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Roles and Clinical Applications of Biomarkers in Cardiovascular Disease 2020

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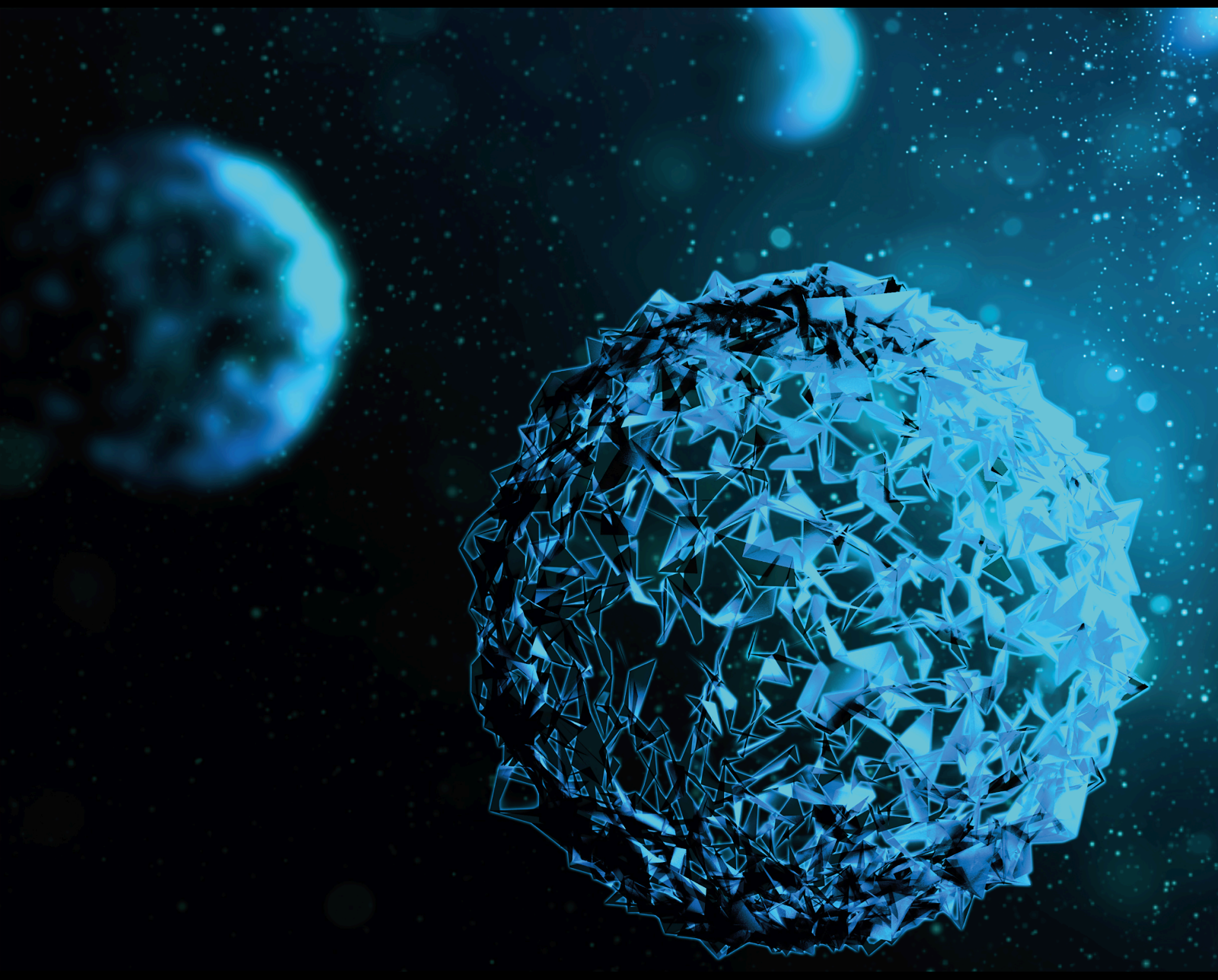

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Research Article

Chronic Venous Disease Patients Showed Altered Expression of IGF-1/PAPP-A/STC-2 Axis in the Vein Wall

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Chronic venous disease (CVeD) has a remarkable prevalence, with an estimated annual incidence of 2%. It has been demonstrated how the loss of homeostatic mechanisms in the vein wall can take part in the physiopathology of CVeD. In this regard, it has been described how different axis, such as IGF-1/PAPP-A/STC-2 axis, may play an essential role in tissue homeostasis. The aim of this research is to study both genetic and protein expressions of the IGF-1/PAPP-A/STC-2 axis in CVeD patients. It is a cross-sectional study in which genetic (RT-qPCR) and protein (immunohistochemistry) expression analysis techniques were accomplished in saphenous veins from CVeD patients ($n = 35$) in comparison to individuals without vascular pathology (HV). Results show a significant increase in both genetic and protein expressions of PAPP-A and IGF-1, and a decrement STC-2 expression at the same time in CVeD patients. Our study is a pioneer for demonstrating that the expression of the different components of the IGF-1/PAPP-A/STC-2 axis is altered in CVeD patients. This fact can be a part of the loss of homeostatic mechanisms of the venous tissue. Further research should be destined to deepen into alterations of this axis, as well as to evaluate the usage of these components as therapeutic targets for CVeD.

1. Introduction

Chronic venous disease (CVeD) is described as a complex of disorders that affect the venous system, in which the presence of varicose veins (VV) implies the most important clinical manifestation [1]. It is a condition of great prevalence, more common amongst women and people aged 40-80 years, with an estimated annual incidence of 2% [2]. Amongst the risk factors associated with chronic venous disease, family history, aging, obesity, sedentarism, pregnancy, hormonal factors, and greater height are of great significance [3, 4]. Furthermore, CVeD implies a great cost to the health system,

especially in occidental societies [3]; consequently, it is necessary to keep expanding the knowledge of this disease towards a more appropriate management.

Usually, CVeD affects lower limbs, producing venous hypertension which the difficulties of the venous blood return to the heart, having been described as the valvular failure as a key factor of this process [5, 6]. Several authors propose that vein wall alterations can precede those processes. The mechanisms, described in regard to varicose veins, are of great importance: remodelling of the extracellular matrix [7], cellular hypoxia mechanisms [8], and multiple hemodynamic changes which, amongst other effects, conduce to secondary inflammation and oxidative stress, globally affecting to the different layers of the vein wall [9–11]. Therefore, it is known that a change in the normal homeostasis of the tissue conforming the vein wall occurs. Hereof, it has been described the potential determinant role of different axis such as the IGF-1/PAPP-A/STC-2 axis in tissue homeostasis [12].

Insulin-like growth factor-1 (IGF-1) is a growth factor implied in a great variety of metabolic processes of proliferation, growth, and cellular differentiation. Its levels are directly correlated to growth hormone (GH) levels, accomplishing all these effects through bounding to IGF type 1 receptor (IGF1R) [13]. Insulin and IGF-1 share a great structural similarity, even though they bound to different receptors; cellular signalisation mediated by both of them is almost identical, highlighting their interaction with some factors like Akt or FoxO [14].

The role of the pregnancy-associated plasma protein A (PAPP-A) has been described as a negative regulator of the levels of IGFBPs, especially of IGFBP 2, 4, and 5. PAPP-A indirectly controls the effects of IGF-1 from the sectioning of IGFBPs, potentiating their cellular effects by increasing their bioavailability [15]. The role of PAPP-A in different vascular conditions, such as atherosclerosis, has been demonstrated, having been associated with an increase of inflammation and endothelial dysfunction along with proliferation and migration of smooth muscular cells [16], processes that are as well important for the pathogenesis of varicose veins. In addition, PAPP-A is a protein which can be determined in serum, where its usage as a biomarker for several vascular pathologies has been demonstrated [17–19]. On the other hand, stanniocalcin 2 (STC-2) has been depicted as an inhibitor of the proteolytic activity of PAPP-A, having been demonstrated multiple alterations of the PAPP-A/Stc-2 axis in a great variety of conditions, such as diabetes mellitus, cancer, and cardiovascular disorders [12].

Due to all of these reasons, this study is aimed at analyzing the expression of the different components of this axis in the vein wall from CVeD patients in comparison to the control patients without venous disease (HV), using immunohistochemistry techniques (IHC) and real-time PCR (RT-qPCR).

2. Patients and Methods

2.1. Study Design. This is a cross-sectional study of 35 patients clinically diagnosed with CVeD (47,000 [27,000-68,000] years old) in comparison to 27 control patients without venous disease (HV) (45,000 [23,000-66,000] years old), $p = 0.198$. In the CVeD group, we had 22 women and 13 men, while in the HV group, we had 20 women and 7 men, $p = 0.349$. We observed that in CVeD patients, there were 22 patients with a family history of CVeD, while in HV, there were 18 patients, $^{*}p = 0.021$.

The inclusion criteria were the following: women and men diagnosed with CVeD, with a body mass index (BMI) less than or equal to 25; with and without venous reflux in the greater saphenous vein; signed informed consent; and commitment to undergo follow-up during the pre- and postoperative periods, as well as to provide tissue samples. The exclusion criteria were patients with venous malformations or arterial insufficiency, patients without access to their clinical history, patients with pathologies that could affect the cardiovascular system (infectious diseases, diabetes, hypertension, and dyslipidemia), patients with toxic habits, and those uncertain of participating in the monitoring. The clinical diagnosis of CVeD and the evaluation of venous reflux were based on a noninvasive color Doppler ultrasound (7.5 to 10 MHz) of the superficial and deep vein systems. The saphenous vein segments for the HV group were verified during organ extraction for bypass surgery.

This study was conducted according to basic ethical principles (autonomy, harmless, benefit, and distributive justice); its development follows the standards of Good Clinical Practice and the principles enunciated in the last Declaration of Helsinki (2013) and the Oviedo Convention (1997). The project was approved by the ethics committee of the Gómez-Ulla Military Hospital (37/17).

2.2. Sample Processing. The totality of the mayor saphenous vein was extracted via saphenectomy. These fragments were introduced in two sterile tubes, one containing a RNA later solution (Ambion, Austin, TX, USA) and another containing MEM medium (Minimum Essential Medium) with 1% of antibiotic/antimycotic (Thermo Fisher Scientific, Waltham, MA, USA). Samples were processed in a Telstar AV 30/70 Müller class II 220 V 50 MHz laminar flow bell (Grupo Telstar SA, Terrassa, Spain) in a sterile environment. Samples collected in the RNAlater solution were kept at -80°C until been processed for the analysis of gene expression. Samples preserved in MEM were used to carry out histological studies. Samples were washed and hydrated multiple times with MEM without antibiotic to remove the erythrocytes and isolate the venous tissue. Subsequently, the venous tissue was sectioned in fragments and samples were preserved in F13 fixative (60% ethanol, 20% methanol, 7% polyethylene glycol, and 13% distilled water). After the time needed to fix it, samples were dehydrated in increasing concentrations of alcohol and were subjected to paraffin inclusion process. Once the tissue is impregnated, a paraffin block is confectioned. From these blocks, fine five-micron sections were made in a HM 350 s rotation microtome (Thermo Fisher Scientific, Waltham, MA, USA) and collected in slides impregnated with a 10% polylysine solution in order to accomplish the adherence of the sections to the slides.

