31* gene polymorphism was conducted by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). We designed the PCR primers with software Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) [27] as shown in Table 1. The 10 μl PCR reaction system was consisted of 1.0 μl DNA and 5 μl 2× Power Taq PCR Master Mix (BioTeke, Peking, China), forward and reverse primer 0.1 μl, respectively, and reserved volume was made up to 10 μl by sterilized water. The PCR condition was designed as 95°C for 4 min firstly, then 33 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and finally, 72°C for 10 min. Furthermore, the PCR products were digested in 37°C stable incubation by distinguished restriction enzyme MboII (New England Biolabs, Peking, China) for 30 minutes of rs4758680 and ScrFI (New England Biolabs, Peking, China) for 2 hours of rs7977932 as shown in Table 1, separately. Ultimately, the results were visually analyzed by 6% polyacrylamide gels in silver staining. To verify the genotyping results, DNA sequencing was performed in about 20% PCR-amplified DNA samples randomly.

2.3. mRNA Isolation, Reverse Transcription, and Quantitative Real-Time PCR (qPCR). Quantitative real-time PCR of IL-31 was conducted in 41 DCM patients and 49 controls. Total RNA was isolated from white blood cells (WBCs) with TRIzol reagent (Invitrogen, Karlsruhe, Germany) and then was reverse-transcribed into cDNA, using the Bioneer kit (R&D Center, Korea) following the manufacturer's protocols. Actin was chosen as a reference parameter to normalize the data. The sense and anti-sense primers for *IL-31* and *Actin* were 5′-CTCACTCAGGCCCCTCG AC-3′ and 5′-GTCGTAGTAAACGGACGGGC-3′, 5′-TGA CGTGGACATCCGCAAAG-3′ and 5′-CTGGAAGGTGGA CAGCGAGG-3′, respectively.

The qPCR settings in triplicate carried on a MasterCycler realplex^4 (Eppendorf, Wesseling-Berzforf, Germany) using SYBR Green were as follows: an initial activation step of 10 min at 95°C, subsequently by two-step cycling for 25 times: denaturation of 15 s at 94°C, and annealing and extension of 20 s at 60°C. Melting curve was added to check the amplification specificity. $2^{-\triangle ct}$ method was used to calculate the mRNA expression levels [28].

Table 2: Baseline characteristics of the DCM patients and the controls.

Variables	DCM patients ($n = 331$)	Controls ($n = 493$)	P value
Age (years)	52 (43–60)	50 (42–57)	0.022
Gender (male/female)	214/117	312/181	0.689
SBP (mmHg)	116 (109–123)	121 (114–127)	<0.001
DBP (mmHg)	73 (68–77)	77 (72–81)	<0.001
NYHA	II: 62; III: 210; IV: 59	I: 395; II: 98	<0.001
LVEDD (mm)	67 (62–73)	46 (44–49)	<0.001
LVEF (%)	30 (25–37)	62 (58–66)	<0.001
BNP (pg/ml)	2787 (1600–3836)	95 (83–108)	<0.001

Data are exhibited as the median ± interquartile range(IQR: Q25%–Q75%) or number; DCM: dilated cardiomyopathy; SBP: systolic blood pressure; DBP: diastolic blood pressure; NYHA: New York Heart Association; LVEDD: left ventricular end-diastolic diameter; LVEF: left ventricular ejection fraction; BNP: brain natriuretic peptide.

2.4. Patients' Clinical Characteristics and Follow-Up. One hundred and fifty-nine DCM patients who have reserved contact information were brought into follow-up plan every three months until September 13, 2016. Basic clinical materials of enrolled DCM patients were obtained from the medical records (age, gender, etc.) and echocardiographic measurement using a S5-1 broadband phased-array transducer (1–5 MHz). According to the recommendations of the American Society of Echocardiography, we conducted a comprehensive 2D and Doppler echocardiography. The echocardiographic indicators such as the left ventricular end-diastolic diameter (LVEDD) were calculated with M-mode echocardiography with the left parasternal window while the left ventricular ejection fraction (LVEF) was accessed by apical two- and four-chamber views with the modified Simpson rule. The follow-up end point was patient's death because of heart failure or sudden cardiac events. A blind manner about patient's genetic status was applied during clinical follow-up.