2.3. Genetic Expression Study. The expression of the genes of interest was studied by real-time PCR method (RT-qPCR), in which the amount of cDNA was quantified in each of them. The RNA extraction was carried out by the guanidine-phenol-chloroform isothiocyanate method, following the procedure described by Ortega et al. [20]. Used primers were designed by the Primer-BLAST tool [21] and the AutoDimer application [22]. The StepOnePlus™ system was used with the relative standard curve method to carry out the quantitative PCR (qPCR). 5 μL from each sample, previously diluted in nucleases free water, was mixed with 10 μL of the intercalant agent IQ™ SYBR® Green Supermix (Bio-Rad Laboratories), 1 μL of forward primer, 1 μL of reverse primer, and 3 μL of free DNases and RNases water in a MicroAmp® 96-well plate (Applied Biosystems-Life Technologies), obtaining a final volume of 20 μL. Final results were normalized and compared to the GAPDH constitutive gene expression (Table 1). Data obtained from each gene were interpolated in a standard curve. In the 96-well plate, samples were triplicated and duplicated in a standard curve, and the two remaining wells were filled with negative controls.

2.4. Immunohistochemistry Studies. The antigen-antibody reaction was detected by the ABC method (avidin-biotin complex) with peroxidase or alkaline phosphatase as chromogen, in accordance with the protocol described by Ortega et al. [23]. The blocking of nonspecific bounding sites was carried out with 3% bovine serum albumin (BSA) and PBS overnight at 4°C (Table 2(a)). The incubation with the secondary antibody bounded to biotin was diluted in PBS for one and a half hour at room temperature (Table 2(b)).

An incubation with the ExtrAvidin®-Peroxidase avidin-peroxidase conjugate (Sigma-Aldrich, St. Louis, MO, USA) was carried out for 60 minutes at room temperature (diluted to 1 : 1200 on PBS). The incubation was revealed with the diaminobenzidine chromogenic substrate (DAB Kit, SK-4100) (Vector Laboratories, Burlingame, CA, USA). The chromogenic substrate was prepared immediately before exposition (5 mL of distilled water, 2 drops of tampon, 4 drops of DAB, and 2 drops of hydrogen peroxide). This technique allows a brown colored stain. In order to obtain contrast with the nuclei stain, incubation in Carazzi's haematoxylin was carried out for 5-15 minutes mount in aqueous medium with Plasdone. In every genetic study, sections from the same tissue were used as negative control, in which the incubation with the primary antibody was substituted with incubation with blocking solution.

2.5. Statistical Analysis in Interpretation of Results. For the purpose of the statistical analysis, the GraphPad Prism® 5.1 statistical program was used, and the Mann–Whitney U and χ^2 Pearson's test were applied. The data are expressed as the median with interquartile range (IQR). Error bars on the figures are expressed with IQR. The significance values were set at $^{*}p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.001$.

The preparations were examined under a Zeiss Axiophot optical microscope (Carl Zeiss, Oberkochen, Germany). Given the important role of the proteins involved, the assessment of the histological results was performed by the intensity of expression for immunohistochemical staining with a score of 1 to 3. Henceforth, histological samples from patients were classified as negative (0) or positive (1-3) IRS score method [24]. For each established group of subjects, 7 randomly selected microscopy fields were examined in each of the 5 sections. Subjects were classified as positive when the average proportion of the labelled sample was greater or equal to 5% of the total sample. This was done by calculating the total percentage of tissue marked in each microscopy field to obtain the value of mean for the study sample as described by Cristóbal et al. [25]. The observation and quantification of the samples were independently performed by two investigators.

3. Results

3.1. Increased Expression of IGF-1 in the Vein Wall of CVeD Patients. Genetic expression study of IGF-1 showed no significant differences between the study groups, although it did show an upward trend in CVeD patients (CVeD = 2.250 [0.630 – 4.013] vs. HV = 2.014 [1.036 – 3.068], $p = 0.5559$) (Figure 1(a)).

In contrast, a significant increase in the IGF-1 protein expression was observed in the vein wall from CVeD patients (CVeD = 2.000 [0.500 – 3.000] vs. HV = 1.000 [0.000 – 2.250], $^{*}p = 0.0104$). The microscopic analysis showed how the expression of IGF-1 was located in the three tunics of the vein wall (Figure 1(b)). An increased intensity was observed in the smooth muscular fibres and extracellular matrix of the vein wall from CVeD patients (Figure 1(c)).

3.2. CVeD Patients Show a Significant Increase of PAPP-A. Genetic expression study of PAPP-A in the vein wall showed a significant increase in CVeD patients in comparison to HV (CVeD = 10.230 [7.846 – 11.726] vs.HV = 9.089 [7.324 – 10.013], $^{**}p = 0.0029$) (Figure 2(a)).

Protein expression of PAPP-A was significantly increased in the vein wall from CVeD patients (CVeD = 1.500 [0.250 – 3.000] vs.HV = 0.500 [0.000 – 1.500], $^{***}p = 0.0002$). PAPP-A was located in the three tunics of the vein wall from CVeD patients (Figure 2(b)). It is worth noting how the expression of PAPP-A was intensely located in the smooth muscular fibres of the vein wall from these patients (Figure 2(c)).

3.3. Significant Decrease in the Expression of STC-2 amongst CVeD Patients. A significant decrease in the genetic expression of STC-2 was observed amongst CVeD patients in comparison to HV by RT-qPCR (CVeD = 7.190 [3.296 – 11.324] vs.HV = 10.845 [9.079 – 13.389], $^{***}p < 0.0001$) (Figure 3(a)).

Protein expression study performed by immunohistochemistry techniques showed in a similar fashion a significant decrease (CVeD = 0.250 [0.000 – 1.000] vs.HV = 1.000 [0.000 – 2.500], $^{**}p = 0.0056$). The microscopic analysis showed SCT-2 was mainly located in the medium tunic of the vein wall from HV patients (Figure 3(c), asterisk).

Table 1: Primer sequences used in RT-qPCR and temperature (Tm).

Gene	Sec. Fwd ($5' \rightarrow 3'$)	Sec. Rev ($5' \rightarrow 3'$)	Tm
GAPDH	GGAAGGTGAAGGTCGGAGTCA	GTCATTGATGGCAACAATATCCACT	60
IGF-1	GCTCTTCAGTTCGTGTGTGG	CGCAATACATCTCCAGCCTC	69
STC-2	GCTCTCGGTCCCGTCAC	GACTCAGGAGAGCTCGACAC	51
PAPP-A	CCCAGGCAGTCAGATCATCTTC	AGCTGCCCCTCAGCTTGA	52

Table 2: Primary (A) and secondary (B) antibodies used in the immunohistochemical studies performed, showing the dilutions used and the protocol specifications.

(a)

Antigen	Species	Dilution	Provider	Protocol specifications
IGF-1	Rabbit	1 : 100	Abcam (ab263903)	10 mM sodium citrate, pH = 6 before incubation with blocking solution
PAPP-A	Mouse	1 : 500	Abcam (ab52030)	0.1% Triton in PBS, 10 minutes before incubation with blocking solution
STC-2	Rabbit	1 : 300	Abcam (ab261915)	—

(b)

Antigen	Species	Dilution	Provider	Protocol specifications
IgG (mouse)	Goat	1 : 300	Sigma (F2012/045k6072)	—
IgG (rabbit)	Mouse	1 : 1000	Sigma (RG-96/B5283)	—

4. Discussion

CVeD causes a severe alteration in the homeostasis of the vein wall, unchaining the activation of both cellular and systemic responses [11]. It has been found that this process is associated with different pathologic mechanisms which are key in the development of the disease such as an increase in the process of apoptosis and cellular death [26], deregulation in the synthesis of some cytokines [27], and even with some cell signaling pathways and changes in the extracellular matrix [28].

The impact that IGF-1 signaling can have on the aging process has long been known. Various animal models ranging from yeasts to human beings have demonstrated that lower levels of IGF-1 are associated with an increased longevity and a deceleration of the aging process [29]. However, it is known that the effects and levels of IGF-1 both at a physiologic level and a pathologic level depend on tissue, gender, and age of the subject [30], and it has also been demonstrated how the alteration of its signaling is associated with the appearance of several aging-associated diseases [31].

Previous studies have shown the existence of an increase in IGF-1 levels and its receptor, IGF-1R in varicose veins, in comparison to control veins, thus demarcating the important role it can play in the pathogenesis of the disease [32]. Moreover, the role of IGF-1 regarding the activation of the PI3K/Akt/mTOR pathway has been demonstrated and how these components were jointly involved in aging process, as well as its associated diseases [33]. Jia et al. [34] reported how IGF-1-mediated signaling promoted the phosphorylation of PI3K and Akt in smooth muscular cells of saphenous veins, promoting their proliferation *in vitro*. Along the same lines, our previous results showed that the PI3K/Akt/mTOR pathway was altered in CVeD patients, showing a direct association between greater involvement of this pathway and both premature and asynchronous aging of the venous tissue [28]. There are also more studies which show how IGF-1 promotes the activation of different signaling pathways, such as MAPK [35]. It has been demonstrated the existence of a greater activation of some components in this pathway, such as ERK1/2 in CVeD patients, especially in those who present venous reflux [36]. Our results show how the increase of IGF-1 could be associated with these statements, collaborating with the detriment suffered by venous tissue.

Amongst the different regulatory mechanisms of IGF-1, pregnancy-associated plasma protein A (PAPP-A) is one of the most studied elements vitally important in the regulation of this factor [37]. Although PAPP-A was initially found and identified in trophoblasts of the placenta from pregnant women, it has been demonstrated how it is also expressed in a vast range of cells and tissues, including fibroblasts and smooth muscular cells, both of great relevance in the pathogenesis of the varicose vein [7, 38].