2.5. Statistical Analysis. Quantitative variables were presented as median and interquartile range (IQR: Q25%–Q75%) and categorical variables as number of observations. Normality was tested using Shapiro-Wilk's test for normality. Differences between two independent samples for continuous data were analyzed using the Mann–Whitney U test since the distributions were different from normal. For categorical variables, statistical analysis was based on Pearson's chi-square test.

The allelic and genotype frequencies were obtained by number counting. The differences of genotypes between the DCM and control groups including codominant, dominant, recessive, and overdominant genetic models were analyzed by using SNPStats online program; meanwhile, odds ratio and 95% confidence intervals were obtained accordingly [29].

Allelic association and Hardy-Weinberg equilibrium were assessed with chi-square test. IL-31 WBC mRNA expression level was compared using the Mann–Whitney test (for two independent groups) and Kruskal-Wallis H test with multiple comparisons post hoc tests according to the results from Shapiro-Wilk's test for normality.

Kaplan-Meier curve and Cox proportional hazard models were applied to evaluate the role of IL-31 SNPs on prognosis in DCM patients. P value less than 0.05 was regarded as statistically significant.

3. Results

3.1. Baseline Characteristics of DCM Patients and the Controls. As shown in Table 2, between the DCM patients and the controls, gender did not exhibit statistically significant differences ($P = 0.689$). Compared to controls, DCM patients were older ($P = 0.022$) and had lower systolic blood pressure (SBP), diastolic blood pressure (DBP), left ventricular ejection fraction (LVEF), higher left ventricular end-diastolic diameter (LVEDD), and brain natriuretic peptide (BNP) ($P < 0.001$, resp.) as well as more severe NYHA functional class ($P < 0.001$). All DCM patients accepted medication treatment according to the clinical guidelines for DCM and heart failure.

3.2. Associations between IL-31 SNPs and Susceptibility for DCM and DCM Patients' Characteristics. The gene polymorphisms of *IL-31* rs4758680 and rs7977932 were identified to compare the allelic and genotype frequencies of 331 DCM patients to 493 controls through direct counting. About 20% PCR-amplified DNA samples were randomly evaluated by DNA sequencing, and the PCR-RFLP results were manifested as 100% accurate. The distribution of both rs4758680 and rs7977932 alleles in control groups was line with the postulation of Hardy-Weinberg equilibrium ($\chi^2 = 1.876$, $P = 0.17$ for rs4758680; $\chi^2 = 0.004$, $P = 0.95$ for rs7977932).

As shown in Table 3, the strikingly statistical difference was discovered at rs4758680. The allele frequency of C of SNP rs4758680 in DCM patients was significantly elevated compared with that in controls (89.6% versus 84.8%); in contrast, the allele A frequency was declined (10.4% versus 15.2%, $P = 0.005$, OR = 0.65, 95%CI = 0.48–0.88) in case group. In codominant model, the frequencies of the CC, CA, and AA genotypes of rs4758680 were 83.1%, 13.0%, and 3.9% in cases and were 73.2%, 23.1%, and 3.6% in controls, respectively. The differences among genotype frequencies were statistically significant ($P = 0.001$). In dominant model, compared with CC genotype, a notably decreased DCM risk was related with CA/AA genotypes ($P < 0.001$,

Table 3: Distributions of *IL-31* SNPs among cases and controls and their associations with DCM susceptibility.