PAPP-A is a protein which undergoes very strict regulation in the cell. On the one hand, it is known that its expression can be modulated by the synthesis of some proinflammatory cytokines, especially by IL-1β and TNF-α [37]. The role of inflammation in CVeDs has been extensively studied. It is known that the inflammatory process accompanies the development of the diseases as a consequence of the tissue damage associated with the situation of venous hypertension and valvular incompetence, observing an increase in leukocyte infiltration, as well as in the synthesis of proinflammatory cytokines [39]. It has been demonstrated that in atheromatous coronary arteries, this macrophage-mediated proinflammatory environment induces the

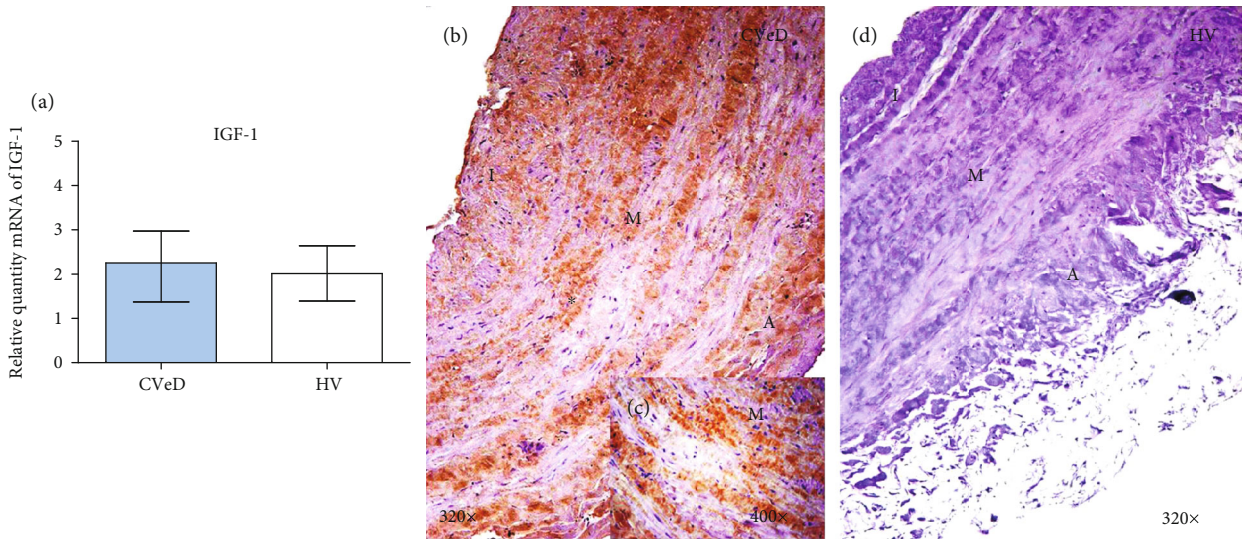

Figure 1: (a) Expression of mRNA for IGF-1 in CVeD (chronic venous disease) and HV (healthy controls) in arbitrary units. (b–d) Images showing immunostaining for IGF-1 in CVD patients in the three venous wall robes (asterisk) and CV. I: intimate robe; M: middle robe; A: adventitious robe.

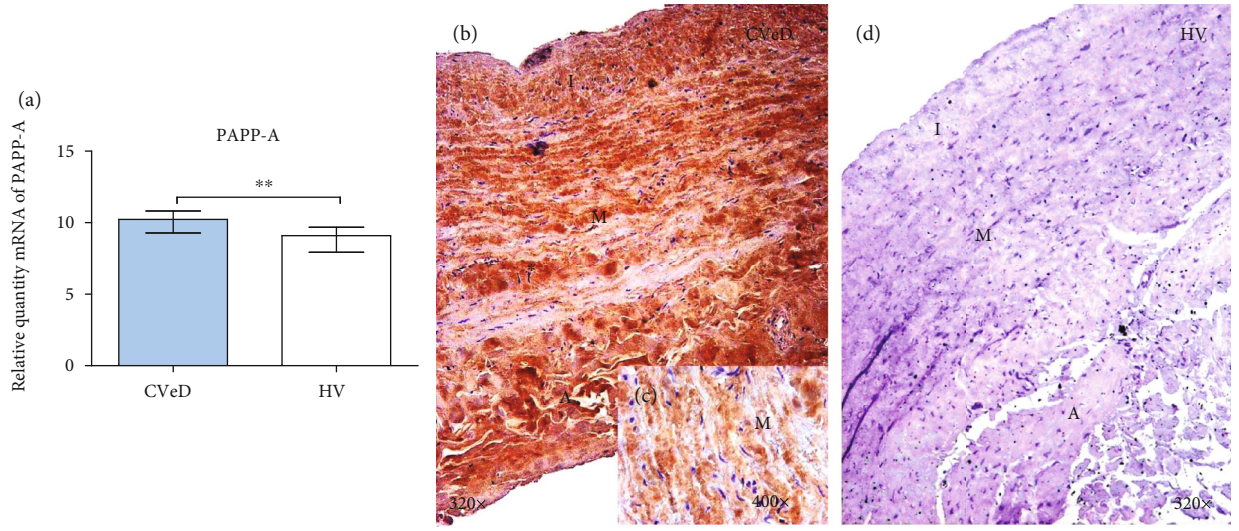

Figure 2: (a) Expression of mRNA for PAPP-A in CVeD (chronic venous disease) and HV (healthy controls) in arbitrary units. (b–d) Images showing immunostaining for PAPP-A in CVD patients in the three tunics of the venous wall (asterisk) and CV. I: intimate robe; M: middle robe; A: adventitious robe. $^{**}p < 0.005$.

expression and secretion of PAPP-A in both endothelial and smooth muscular cells [40]. In addition, as previously mentioned, PAPP-A increases the bioavailability of IGF-1 and the signaling mediated by its receptor in an autocrine and paracrine manner in the tissue [41], having been described the role of IGF-1 in the stimulation of the inflammatory response and the synthesis of cytokines [42].

Our results suggest that in the vein wall from CVeD patients, the inflammation associated with this condition could be acting similarly by increasing the expression of PAPP-A, in a positive feedback process. This fact is involved in promoting other main pathogenic processes, such as endothelial dysfunction [43].

On the other hand, there are different mechanisms which negatively regulate PAPP-A levels in the cell, those that are carried out by a series of proteins depicted as stanniocalcins 1 and 2 [44]. Interestingly, it is known that Stc-2, contrarily to Stc-1, covalently bounds to PAPP-A, performing both essential functions in the regulation of calcium levels and cellular response to different conditions as oxidative stress or endoplasmic reticulum stress, hence being fundamental in cellular homeostasis [45, 46]. We previously demonstrated the existence of an increase in oxidative stress damage markers and in the lipidic peroxidation process in CVeD patients [11]. Our results show how decreasing Stc-2 levels could support this fact, in addition to implying the loss of

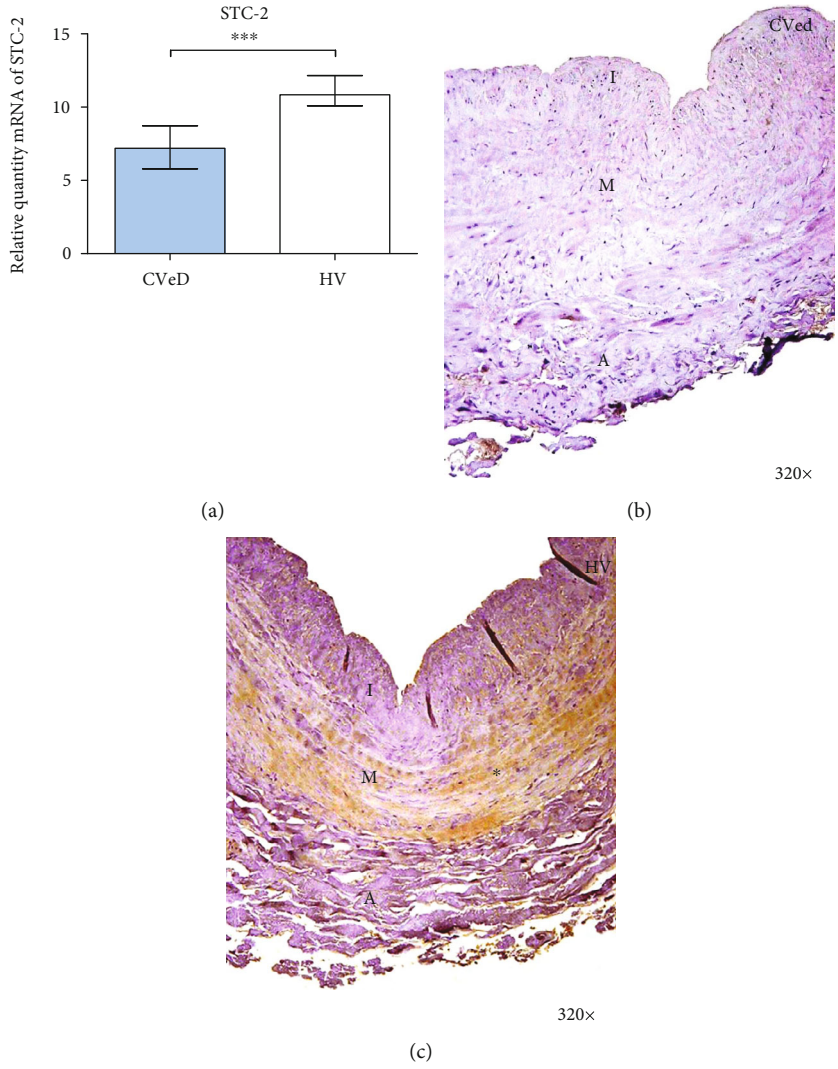

Figure 3: (a) Expression of mRNA for STC-2 in CVeD (chronic venous disease) and HV (healthy controls) in arbitrary units. (b, c) Images showing the immunostaining for STC-2 in CVD patients in the three tunics of the venous wall (asterisk) and CV. I: intimate robe; M: middle robe; A: adventitious robe. $^{***}p < 0.001$.

an inhibition mechanism of the proteolytic activity of PAPP-A and, therefore, of the signaling regulation mediated by IGF-1 in the venous tissue that appears in CVeD patients.

The analysis of the serum levels of these components and their possible use as biomarkers is also currently in progress. It is important to note that circulating PAPP-A can be found in 2 different forms: a first form associated with pregnancy, covalently bound to the proteolytically inactive eosinophil major basic protein (proMPB), and a second form produced by vascular cells that are not covalently bound to proMBP and that do exhibit proteolytic activity, which this distinction is being important for its measurement and interpretation under different conditions [47]. The presence of Stc-2 in serum has also been identified, as well as IGFBP-4, which is regulated by PAPP-A [48]. Although PAPP-A also modulates other IGFBPs, such as IGFBP 2 and 5, IGFBP-4 exclusively appears to be regulated by the proteolytic activity of PAPP-A. Taking into account all of these reasons, the Stc-2/PAPP-A/IGF-1 axis is necessary to know all the physiopathologic mechanisms involved in vascular diseases [49–54].

5. Conclusions

Our study is pioneering in showing how CVeD patients have a significant increase of PAPP-A expression in their vein wall. These markers may be quite relevant as CVeD markers. Further studies should be oriented to the measurement and assessment of different components of this axis which may serve as serum biomarkers in venous disease patients. However, our study is the first to highlight the presence and importance of IGF-1/PAPP-A/STC-2 markers in CVeD.

Data Availability

The data used to support the findings of the present study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Miguel A Ortega and Oscar Fraile-Martínez contributed equally in this work. Julia Buján, Natalio García-Honduvilla, and Felipe Sainz shared senior authorship in this work.

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Review Article

The Role of Prognostic and Predictive Biomarkers for Assessing Cardiovascular Risk in Chronic Kidney Disease Patients

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Chronic kidney disease (CKD) is currently defined as the presence of proteinuria and/or an eGFR < 60 mL/min/1.73m^2 on the basis of the renal diagnosis. The global dimension of CKD is relevant, since its prevalence and incidence have doubled in the past three decades worldwide. A major complication that occurs in CKD patients is the development of cardiovascular (CV) disease, being the incidence rate of fatal/nonfatal CV events similar to the rate of ESKD in CKD. Moreover, CKD is a multifactorial disease where multiple mechanisms contribute to the individual prognosis. The correct development of novel biomarkers of CV risk may help clinicians to ameliorate the management of CKD patients. Biomarkers of CV risk in CKD patients are classifiable as prognostic, which help to improve CV risk prediction regardless of treatment, and predictive, which allow the selection of individuals who are likely to respond to a specific treatment. Several prognostic (cystatin C, cardiac troponins, markers of inflammation, and fibrosis) and predictive (genes, metalloproteinases, and complex classifiers) biomarkers have been developed. Despite previous biomarkers providing information on the pathophysiological mechanisms of CV risk in CKD beyond proteinuria and eGFR, only a minority have been adopted in clinical use. This mainly depends on heterogeneous results and lack of validation of biomarkers. The purpose of this review is to present an update on the already assessed biomarkers of CV risk in CKD and examine the strategies for a correct development of biomarkers in clinical practice. Development of both predictive and prognostic biomarkers is an important task for nephrologists. Predictive biomarkers are useful for designing novel clinical trials (enrichment design) and for better understanding of the variability in response to the current available treatments for CV risk. Prognostic biomarkers could help to improve risk stratification and anticipate diagnosis of CV disease, such as heart failure and coronary heart disease.