		rs4758680					rs7977932			
	Genotype	Cases *n* (%)	Controls *n* (%)	OR (95%CI)	*P* value	Genotype	Cases *n* (%)	Controls *n* (%)	OR (95%CI)	*P* value
Model										
Codominant	CC	275 (83.1%)	361 (73.2%)	1.00	—	CC	271 (81.9%)	401 (81.3%)	1.00	—
	CA	43 (13.0%)	114 (23.1%)	**0.50 (0.34–0.72)**	**0.001**	CG	56 (16.9%)	87 (17.6%)	1.05 (0.73–1.52)	0.930
	AA	13 (3.9%)	18 (3.6%)	0.95 (0.46–1.96)	—	GG	4 (1.2%)	5 (1.0%)	0.84 (0.22–3.17)	—
Dominant	CC	275 (83.1%)	361 (73.2%)	1.00	—	CC	271 (81.9%)	401 (81.3%)	1.00	—
	CA/AA	56 (16.9%)	132 (26.8%)	**0.56 (0.39–0.79)**	**<0.001**	CG/GG	60 (18.1%)	92 (18.7%)	1.04 (0.72–1.49)	0.850
Recessive	CC/CA	318 (96.1%)	475 (96.3%)	1.00	—	CC/CG	327 (98.8%)	488 (99.0%)	1.00	—
	AA	13 (3.9%)	18 (3.6%)	0.93 (0.45–1.92)	0.840	GG	4 (1.2%)	5 (1.0%)	0.84 (0.22–3.14)	0.790
Overdominant	CC/AA	288 (87.0%)	379 (76.9%)	1.00	—	CC/GG	275 (83.1%)	406 (82.4%)	1.00	—
	CA	43 (13.0%)	114 (23.1%)	**0.50 (0.34–0.73)**	**<0.001**	CG	56 (16.9%)	87 (17.6%)	1.05 (0.73–1.52)	0.790
	Allele					Allele				
	C	593 (89.6%)	836 (84.8%)	1.00	—	C	598 (90.3%)	889 (90.2%)	1.00	—
	A	69 (10.4%)	150 (15.2%)	**0.65 (0.48–0.88)**	**0.005**	G	64 (9.7%)	97 (9.8%)	1.02 (0.73–1.42)	0.909

OR: odds ratio; CI: confidence interval; SNP analysis adjusted for age, gender, LVEDD, and LVEF.

OR = 0.56, 95%CI = 0.39–0.79). Subjects with heterozygous genotype (CA genotype) of rs4758680 had distinctly decreased risks for DCM compared with the CC/AA genotypes in overdominant model ($P < 0.001$, OR = 0.50, 95%CI = 0.34–0.73). As shown in Table 3, there was no significant difference described between DCM patients and controls in rs7977932 gene polymorphism analysis. To provide insights into the effects of *IL-31* SNPs on DCM, we exhibited the stratified analyses among 331 DCM patients. After adjusted by age and gender, the association between rs4758680 of *IL-31* and LVEF was shown in Table 4 which revealed that the heterozygote CA was the protect factor for DCM patients whose LVEF was <30% compared with those LVEF was ≥30% ($P = 0.042$). There were no statistically significant differences between the two SNPs of *IL-31* (rs4758680 and rs7977932) and LVEDD.

3.3. IL-31 WBC mRNA Expression Level. The median and IQR of $2^{-\triangle ct}$ result among 41 DCM patients were 0.072 (0.044–0.144), while it was 0.036 (0.020–0.052) among 49 controls, and the difference for IL-31 WBC mRNA expression between DCM group ($n = 41$) and control group ($n = 49$) was statistically significant ($P < 0.001$) as shown in Figure 1(a). To probe into the functional influence of the IL-31 (rs4758680) genotype polymorphism (CC, CA, and AA genotypes) on the IL-31 WBC mRNA expression, the quantitative IL-31 mRNA results among different genotypes were analyzed. The IL-31 mRNA results for CA/AA genotypes in DCM ($n = 21$) and controls ($n = 22$) were 0.087 (0.044–0.223) and 0.038 (0.021–0.050), and those for CC genotype in DCM ($n = 20$) and controls ($n = 27$) were 0.065 (0.044–0.124) and 0.033 (0.018–0.065). As Figure 1(b) delineated, the P value was less than 0.001 for Kruskal-Wallis H test and the results of multiple comparisons post hoc tests revealed that there were statistically significant differences for IL-31 mRNA level between CA/AA genotypes in DCM and CA/AA genotypes in controls, CC genotype in DCM and CA/AA genotypes in controls, CA/AA genotypes in DCM and CC genotype in controls, and CC genotype in DCM and CC genotype in controls ($P < 0.001$, resp.). There were no statistically significant differences between CA/AA genotypes and CC genotype in DCM, as well as in controls ($P = 0.191$, 0.389, resp.).