1. Introduction

According to the latest classification, edited by the Kidney Disease: Improving Global Outcomes Work Group (KDIGO) in 2012, chronic kidney disease (CKD) is defined as the presence of a reduced kidney function (i.e., an estimated glomerular filtration rate (eGFR) < 60 mL/min/1.73 m^2) and/or albuminuria, a strong marker of kidney damage [1]. The cause of CKD was also included in the KDIGO classification, since different causes are associated with disparate

outcomes and need specific treatments [1, 2]. An important aspect that has drawn attention to this topic, in the past decades, is the global impact of CKD. The 2017 Global Burden of Disease study has shown that the number of deaths attributable to CKD increased by 33.7% over the 2007-2017 period and that this trend was higher than that of mortality due to neoplasms (+25.4%) and cardiovascular diseases (+21.1%) and close to that of diabetes mellitus (+34.7%) [3]. These general epidemiologic evidences are even more impressive when considering that from 1990 to 2016 the incidence and prevalence substantially doubled worldwide, rising by 88.76% and 86.95%, respectively [4]. The main reasons that have been considered to explain the increase in CKD burden are population growth and aging together with the decrease in age-standardized mortality and morbidity rates in most regions. Furthermore, the tide of type 2 diabetes in high-income countries has also driven the increasing trend of CKD and was confirmed as a leading cause of CKD and the more severe clinical condition of end-stage kidney disease (ESKD) [4, 5]. The result of the global dimension, when translated into clinical practice, is that a growing number of patients are exposed to both severe cardiovascular (CV) and renal risks [6, 7]. In the attempt to improve the management of CKD patients as well as to optimize the individual treatment, a large number of studies have been carried out in the past decades. Indeed, observational analyses have provided clinicians with important evidence on the predictors of poor prognosis in CKD patients, thus improving their risk stratification [8–10]. In addition, a large number of intervention studies testing the effect of antihypertensive drugs, diuretics, albuminuria-lowering agents, sodium-glucose cotransporter 2 inhibitors (SGLT-2i), and endothelin receptor antagonists on CV risk reduction in CKD patients have been conducted [11–14]. However, despite the relevant protective effects that these drugs exert against CV events, they also showed a large variability in individual response, thus determining that a considerable proportion of patients do not respond to the scheduled treatment and remain at very high risk of developing CV events [15]. To overcome individual response variability and to reduce the residual CV risk in CKD patients, several strategies have been adopted in clinical research. The first consists in designing new clinical trials that allow to understand what patient is likely to respond to a specific treatment (ClinicalTrials.gov identifier: NCT03504566), whereas the second is focusing attention on the identification, validation, and implementation of novel CV risk biomarkers that may improve risk stratification of CKD patients and identify aspects of renal disease that are not detected by albuminuria or eGFR such as inflammation, tubular damage, and fibrosis. In general, the term biomarker refers to a defined characteristic that can be measured accurately and reproducibly and evaluated as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions [16]. They have been investigated and also used for several diseases or pathologic conditions, including the renal ones [17–19]. The aim of this review is to summarize the strong association between CKD and CV disease and to examine the role of novel biomarkers of CV risk in CKD, dealing with biomarkers' function, clinical application, and future perspectives.

2. Cardiovascular Disease in CKD Patients

The association between CV abnormalities and CKD is an old concept. Indeed, the first scientist who described the interconnection between heart failure and the degree of renal fibrosis was Richard Bright in 1836, in a fascinating manuscript that is still available in PubMed [20]. Many studies have since confirmed this association, and explanations have been sought in terms of epidemiology, pathophysiology, and clinical perspective. From observational analyses emerged that either low eGFR or increased proteinuria, which are considered the two main kidney measures, is associated with the onset of CV complications, such as CV mortality, heart failure (HF), coronary heart disease (CAD), and stroke (Figure 1) [5, 10, 21–23]. Although results of previous studies are controversial, a recent individual-level meta-analysis of the CKD Prognosis Consortium provided strong evidence by analyzing uniformly more than 600,000 CKD patients [21]. In that meta-analysis, both eGFR and proteinuria (measured as albumin-to-creatinine ratio) predicted CV endpoints even after accounting for traditional risk factors (i.e., blood pressure, serum cholesterol levels, smoking habit, age, and gender). Interestingly, the contribution of either eGFR or proteinuria to the CV risk prediction was equal, or even greater, than any traditional CV risk factor. Moreover, for eGFR, a cut-off point of 60 mL/min/1.73m^2 has been identified as the level below which the CV risk starts to increase, while there is no specific threshold for proteinuria. This means that an increase in proteinuria, even within the normal range, confers CV risk.

These data suggest that eGFR and proteinuria should be considered before estimating the CV risk in patients with CKD, especially if considering that the already available risk scores, such as the Framingham or the Atherosclerotic Cardiovascular Disease (ASCVD), failed in predicting CV risk in CKD [6, 7, 21]. The linkage between CV disease and CKD measures has also been recently extended to the peripheral vascular disease (PVD). It has been demonstrated that even slight increases in proteinuria, as well as moderate reductions in eGFR, were found significant predictors of PVD (i.e., peripheral artery disease and leg amputation) beyond traditional CV risk factors [24]. Taken together, CV events are prevalent in CKD patients and are also responsible for most of the unfavorable outcomes. In the Kidney Early Evaluation Program (KEEP), which enrolled subjects at high risk of developing CKD, the overall prevalence of CV disease (CVD) was 22.1% and rose to 30-50% in CKD populations of MASTERPLAN (Netherlands), Chronic Renal Impairment in Birmingham, United Kingdom (CRIB), African Americans Study (AASK), and CKD Multicohort [8, 25–28]. Once CKD is established, up to 50% of patients are reported to die of cardiovascular causes over time [29]. Indeed, in the CKD populations of Kaiser Permanente Northwest, a healthcare service of the United States of America (USA), as well as among CKD diabetic and nondiabetic patients in the USA Medicare system, patients have died or developed CV disease

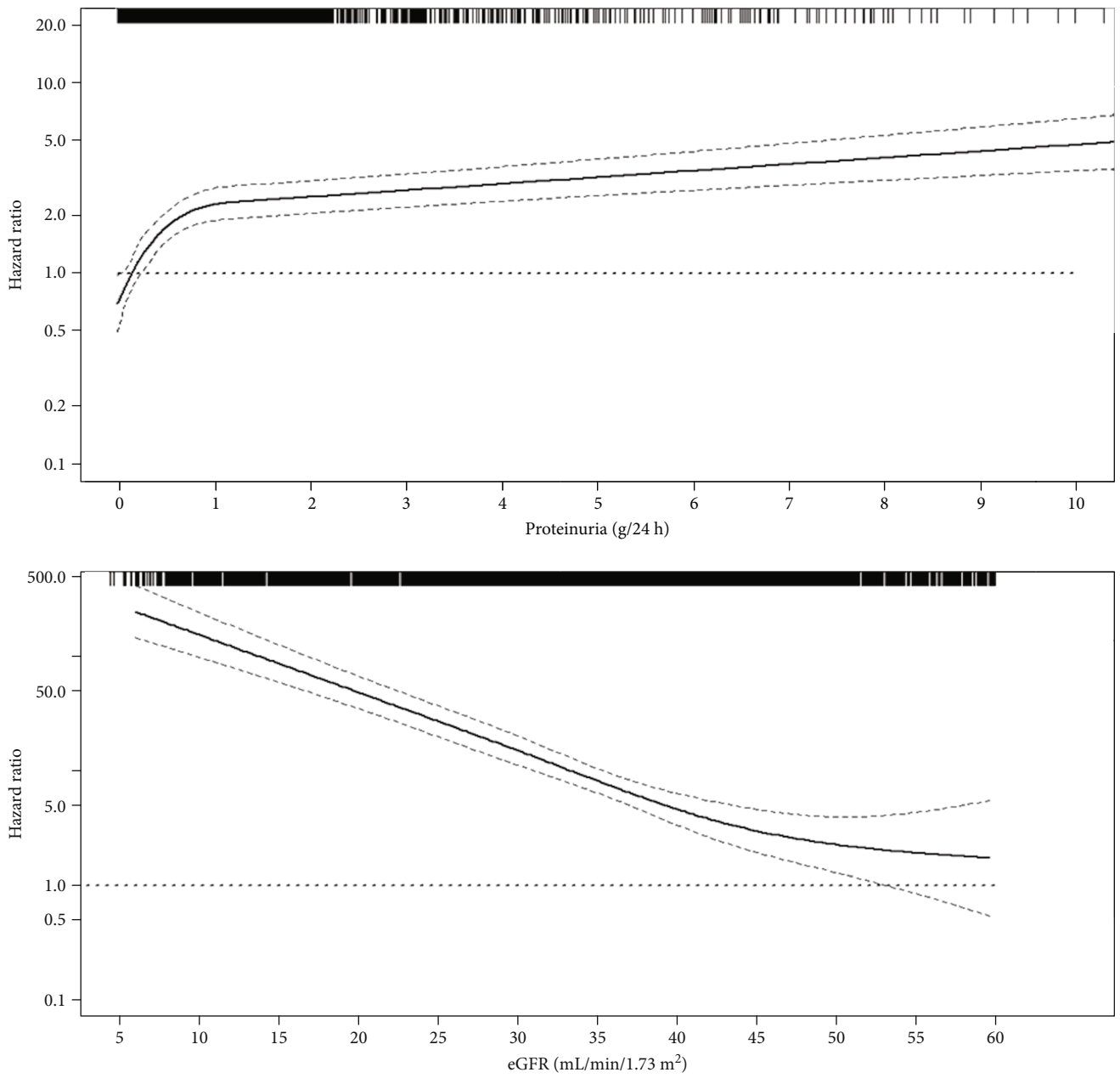

Figure 1: Adjusted associations between eGFR, proteinuria, and risk for cardiovascular (CV) fatal and nonfatal events (i.e., myocardial infarction, congestive heart failure, stroke, revascularization, peripheral vascular disease, nontraumatic amputation, or CV death). Solid line represents hazard ratio (HR), whereas dashed lines represent the 95% confidence intervals. HR is adjusted for the main predictors of CV events (age, gender, type 2 diabetes, history of cardiovascular disease, body mass index, hemoglobin, smoking habit, systolic blood pressure, serum phosphorus, and use of RAAS inhibitors). Knots were located at the 25th, 50th, and 75th percentiles for both proteinuria and eGFR. Rug plots at the top of the x-axis represent the distribution of observations. Data source: CKD Multicohort, a pooled analysis of 3,957 patients referred to Italian nephrology clinics [8].

with a higher rate than ESKD and the two-year survival probability in patients with previous CV disease was modified by the presence/absence of CKD (Figure 2) [30, 31]. In the Italian CKD Multicohort, which included CKD patients under stable nephrology care, the incidence rates of ESKD and CV events before ESKD were similar (5.26 vs. 4.52 per 100/pts/year), thus confirming that the CV risk remains a major complication for these patients [5].