3.4. Survival Analysis of IL-31 Genotypes in DCM Patients. The prognosis of DCM associated with two SNPs of *IL-31* gene was carried out by survival analysis. 159 DCM patients (mean age, 51.03 ± 13.43 years; male/female, 107/52) were tracked for a mean period of three months. 20 DCM patients were lost during the follow-up. During the follow-up, all included patients accepted consecutive medication treatment and none underwent heart transplantation.

When ending up the follow-up, 104 (65.4%) DCM patients died ascribable to cardiac events. The baseline characteristic differences between 104 dead DCM patients and 35 survival DCM patients were described in Supplementary Table available online at https://doi.org/10.1155/2017/4191365, and there were no statistically significant differences between the two analyzed groups for gender, SBP, DBP, and LVEDD ($P = 0.251$, 0.789, 0.431, and 0.817, resp.). By contrast, dead DCM patients were younger ($P = 0.036$) and had worse NYHA functional class ($P < 0.001$), lower LVEF ($P = 0.025$), and higher BNP ($P = 0.020$).

Kaplan-Meier curves indicated that CC homozygote and CC/AA genotypes of *IL-31* rs4758680 were implicated in worse prognosis for DCM patients, respectively (Log-rank: $P = 0.005$, Figure 2; Log-rank: $P = 0.009$, Figure 3). Cox univariate survival analysis revealed A carriers (CA/AA genotypes) were correlated with better prognosis compared to CC genotype in genetic dominant model for DCM patients (HR = 0.530, 95%CI = 0.337–0.834, $P = 0.006$, Table 5). Similarly in overdominant model of rs4758680, compared to CC and AA homozygotes, heterozygote CA accounted for better prognosis for DCM patients (HR = 0.516, 95%CI = 0.310–0.861, $P = 0.011$, Table 5). After adjusting for age, gender, LVEDD, and LVEF, the associations between A carriers (CA/AA genotypes), CA heterozygote of rs4758680, and prognosis of DCM were still statistical significant for both dominant and overdominant models in multivariate Cox proportional hazard model analysis (HR = 0.548, 95%CI = 0.345–0.869, $P = 0.011$ and HR = 0.503, 95%CI = 0.297–0.852, $P = 0.011$, Table 5, resp.). There were no statistical differences between *IL-31* rs4758680 recessive model as well as rs7977932 gene polymorphism and overall survival time in univariate and multivariate Cox proportional hazard models as shown in Table 5.

4. Discussion

As previously reported, although the etiology and pathogenesis of DCM were complicated, chronic inflammation might contribute to cardiac remodeling and the development of DCM [30], and abnormal immune responses were proposed to be prominent factors in the DCM process especially after myocarditis [31]. Inflammatory cytokines like IL-6 in conjunction with TNF-α participated in myocyte apoptosis and myofibrosis which were involved in DCM [15, 32].

Beyond that, the established studies validated that IL-31 as a novel inflammatory cytokine was a potent inducer of proinflammatory mediators such as IL-6 in various cell types, including epithelial cells, colonic subepithelial myofibroblasts, PBMCs, macrophages, and eosinophils [33]. Matrix metalloproteinases (MMPs) also can be induced by IL-31 in colonic subepithelial myofibroblasts of IBD [14] and their higher serum level was involved in continuous extracellular matrix remodeling and increased collagen turnover of DCM with mildly dilated left ventricle [34, 35]. Shen et al. revealed that inhibition of MMPs, especially MMP-2, could reduce apoptosis from TNF-α in cultured cardiac myocytes [36]. In the past few years, IL-31 receptor was principally found in the skin, brain, lung, skeletal muscle, ovary, testis, prostate, spleen, thymus, bone marrow, and more [10]. In coincident with IL-31 receptor distribution, IL-31 was already identified to be associated with immune-dysfunction diseases such as atopic dermatitis, SLE, and asthma.