Hence, the presence of kidney-specific mechanisms contributes to the raised CV risk beyond traditional risk factors and individual comorbidities. It has been shown that in CKD the expression of endothelial nitric oxide synthase is downregulated [32]. This mechanism has been hypothesized as the main cause of endothelial dysfunction in CKD patients in association with the increased levels of asymmetric dimethylarginine (ADMA). Indeed, ADMA acts by

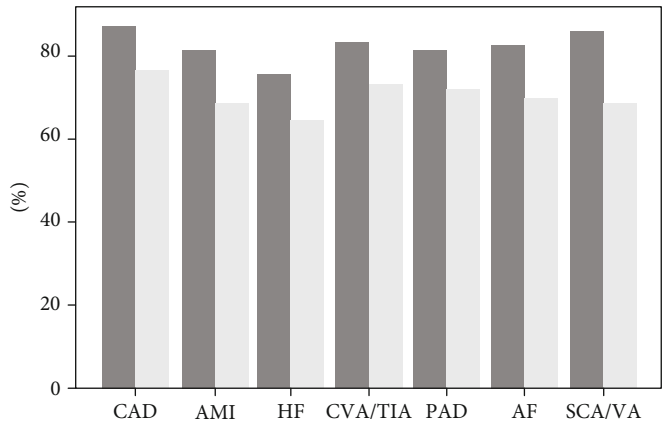

Figure 2: Two-year survival (%) of patients with cardiovascular disease (CVD) by chronic kidney disease (CKD) status. Columns in dark gray depict patients without CKD whereas columns in light gray depict patients with CKD. AF: atrial fibrillation; AMI: acute myocardial infarction; CAD: coronary artery disease; CVA/TIA: cerebrovascular accident/transient ischemic attack; HF: heart failure; PAD: peripheral arterial disease; SCA/VA: sudden cardiac arrest and ventricular arrhythmias. Data source: United States Medicare Population [30].

inhibiting generation of nitric oxide and increasing systemic vascular resistance and blood pressure [33]. The endothelial stretch and the increase in ADMA lead to an impairment in coronary vascular resistance and left ventricular hypertrophy [34]. Moreover, although arterial hypertension is present in a large number of CKD patients, it seems that the renal anemia and the increased vascular stiffness mainly contribute to the onset of left ventricular hypertrophy in combination with the endothelial dysfunction [35]. CKD also causes dyslipidemia. In the presence of impaired kidney function, an excessive oxidation of low-density lipoprotein (LDL) cholesterol has been observed together with a defective high-density lipoprotein (HDL) function. The lipid profile becomes, thus, atherogenic [36, 37]. Accordingly, an observational analysis of 1,162 subjects who died between 1988 and 2005 in a suburban community adjacent to Fukuoka City, in southern Japan, showed that the entity of coronary artery stenosis was raised from state I-II to stage V of CKD and that the vascular stenosis was attributable to a worsening atherosclerosis in advanced stages of CKD [38]. CKD is also associated with the presence of systemic inflammation which is, in turn, a trigger for CV damage. The increased oxidative stress and accumulation of toxins, normally excreted in the case of normal kidney function, favor the onset of an imbalance of inflammatory factors. In CKD patients, levels of IL-6 and matrix metalloproteinases (MMPs) have also been found to have increased [39, 40]. Interestingly, it has been shown that MMPs play an important role in expanding inflammatory response and in the inflammation and rupture of atherosclerotic plaques [40]. Another key factor of endothelial dysfunction is proteinuria (or albuminuria). A group of researchers from the Steno Memorial Hospital, in Denmark, described for the first time in 1989 that in diabetic patients with proteinuria the presence of proteinuria was strongly associated with raised levels of von Willebrand factor and transcapillary escape rate of fibrinogen, thus testifying that proteinuria is most likely a marker of systemic vascular damage [41]. Further evidence has confirmed that proteinuria exerts prominent toxic effects on all parts of the nephrons including the renal tubules, thus feeding a vicious circle that moves from kidney to systemic damage [42, 43]. Patients with impaired kidney function present a deficiency in vitamin D, because of the weakened function of the 1α-hydroxylase, a renal enzyme which converts the vitamin D precursor to the active hormone. Many studies suggested that vitamin D deficiency is associated with CV risk since the vitamin D pathway directly works in modifying cardiac function [44, 45]. Other factors have been considered as CV risk factors in the early phase of CKD, such as hyperphosphataemia, parathyroid hormone (PTH), and leptin which worsen atherosclerosis, vascular calcifications, and cardiorenal prognosis [45–47].

3. Rationale to Incorporate Novel Biomarkers of CV Risk in CKD Patients

Owing to the great burden of CV events in patients with CKD, much effort has been initiated to improve prognosis of these patients. One strategy, which we previously mentioned, is to test novel drugs that would probably represent the best possible treatment in the near future. In this context, SGLT-2i have been shown to reduce the rate of CV events in patients with CKD and diabetes [13, 14] and the results were so promising to the point that new trials have been started testing the effect of SGLT-2i in patients with nondiabetic CKD (ClinicalTrials.gov identifier: NCT03036150). One major concern of these trials is that they answer the question whether one treatment is able to reduce on average the CV risk compared with the standard treatment (control group) without considering the individual response to treatments. Indeed, a variability in response has already been described for drugs intervening in the renin-angiotensin-aldosterone system (RAAS), but also with respect to SGLT-2i, thus meaning that a consistent proportion of patients continue to remain at increased risk if the response to treatment is suboptimal. Another strategy that has been considered is to evaluate, develop, and implement novel biomarkers of CV risk. Biomarkers may improve the management of CKD patients in several ways. Although the increase in proteinuria and

the falling of eGFR define CKD, their presence is often the marker of an already established and possibly irreversible kidney damage. In this context, novel biomarkers would be desirable for several reasons:

(1) Novel biomarkers that are able to anticipate the diagnosis of kidney damage (at early stage of the disease) would be extremely useful in clinical practice since they help in adopting timely strategies to prevent the progression of kidney disease and CV risk

(2) Novel biomarkers can reveal aspects of kidney disease that are not directly captured by eGFR or proteinuria, for example, by informing about the degree of fibrosis, renal inflammation, or tubular damage

(3) The combination of the novel biomarker measurement and renal biopsy could be useful in the case whether eGFR and proteinuria are noninformative, such as in nonproteinuric CKD [47]

(4) Novel biomarkers must be studied in those fields of research where therapeutic strategies are not yet adequately improved. For example, it has been shown that, among CV diseases, CKD patients are more likely at risk of developing HF than CAD, probably due to left ventricular hypertrophy and the impaired preload that are commonly observed in advanced CKD [21]. It is thus remarkable that, since proteinuria and eGFR may be suboptimal in predicting HF, the role of novel biomarkers in anticipating the clinical diagnosis in order to plan proper therapeutic strategies would be determinant

(5) Novel biomarkers could also reveal more information on pathophysiological mechanisms of kidney and CV damage

(6) The assessment of clinical utility of biomarkers in large cohorts with proper follow-up is essential in order to understand whether a specific biomarker can be transportable to clinical practice, since it would help to improve monitoring the disease trend over time (prognostic biomarker) or predicting the individual response to a treatment or intervention (predictive biomarker)

4. Principally Investigated CV Biomarkers in CKD

Biomarkers have been differently classified in previous available studies. As far as we know, at least three classification systems exist [48–50]. The first considers the anatomic origin or the mechanisms of damage and thus identifies kidney and cardiac markers [49]. A second classification encompasses filtration markers, namely, biomarkers that give a better estimation of GFR as compared to creatinine eGFR and nontraditional biomarkers that were derived from imaging techniques (i.e., coronary artery calcium score) or laboratory measurements [48]. A third classification is based on the clinical "intended use" of the biomarker and distinguishes prognostic and predictive biomarkers [50, 51]. A prognostic biomarker is used to identify the likelihood of the patient to develop a clinical outcome regardless of treatment. Indeed, it can be evaluated in untreated patients or patients who undergo heterogeneous treatments that often happen under the standard of care. Such a measure may improve the physician's ability to identify patients with a poor prognosis. On the other hand, predictive biomarkers are used to determine whether the patient is likely to benefit from a specific treatment. In this context, the clinical benefit is interpretable as either a good response to a drug that can be used if the biomarker is positive or, alternatively, a resistance to the same drug that can save a patient from drug toxicity or pointless side effects. Since we are interested in the clinical utility of the biomarkers, we adopt and follow the latter classification.

4.1. Prognostic Biomarkers. In patients with already established CKD, many biomarkers have been shown to improve prediction of CV events. The use of cystatin C to estimate GFR ($eGFR_{cys}$) was able to refine risk stratification of CKD patients as compared to creatinine-based GFR ($eGFR_{crea}$) [1]. $eGFR_{cys}$ affords estimates of kidney function levels that are slightly different from those estimated by $eGFR_{crea}$. A meta-analysis of the CKD Prognosis Consortium showed that the reclassification of patients according to $eGFR_{cys}$ versus $eGFR_{crea}$ is accurate in the sense that patients with lower and higher $eGFR_{cys}$ than $eGFR_{crea}$ levels were, respectively, at higher and lower risk for all endpoints, including CV events [52]. β_2-Microglobulin is another filtration marker that was found to improve prediction of CV events to an extent similar to cystatin C [53]. Strong pieces of evidence toward the utility of cardiac troponins (high-sensitivity cardiac troponin (hs-cTnT)) and natriuretic peptides (N-terminal pro-B-type natriuretic peptide (NT-proBNP)) have been recently published [54, 55]. Blood levels of hs-cTnT and NT-proBNP are routinely used for diagnosing CAD and HF, respectively, and reflect subclinical abnormalities in the heart. Interestingly, in CKD patients, both hs-cTnT and NT-proBNP are more consistently associated with the development of HF than CAD over time. More importantly, this association is true even after accounting for the kidney function level which *per se* alters the serum concentrations of the two biomarkers [56]. The importance of such evidence is enormous when considering that HF is the most represented CV disease in CKD patients and for whom the two kidney measures of CV risk, proteinuria and eGFR, show a suboptimal prediction. The clinical implication is also relevant as these novel biomarkers could be used in the future to identify CKD patients at increased CV risk who could be prescribed with preventive treatments (e.g., statins and/or aspirin therapy) [57]. In the context of HF, two further biomarkers are of particular interest: soluble suppressor of tumorigenicity (sST2) and galectin-3. sST2 is a protein produced by the endothelial cells lining the left ventriculus in response to mechanical strain. It has shown to have an incremental value to NT-proBNP to predict deaths and hospitalizations due to HF, irrespective of kidney function [58]. Galectin-3 is a member of the β-galactoside-binding lectin family that interacts with laminin, synexin, and other extracellular matrix proteins. In

observational analyses which included patients with HF, serum galectin-3 levels were independent predictors of hospitalizations due to HF and CV mortality, regardless of kidney measures (proteinuria and eGFR) [59, 60]. Markers of inflammation or tissue remodeling have also sparked interest in assessing CV risk in CKD patients. Among these, levels of MMPs have been considered as possible biomarkers. Serum levels of MMP-2, MMP-8, and MMP-9 have been found increased in CKD patients and diabetic patients, being correlated, respectively, with serum phosphate (MMP-2), fibroblast growth factor-23 (FGF-23), and the degree of proteinuria (MMP-8 and MMP-9), two relevant predictors of oxidative stress and CV risk [61–63]. Moreover, MMP-2 has been directly correlated with vascular calcification, atherosclerotic plaque rupture, and carotid intima-media thickness (cIMT), thus playing an important role in atherogenesis [64]. Higher serum levels of MMP-9 and tissue inhibitor of metalloproteinases-1 (TIMP-1) are involved in the pathogenesis of left ventricular hypertrophy by cleaving intracellular myosin filaments [65, 66]. Several MMPs, such as MMP-2, MMP-3, and MMP-9, are also implicated in the pathogenesis of vascular aneurysm and their levels after surgical interventions for lower extremity bypass were an independent predictor of CV death [40]. All these mechanisms of damage are made even worse by the presence of an inflammatory milieu in patients with CKD and by the raised serum concentration of MMPs due to the reduction of GFR. The assessment of measures of CV disease process has been also evaluated as biomarkers of CV risk in CKD. Among these, the coronary artery calcium (CAC) score has been used. CAC score is computed using either an electron beam or multidetector cardiac computed tomography (CT). Afterward, a semiautomated tool called Agatston score is used to create a risk score based on the degree of plaque densities and their areas in all coronary arteries [67]. CAC score has shown to be a reliable predictor of atherosclerotic CV disease among the general population and in patients with moderate and advanced CKD beyond traditional risk factors and with a discrimination ability that is greater than of other filtration markers such as cystatin C [48, 68].

4.2. Predictive Biomarkers. One fascinating and advantageous aim of the biomarkers is to identify individuals who will likely respond to a drug of interest. These biomarkers are commonly defined "predictive" biomarkers. The baseline level of a predictive biomarker could also change over time (dynamic predictive biomarker) as a treatment-induced effect, so it can be used for monitoring the course of the disease and its treatment efficacy [50]. Predictive biomarkers can be genes, proteins, metabolites, or others. The most used predictive biomarker in nephrology is the presence of proteinuria. Several clinical trials have shown in the past three decades that the drug-induced reduction in proteinuria is associated with a protection from CV risk over time both in diabetic and nondiabetic CKD patients [12–14, 69, 70]. Treatments tested in these trials were disparate and included antihypertensive, diuretics, and oral hypoglycemic agents. However, the common pieces of evidence derived from these studies were that (1) the magnitude of treatment effect, i.e., risk reduction for fatal and nonfatal CV events, was greater in patients with proteinuric CKD phenotype as compared to those without CKD and (2) the extent of CV risk reduction after interventions was strictly correlated with the reduction in proteinuria [69, 70]. Two post hoc analyses of clinical trials enrolling CKD patients, the Reduction in Endpoints in Noninsulin-dependent diabetes mellitus with the Angiotensin II Antagonist Losartan (RENAAL) and the Irbesartan Diabetic Nephropathy Trial (IDNT) study, have shown that the greatest protective effect was found in patients with the larger reduction of proteinuria after 6 months from randomization visit that corresponds to the start-of-treatment visit [70, 71]. There is now a general agreement, confirmed by KDIGO guidelines, that proteinuria should be measured in CKD patients to monitor the progression of the disease. However, although further studies are needed to establish how often it should be measured and what the correct threshold that confers a strong protection against CV disease, it is reasonably accepted based on previous trials that a 30% reduction of proteinuria after 6 months is a sufficient target [42, 43]. Presently, in clinical research in nephrology, additional predictive biomarkers that are able to predict the response to nephroprotective treatments beyond proteinuria would allow to better control the CV risk and refine the treatment decision toward "the right drugs for the right patient" perspective. There is interesting evidence that MMPs could play a predictive, other than prognostic, role in CKD patients [40]. In fact, a reduction in serum concentration of MMPs in response to the antibiotic doxycycline and the nonselective inhibitors of MMPs Batimastat and Marimastat has been associated with a reduction of detrimental vascular tissue remodeling and to a significant reduction of proteinuria in patients with CKD [40, 72]. Even more importantly, the novel SGLT-2i medications, which have been widely demonstrated to reduce the cardiovascular risk in CKD patients in several clinical trials, may exert part of their CV and renal risk reduction effect through a mechanism that is independent from the level of proteinuria and is possibly based on the activation of an endogenous inhibitor of MMPs, the reversion-inducing cysteine-rich protein with kazal motifs (RECK) [73]. This is important for improving clinical trial design in CKD, since novel drugs may be also tested in non-proteinuric subjects, which represent a nonnegligible part of the CKD cohort [47]. A growing body of evidence is emerging around the role of renal resistive index (RI) as a dynamic biomarker of CV risk. It is well known that impaired RI levels reflect both kidney and systemic vascular damage [74, 75]. Moreover, RI also predicts CV events in high-risk patients regardless of eGFR and proteinuria [76]. Interestingly, recent studies showed that RI can change over time and in response to treatments. Solini and colleagues have demonstrated that the SGLT-2i dapagliflozin improves endothelial function, vascular damage, and RI in type 2 diabetic patients [77]. A similar effect is determined by the RAAS inhibitors [78]. Hence, novel studies should assess whether the dynamic changes in RI and its trajectory over time could influence prognosis. An insertion/deletion polymorphism of the angiotensin-converting enzyme gene was able to predict the response to losartan in type 2 diabetic patients enrolled in

the RENAAL study trial [79]. This evidence was also replicated in nondiabetic patients, thus testifying that intrarenal RAAS activity has a role in CV risk prediction as well as in response to treatment prediction [80]. Among complex biomarkers, a panel of 185 serum metabolites, including amino acids, energy/sugar lysophosphatidylcholines, phosphatidylcholines, and sphingomyelins, was analyzed to select a subset of metabolites, which predicts accurately the response to the angiotensin receptor blocker (ARB) therapy in diabetic patients. That prediction ability was also independent from main confounding covariates such as age, gender, eGFR, and proteinuria [81]. Similarly, another classifier has been developed from the PREVEND study, using plasma proteomic profiles which have been shown to predict the change in albuminuria stage and to improve the prediction ability of standard risk factors like albuminuria, eGFR, and RAAS inhibitor use [82]. A summary of the principal prognostic and predictive biomarkers of cardiovascular risk in chronic kidney disease patients is provided in Table 1.

5. Strategies for Implementing Novel Biomarkers of CV Risk in the CKD Setting

CV disease is a major complication of CKD patients. Despite the introduction of novel treatments and a stricter monitoring of patients, the frequency of CV fatal and nonfatal events remains disproportionately high [84, 85]. Moreover, the risk of CV events among these patients equals or even overcomes the competing risk of CKD progression or ESKD [5]. The correct detection, assessment, and implementation of novel biomarkers may certainly support the aim of improving CV risk management in the CKD setting. As we previously discussed, several biomarkers have been demonstrated to play a prognostic or predictive role but just a few biomarkers have made it from the discovery phase to clinical use. With the exception of cystatin C, whose adoption allowed a refinement in the estimation of GFR and CV risk prediction, the risk markers widely used currently in CKD patients are eGFR and proteinuria. Although they convey a great part of information for individual prognosis and treatment decision as well, several concerns have been recently raised. Yoshio Hall and Jonathan Himmelfarb, in a recent Editorial, reported in the *Clinical Journal of the American Society of Nephrology*, defined the eGFR/proteinuria-based classification a "reductionist" approach, since it does not consider that CKD could manifest through a myriad of clinical and histological phenotypes and that each renal diagnosis deserves a proper comprehensive investigation [86]. The major limitations to the development of previous biomarkers are represented by the small sample sizes, the heterogeneous results from a specific biomarker assessment, and the lack of result validation [87]. The framework for the development of a prognostic biomarker includes a series of steps [88]. Briefly, to determine if a biomarker improves the clinical prediction on top of already available variables included in risk prediction models, it is recommended to report model *calibration*, meaning that the event rates predicted by the model correspond to those rates observed in a clinical setting; the *significant association* of the biomarker with a clinical outcome that should be independent from other main confounders (the effect size of the biomarker with the outcome after multiple adjustments and the p value should be considered); *discrimination*, a measure according which a model has a good performance if it classifies at high-risk patients who develop the outcome of interest and at low risk those who do not. Although sensitivity and specificity are the proper measures for a precise threshold of the biomarker, a summary measure that depicts sensitivity and specificity for all possible thresholds is the Receiver Operating Characteristic (ROC) curve. It is thus suggested to present the ROC derived from the model together with the Area Under the Curve (AUC) that in these cases is also called c-statistic [89]. If the model with the biomarker c-statistic is significantly higher than the model without the biomarker, it could be clinically useful; *reclassification measures*, such as net reclassification improvement (NRI) and integrated discrimination index (IDI). Indeed, if the prediction model with the standard covariates (e.g., a model with eGFR and proteinuria in nephrology) accounts for most of prognostic information, it is hard to find a significant improvement of c-statistic, following the statement "it is hard to improve an already good thing." For this reason, measures of reclassification could give useful information on the frequency (%) of patients that are reclassified in the true risk category (lower or higher) with the addition of the new biomarker as compared to the traditional model [90]. During all these phases, it is important to keep in mind the intended use of the biomarker (e.g., what kind of outcome it may predict) and the clinical setting (CKD, general population, and high-risk population), since different clinical settings may give disparate results and the variables that influence the effect size of the biomarker. To this aim, it is useful to run subgroup analyses (e.g., by age, gender, race, eGFR, or proteinuria categories). After computing and depicting the measures of performance, a crucial step forward is to validate biomarker performance. Indeed, if biomarker performance is measured on the same cohort from which it was developed, this performance is likely overestimated. Two strategies to assess a correct validation and avoid overfitting are the internal and external validation [89]. The internal validation consists in splitting the cohort in multiple samples so that it is possible to develop and validate the biomarker in different samples of the same cohort. Alternatively, cross-validation and bootstrap-based methods can be used [91]. External validation allows one to transport and apply the model to different populations. The biomarker performance may be poor in other populations because the baseline characteristics (frequency of diabetes, CV disease, and degree of kidney impairment) are often different, thus varying the baseline risk of the new population. However, strategies to recalibrate and adapt the performance measures to the new population are applicable [92]. Hence, external validation is considered the most effective way to validate a biomarker. Predictive biomarker performance should be assessed following the same scheme used for the prognostic biomarkers. However, predictive biomarkers are also useful in research to select patients for new trials testing drugs for CV protection. A strategy that follows this concept is the adaptive enrichment design [83]. This design consists in enrolling patients who respond to a drug

Table 1: Summary of the principal prognostic and predictive biomarkers of cardiovascular risk in chronic kidney disease patients.