The established studies suggested that soluble IL-31RA might expand the range of responsive cells and tissues because of the transsignaling for IL-6 [37]; meanwhile,

Table 4: Associations between *IL-31* SNPs and DCM patients' characteristics.

	rs4758680					rs7977932				
	Genotype	LVEF <30% *n* (%)	LVEF ≥30% *n* (%)	OR (95%CI)	*P* value	Genotype	LVEF <30% *n* (%)	LVEF ≥30% *n* (%)	OR (95%CI)	*P* value
Model										
Codominant	CC	131 (85.1%)	144 (81.4%)	1.00	—	CC	128 (83.1%)	143 (80.8%)	1.00	—
	CA	14 (9.1%)	29 (16.4%)	0.52 (0.26–1.03)	0.035	CG	24 (15.6%)	32 (18.1%)	1.20 (0.67–2.15)	0.810
	AA	9 (5.8%)	4 (2.3%)	2.56 (0.76–8.33)	—	GG	2 (1.3%)	2 (1.1%)	0.82 (0.11–5.97)	—
Dominant	CC	131 (85.1%)	144 (81.4%)	1.00	—	CC	128 (83.1%)	143 (80.8%)	1.00	—
	CA/AA	23 (14.9%)	33 (18.6%)	0.76 (0.42–1.37)	0.360	CG/GG	26 (16.9%)	34 (19.2%)	1.17 (0.66–2.07)	0.590
Recessive	CC/CA	145 (94.2%)	173 (97.7%)	1.00	—	CC/CG	152 (98.7%)	175 (98.9%)	1.00	—
	AA	9 (5.8%)	4 (2.3%)	2.78 (0.84–9.09)	0.081	GG	2 (1.3%)	2 (1.1%)	0.79 (0.11–5.75)	0.820
Overdominant	CC/AA	140 (90.9%)	148 (83.6%)	1.00	—	CC/GG	130 (84.4%)	145 (81.9%)	1.00	—
	CA	14 (9.1%)	29 (16.4%)	**0.50 (0.25–0.99)**	**0.042**	CG	24 (15.6%)	32 (18.1%)	1.20 (0.67–2.16)	0.530
	Allele					Allele				
	C	276 (90.0%)	317 (90.0%)	1.00	—	C	280 (91.0%)	318 (90.0%)	1.00	—
	A	32 (10.0%)	37 (10.0%)	1.00 (0.64–1.56)	0.99	G	28 (9.0%)	36 (10.0%)	1.12 (0.67–1.88)	0.670

LVEF: left ventricular ejection fraction; OR: odds ratio; CI: confidence interval; SNP analysis adjusted for age and gender.

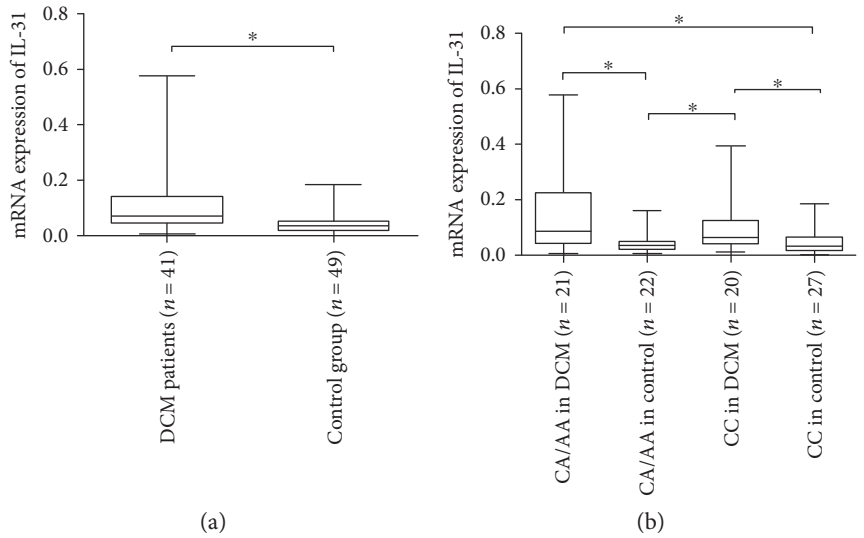

Figure 1: Comparsion of WBC IL-31 mRNA expression level between the DCM patients and the control group (a); comparsions of WBC IL-31 mRNA expression levels among rs4758680 different genotypes (b). The results were presented on box plots (median, IQR, range), $^{*}P < 0.001$.