Biomarkers	Characteristics	Prognostic value	Predictive value
Cystatin C	Protein produced by all nucleated cells mainly used as marker of kidney function	Cystatin C improves the estimation of eGFR and risk prediction of CV events; it also allows to reclassify patients into more accurate CV risk categories [52]	—
β2-Microglobulin	Component of MHC class I molecules and expressed on all nucleated cells in humans	Improves risk prediction in CKD patients beyond traditional risk factors [53]	—
hs-cTnT	Regulatory protein that is integral to cardiac and skeletal muscle contraction	Improves the risk prediction of CV events, particularly heart failure regardless of the level of kidney function [54–56]	—
NT-proBNP	Prohormone with a 76-amino acid N-terminal inactive protein	Improves the risk prediction of CV events, particularly heart failure regardless of the level of kidney function [54–56]	It has been used as predictive biomarker in the SONAR trial during the run-in phase, in order to exclude patients with sodium retention after treatment with atrasentan [83].
sST2	Member of the IL-1 receptor family, which is produced by cardiomyocytes and cardiac fibroblasts	It is delivered in response to mechanical stress conditions and showed incremental prediction ability (over NT-proBNP) for HF-related death and hospitalizations [58]	—
Galectin-3	30 kDa protein that contains a carbohydrate-recognition-binding domain that enables the linkage of β-galactosides	In patients with already established CV disease, galectin-3 is an independent predictor of hospitalizations and death due to CV causes [59, 60]	—
MMPs	Six families of zinc-containing endopeptidases that are involved in regulating tissue development and homeostasis	Serum MMP-2, MMP-8, MMP-9, and TIMP-1 are associated with atherogenesis, the severity of kidney damage, and the onset of left ventricular hypertrophy and peripheral vascular disease [61–66]	MMP levels are modified by selective and nonselective drugs. Changes in MMP levels have been associated with a reduction of CV risk [72, 73].
CAC	CAC is a score measured at cardiac TC based on the entity of calcium depositions on artery plaques.	Improves risk prediction in CKD patients beyond traditional risk factors [48, 68]	—
$eGFR_{crea}$	$eGFR_{crea}$ is an estimation of the kidney function level based on serum creatinine, age, gender, and race.	A reduction of eGFR is a potent predictor of CV endpoints, regardless of age, gender, and other risk factors [1, 2, 5, 8, 22, 23]	Although a treatment-induced reduction of eGFR is considered a surrogate endpoint of ESKD, the predictive role of eGFR change for CV risk is still controversial [67].
Proteinuria	Presence of an abnormal quantity of proteins in urine; it is considered the principal marker of kidney damage.	The increase in proteinuria is strongly associated with the onset of fatal and nonfatal CV events [1, 2, 5, 8, 21, 22]	In clinical trials, patients who develop a significant reduction in proteinuria during the first months after treatment were protected against CV events over time [12–15, 69–71].

Table 1: Continued.

Biomarkers	Characteristics	Prognostic value	Predictive value
RI	Renal resistive index is a sonographic index of intrarenal arteries defined as (peak systolic velocity − end diastolic velocity)/peak systolic velocity .	Raised RI levels above have been shown to predict CV events in hypertensive and CKD patients [75, 76]	Medications as RAAS inhibitors and SGLT-2i reduce RI levels over time and improve vascular damage [77, 78].
ACE ID/DD	Insertion (I)/deletion (D) polymorphism of the angiotensin-converting enzyme (ACE) gene influences the circulating and renal activity of RAAS.	The D allele patients showed a poor CV prognosis in the RENAAL trial [79]	Patients with DD genotype, despite being at high risk of CV events, showed the better response to losartan in the RENAAL study [79].
Classifiers	A classifier is the combination of the informative markers which is able to classify patients according to their risk of developing an outcome or likelihood of response to a treatment.	—	A panel of 185 metabolites and a proteomic-based classifier have shown to predict the proteinuric response to RAAS inhibitors [81, 82].

rather than randomize all the population irrespective of a response/no response. Advantages from this strategy are several. Firstly, patients under study would avoid a long period of ineffective therapy if they were nonresponders. Secondly, since all the patients are treated with the study drug before randomization (the run-in period), the treatment effect is estimated in a proper fashion. Finally, such a design is close to clinical practice since clinicians are used to continuing a treatment only if patients respond to that treatment. Predictive biomarkers could be also used to better understand the phenomenon of variability in response to treatment. The crossover studies and even the single-patient trials, the so-called *n-of-1*, may help to answer this important question. Indeed, in these study designs, patients are randomized to 2 or more sequences of different drugs interspersed with a washout period. With such a design, by measuring a panel of biomarkers before starting each treatment, it is possible to assess what are the characteristics of a patient who responds to the first treatment and does not respond to the second treatment or vice versa. This could also lead in the future to dose a biomarker before selecting the correct treatment as well. One example of such a crossover study is the ROTATE trial (ClinicalTrials.gov identifier: NCT03504566); the results of which are eagerly expected in 2021.

6. Conclusions

Owing to the global dimension of CKD and the high prevalence of CV disease in this setting, great effort is currently ongoing with the aim of reducing CV residual risk. One important strategy that can be pursued to this aim is to develop reliable prognostic and predictive biomarkers. In fact, eGFR and proteinuria, despite their great importance, have shown suboptimal performance in predicting several CV outcomes in CKD patients such CAD and heart failure [93]. Predicting the response to treatments is another important scope of clinical research since it allows to individualize therapies, to improve the clinical trial design, and to better comprehend the variability in the response to different treatments. The implementation of novel biomarkers of CV risk from the discovery to clinical practice should follow a rigorous methodology so that it would be possible to improve the management of patients by clinicians.

Data Availability

The underlying data supporting the results of our study can be asked from the corresponding author.

Conflicts of Interest

The authors declare they have no conflict of interests.

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Research Article

Screening of 22q11.2DS Using Multiplex Ligation-Dependent Probe Amplification as an Alternative Diagnostic Method

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Background. The 22q11.2 deletion syndrome (22q11.2DS) is the most common form of deletion disorder in humans. Low copy repeats flanking the 22q11.2 region confers a substrate for nonallelic homologous recombination (NAHR) events leading to rearrangements which have been reported to be associated with highly variable and expansive phenotypes. The 22q11.2DS is reported as the most common genetic cause of congenital heart defects (CHDs). *Methods*. A total of 42 patients with congenital heart defects, as confirmed by echocardiography, were recruited. Genetic molecular analysis using a fluorescence *in situ* hybridization (FISH) technique was conducted as part of routine 22q11.2DS screening, followed by multiplex ligation-dependent probe amplification (MLPA), which serves as a confirmatory test. *Results*. Two of the 42 CHD cases (4.76%) indicated the presence of 22q11.2DS, and interestingly, both cases have conotruncal heart defects. In terms of concordance of techniques used, MLPA is superior since it can detect deletions within the 22q11.2 locus and outside of the typically deleted region (TDR) as well as duplications. *Conclusion*. The incidence of 22q11.2DS among patients with CHD in the east coast of Malaysia is 0.047. MLPA is a scalable and affordable alternative molecular diagnostic method in the screening of 22q11.2DS and can be routinely applied for the diagnosis of deletion syndromes.

1. Introduction

The 22q11.2 deletion syndrome (22q11.2DS) is the most common genetic disorder caused by deletions of chromosome 22, at the q11.2 locus [1]. Depending on the method of accreting in different countries, prevalence of 22q11.2DS has been reported to range from 1 : 2000 to 1 : 7000 [2].

Approximately 97% of patients with 22q11.2DS were reported to harbour the 3 Mb deletion of DNA, causing a haploinsufficiency in about 30-40 genes within the locus [3, 4]. Common clinical features of 22q11.2DS include dysmorphic facies, congenital heart defects, palatal malformations, learning difficulties, and immunodeficiency. In terms of congenital heart defects (CHDs), the 22q11.2DS has been reported as a common genetic cause, contributing to approximately 1.9% of CHDs at birth [4]. About 70% of CHDs are conotruncal malformations, followed by tetralogy of Fallot (20%), truncus arteriosus (6%), and conoventricular ventricular septal defect (VSD) (14%), which is a type B interruption of the aortic arch (IAA) (13%) [5, 6]. Nevertheless, incidences in atrial septal defects (ASDs), pulmonary valve stenosis (PVS), hypoplastic left heart syndrome (HLHS), double-outlet right ventricle, and transposition of the great arteries (TGA) are less common [4].

The 22q11.2 deletion syndrome presents an expansive phenotype with more than 180 clinical features involving almost every organ and system in the body [2]. Thus, diagnoses through clinical features are unreliable, leading to a heavy reliance on molecular genetic analysis where chromosome 22q11.2 is observed for deletions and/or duplications using

a cytogenetic approach with the method regarded as highly reliable [7].

Fluorescence *in situ* hybridization (FISH), a molecular genetic analysis which detects chromosomes for abnormalities, has been reported as the "gold standard" for diagnosis [8]. FISH utilises fluorescence probes (N25 and *TUPLE1*) located at the proximal part of the typically deleted region (TDR) to determine abnormalities within the 22q11.2 regions. However, FISH probes cannot detect deletions proximal or distal to the particular probe used; besides, it only provides information on targeted locations [9, 10]. Therefore, it does not allow a comprehensive evaluation of the whole genome. In addition, it is a challenge to identify atypical smaller deletions by FISH due to the fact that the probes are unable to cover these regions. Hence, FISH alone cannot provide reliable diagnosis for cases of 22q11.2DS, thus necessitating the need for an alternative molecular genetic diagnostic tool which could provide a scalable and accurate diagnosis in a cost-effective and less labour-intensive manner with practicality for application in small laboratories.

Over the years, new diagnostic methods for the detection of the 22q11.2 deletion syndrome have been developed, including comparative genomic hybridization (CGH) [11, 12], multiplex ligation-dependent probe amplification (MLPA) [13], multiplex quantitative real-time polymerase chain reaction (PCR) [14], and high-resolution single-nucleotide polymorphism (SNP) microarray analysis [14, 15]. Nonetheless, some of these methods are still at the experimental stage, requiring expensive equipment for assay and data analysis as well as trained personnel to conduct the experiments. On the other hand, the MLPA technique can easily be performed in laboratories without such needs. MLPA is a PCR-based technique which can provide a good resolution combined with practicality and affordability, thus providing approximately 98.9% sensitivity and 97.8% specificity [16].

Both FISH and MLPA techniques are locus-specific tests. However, FISH is a qualitative test that indicates the presence or absence of the 22q11.2DS. On the other hand, MLPA provides both qualitative and copy number variation data for the 22q11.2 region and other locus contained in the kit.

Despite the significance and high prevalence of 22q11.2DS as one of the most common frequent genomic disorders [17], to the best of our knowledge, the incidence of 22q11.2DS in Malaysia has not been reported. Therefore, a pilot approach is necessary in determining the incidence of 22q11.2DS in the east coast of Malaysia and in investigating the utility of MLPA as a potential alternative to FISH in diagnosing 22q11.2DS among nonsyndromic patients with CHDs.

2. Material and Methods

2.1. Editorial Policies and Ethical Considerations. The research project was approved by the Research and Ethics Committee, School of Medical Sciences, Universiti Sains Malaysia (USM) Health Campus (USMKK/PPP/JEPeM [252.3(13)]), and the Ministry of Health Malaysia (KKM/NIHSEC/BOO-2/2/2/P13-147) which complies with the Declaration of Helsinki. Written informed consent was obtained from either the parents of patients below 18 years old or directly from the patients who are 18 years old and above. Additionally, all patients/parents of patients must sign a written informed consent form to allow publication of their medical and/or genetic information.