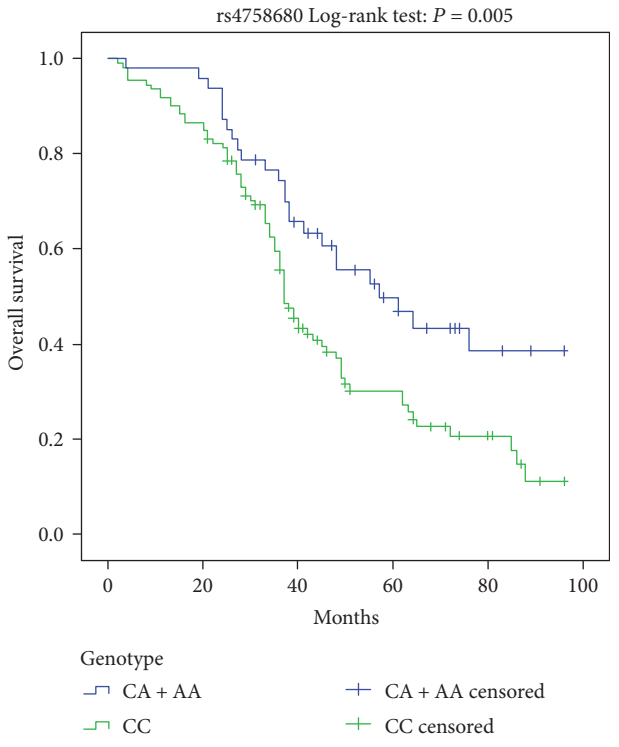

Figure 2: Kaplan-Meier survival curves for the dominant model of IL-31 rs4758680 polymorphism.

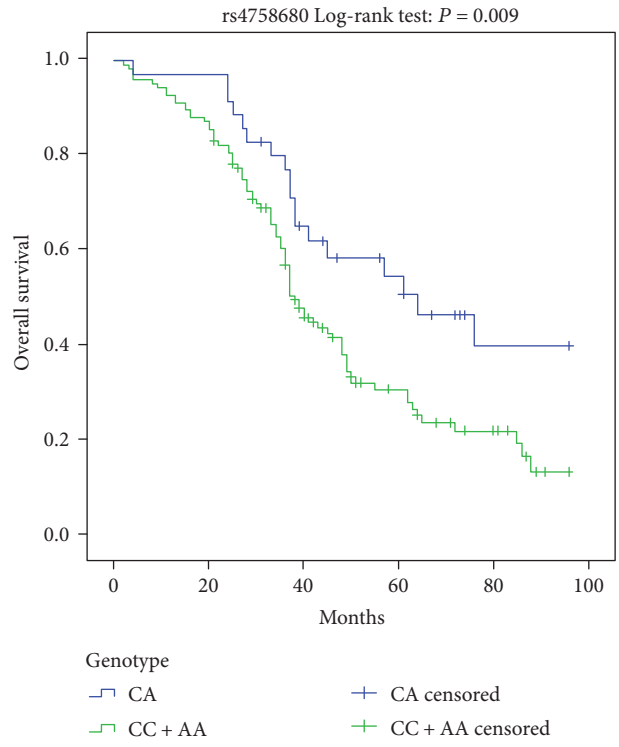

Figure 3: Kaplan-Meier survival curves for the overdominant model of IL-31 rs4758680 polymorphism.

myocarditis had overlapping loci with diabetes and SLE, suggesting that these autoimmune diseases shared genetic traits [1]. Similarly, Doria et al. identified that DCM was one of the most serious complications involved in SLE [38] and D. Y. Chen et al. have verified that the level of Th17-related cytokines (containing IL-6, TNF-α) was elevated in SLE-related DCM [39]. These inferences imply a possible role of IL-31 in immune response and in DCM pathogenesis process.

The present study was the first one to investigate the correlation between IL-31 and DCM in a Han Chinese population. The human IL-31 gene is located on chromosome 12q24.31 and encodes a protein with 164 amino acids. Both rs4758680 (C/A) and rs7977932 (C/G) are in chromosome

Table 5: Associations between *IL-31* SNPs and patients' overall survival.

Characteristics	Genotype	Overall survival					
		Multivariate survival analysis[a]			Univariate survival analysis		
		HR	95%CI	*P* value	HR	95%CI	*P* value
Model							
rs4758680							
Dominant	CC	1	—	—	1	—	—
	CA/AA	**0.548**	**0.345–0.869**	**0.011**	**0.530**	**0.337–0.834**	**0.006**
Recessive	CC/CA	1	—	—	1	—	—
	AA	0.868	0.399–1.888	0.722	0.778	0.361–1.678	0.522
Overdominant	CC/AA	1	—	—	1	—	—
	CA	**0.503**	**0.297–0.852**	**0.011**	**0.516**	**0.310–0.861**	**0.011**
rs7977932							
Dominant	CC	1	—	—	1	—	—
	CG/GG	1.161	0.666–2.024	0.599	1.275	0.736–2.211	0.386
Recessive	CC/CG	1	—	—	1	—	—
	GG	3.739	0.862–16.218	0.078	3.482	0.841–14.414	0.085
Overdominant	CC/GG	1	—	—	1	—	—
	CG	1.096	0.619–1.943	0.753	1.211	0.687–2.133	0.508

[a]Multivariate survival analysis adjusted for age, gender, LVEDD, and LVEF.

12 intron region of *Homo sapiens* which have been implicated in SLE and AD [19, 20, 40].

Our results showed that genotype frequencies in the codominant, dominant, and overdominant models of rs4758680 were associated with DCM susceptibility. The C allele frequency of rs4758680 (C/A) in DCM was elevated, whereas the A allele was declined. We manifested that the C allele was the main predisposing factor and A carriers (CA/AA genotypes) were the protect factors for DCM especially in genetic dominant model. The CC genotype frequency of rs4758680 of *IL-31* was also relevance with DCM worse prognosis in Kaplan-Meier curve and Cox proportional hazard models. Moreover, in accordance with SNP results of rs4758680, the IL-31 WBC mRNA expression level was overtly elevated in DCM group, and the WBC mRNA expression levels of CC and CA/AA genotypes in DCM patients were higher than those in control group. As previously reported, IL-31 acted through the receptor complex of IL-31RA which is gp130-like receptor and OSMR-β. gp130 has been shown to mediate the cardiotrophin-1 (CT-1) in the heart that resulted in LV hypertrophy [41]. OSMR-β has been proved as increasing expression trend in DCM patients [42] and signaling in myocardium that result in loss of sarcomere elements and cardiac fibroblast in mouse cardiac fibroblasts [43]. Kunsleben et al. delineated that the calcium influx was induced by IL-31 in eosinophils mainly through OSMR mediating, which prompt IL-31 may affect the myocardial contraction [44]. Hence, we concluded that rs4758680 of *IL-31* SNPs played a pathogenic role in DCM patients by facilitating IL-31 protein production. In our study, the number of WBC mRNA samples was not large enough, especially the number of AA genotype was only three cases and two cases in DCM and controls, respectively. The precise and intricate mechanisms for protein expression were still unclear, and much more studies will be indispensable. By contrast, the genotype frequency of rs7977932 was absence in the DCM susceptibility. As previously reported, rs7977932 (C/G) genotype polymorphism of *IL-31* was implicated in SLE and AD [20, 40]. The difference of inclusive quantity or disease essence between our study in DCM and the previous one in SLE may account for the inconsistency of rs7977932 effect.

5. Conclusions

In conclusion, IL-31 as scratch factor was a research hot spot before we firstly revealed that rs4758680 (C/A) of *IL-31* was associated with the susceptibility of DCM in the Chinese Han people although larger sample sizes of *IL-31* SNP would be necessary to confirm our findings; moreover, CC genotype was implicated in the worse prognosis in DCM group. Even so, plasma IL-31 protein level and the underlying mechanisms were lack in our study; besides, more SNPs of *IL-31* with DCM susceptibility and prognosis in a variety of ethnic populations need to be investigated in future studies.

Conflicts of Interest

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

Authors' Contributions

Huizi Song and Ying Peng contributed equally to this work.

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