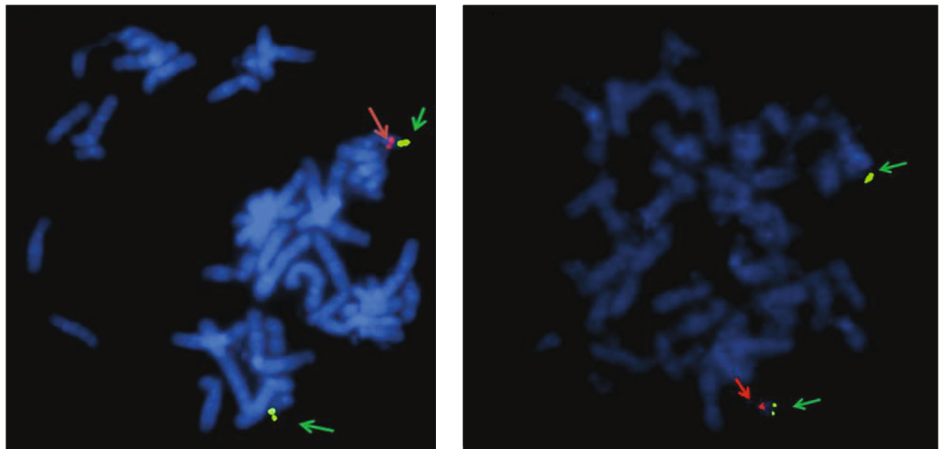
2.2. Study Population and Sample Collection. CHD patients admitted to Hospital Universiti Sains Malaysia (HUSM), which serves as the main tertiary cardiac referral centre in the east coast region of Peninsular Malaysia from January 2013 to November 2014, were recruited ($n = 42$). Patients ranging from newborns to adults confirmed to harbour the defect based on an echocardiogram were recruited. The conditions were identified and confirmed by a paediatric cardiologist in the Echocardiography Unit, HUSM.

Approximately 3 ml of peripheral blood was collected from each patient; 1 ml of the sample was stored in a sodium-heparin tube for culture of lymphocytes, whereas 2 ml was stored in EDTA tubes for DNA extraction.

2.2.1. FISH. FISH analysis of chromosome 22q11.2 was performed on metaphase spreads and on interphase nuclei obtained from the synchronous culture of lymphocytes, using a commercially available DiGeorge/VCFC *TUPLE1* probe (Cytocell, Cambridge, UK). The DiGeorge/VCFC *TUPLE1* region deletion probe measures approximately 120 kb of the gene and covers the entire *TUPLE1* gene as well as the flanking DNA. The 22qter sub-telomere-specific probe (clone N85A3) is located in the *ProSAP2*/*SHANK3* gene, allowing identification of the most distal 22q13.3 deletions. In a normal cell, there should be two red and two green signals (2R and 2G, respectively), while a deletion of the DGCR probe target will result in only the formation of 1R and 2G signals. On the other hand, a deletion of the 22q subtelomeric probe will result in 2R and 1G signals. The slide preparation, denaturation, and hybridization were carried out according to the manufacturer's protocols (http://www.amplitech.net/PDF/microdeletions/LPU004.pdf). Generally, 20 metaphases were examined and 100 interphase nuclei were scored for the number of signals present.

2.2.2. MLPA. Genomic DNA was extracted from peripheral blood using the GeneAll® Exgene™ Blood SV mini kit (GeneAll, Korea) following the manufacturer's instructions. DNA concentration and purity were determined using the NanoQuant spectrophotometer (Tecan, USA). MLPA was conducted using the SALSA MLPA P250-A1 DiGeorge Kit (MRC Holland, Amsterdam, Netherlands). The kit consisted of 48 probes from which 29 are within the 22q11.2 loci while the remaining 19 are within the regions of DiGeorge syndrome (DGS) and DGS type II (all covering chromosomes 22q13, 4q, 8p, 9q, 10p, and 17p).

PCR amplification was carried out at the Human Genome Centre, Universiti Sains Malaysia, Kelantan, Malaysia. The capillary electrophoresis using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) was conducted at First BASE Laboratories Sdn Bhd, Malaysia. The data were analysed using the Coffalyser VBA analysis



(a) (b)

Figure 1: Photographs of FISH assays showing microdeletions of chromosome 22q11.2 in (a) S1 and (b) S2. A metaphase spread indicating the presence of two green signals designating *SHANK3* (indicated by a green arrow) and a single red signal designating the 22q11.2 region (indicated by a red arrow).

software V8 (http://www.mlpa.com/coffalyser). Two healthy controls (as confirmed by echocardiography) were used as positive samples for data normalisation. The threshold for deletion was set at 0.75 while the threshold for duplication was set at 1.30. Samples which showed deletions and/or duplications were reanalysed for further confirmation.

3. Results

To our knowledge, this study is the first to successfully report screening of 42 nonsyndromic CHD patients for deletions and/or duplications in the 22q11.2 locus using both the FISH assay and MLPA tests, as a part of the routine diagnosis of 22q11.2DS in Malaysia. The screening was followed by a MLPA test, which was performed on all patients irrespective of their FISH assay results, thus serving as a confirmatory test. From the 42 cases, two samples (4.76%) showed deletion and duplications within the 22q11.2 regions. Subsequently, the FISH assay using the DiGeorge/VCFC *TUPLE1* probe (Cytocell, Cambridge, UK) in both patients showing deletions within the 22q11.2 regions was reconducted (Figure 1) for further confirmation, whereas 40 cases showed no deletion and/or duplications using both FISH and MLPA techniques.

The 22q11.2 deletions were detected in two patients using the DiGeorge/VCFC *TUPLE1* probe (Cytocell, Cambridge, UK) (Figure 1). The MLPA assay using the SALSA MLPA P250-A1 DiGeorge Kit (MRC Holland, Amsterdam, Netherlands) also confirmed deletion in both patients. However, in contrast to FISH, MLPA detected duplications within the 22q11.2 region indicating that it is a more sensitive tool for detection of duplications.

When the patients' data was further analysed, the first patient (S1) was a 3-week-old baby girl diagnosed with patent ductus arteriosus. The MLPA assay showed deletions in the typically deleted regions (TDR): *LZTR1* (LCR C-D) and *TOP3B* (LCR D-E) and *RTDR1* (LCR D-E). In addition, deletions detected by the probes outside of 22q11.2 regions were also observed: *BID4* (22q11.2 CES), *PPP1R3B* and *MSRA* located within the 8p23.1 locus, *TCEB1P3* (chromosome 10p14), and *RPH3AL* (chromosome 17p13.3) alongside duplication of *EHMT1* (chromosome 9q34.3), *CELF2* (chromosome 10p14), and *YWHAE* (chromosome 17p13.3) (Figure 2).

The second patient (S2) was a month-old baby boy diagnosed with pulmonary atresia with VSD and a major aortopulmonary collateral artery (MAPCAS). The MLPA assay detected a 3 Mb deletion within the TDR from *CLTCL1* (LCR A-B) to *LZTR1* (LCR C-D) as well as duplication of *YWHAE* (chromosome 17p13.3) and *GATA3* (chromosome 10p14) (Figure 3).

4. Discussion

In this study, we successfully screened the 22q11.2DS in 42 nonsyndromic Malaysian CHD patients using both the FISH and MLPA techniques. MLPA confirmed the presence of deletions as detected by the FISH assay in the two nonsyndromic CHD patients. Nevertheless, in both cases, FISH failed to detect deletions located outside the TDR and deletions in probes outside of the 22q11.2 regions as well as duplications indicating that MLPA is superior to FISH as a diagnostic tool. Our findings suggest the possibility of using MLPA as a potential alternative diagnostic method in the screening of 22q11.2DS.

S1 carried a deletion within the distal deletion region and deletions outside of the 22q11.2 TDR. Since the "classical" candidate genes were not deleted, the cardiac malformation observed might be due to the deletion of the other genes. Furthermore, deletions were also observed in *BID4*, a gene associated with the cat eye syndrome, as well as genes outside of chromosome 22q11.2: *PPP1R3B*, *MSRA*, *TCEB1P3*, and *RPH3AL*. This is defined as a typical characteristic of molecular complexity which controls the 22q11.2DS phenotypes. Furthermore, the 22q11.2 hemizygosity alone cannot explain the genetic mechanism of the highly variable phenotypic

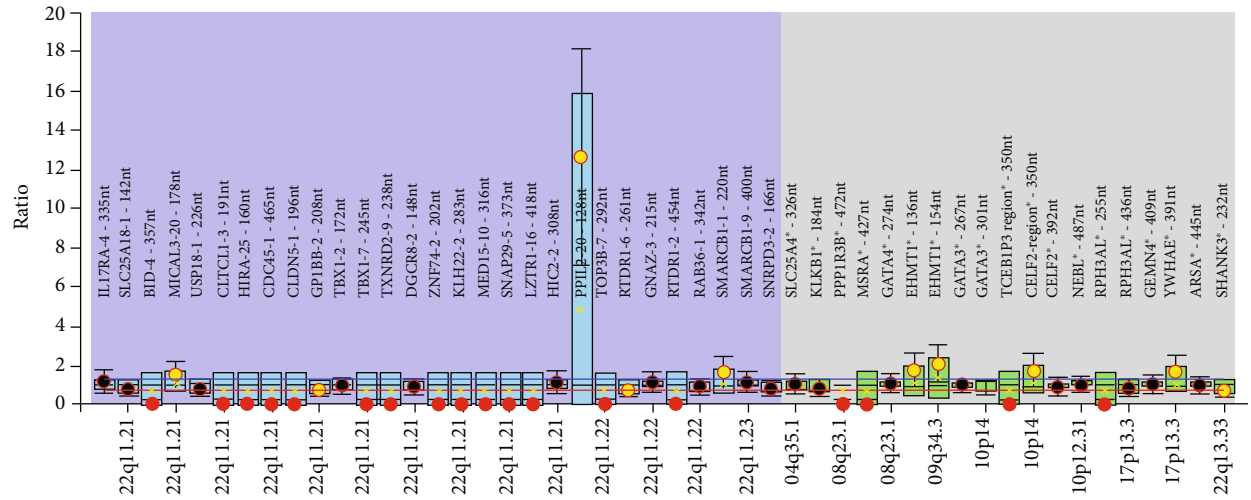


Figure 2: An MLPA ratio chart showing deletions of *LZTR1* (LCR C-D), *TOP3B* (LCR D-E), *RTDR1* (LCR D-E), *BID4*, *PPP1R3B*, *MSRA*, *TCEB1P3*, and *RPH3AL* as well as duplications of *PPIL2*, *EHMT1*, and *YWHAE*. The black dots display the probe ratios and the error bars with 95% confidence intervals. The red dots display deletion, and the yellow dots display duplication. The blue box plots display genes within the 22q11.2 region whereas the green box plots display other regions associated with 22q11.2DS. A map view of all the locations are displayed on the *x*-axis while the *y*-axis shows the ratio. The red and blue horizontal lines indicate the arbitrary borders for loss and gains at a ratio of 0.7 and 1.3, respectively.

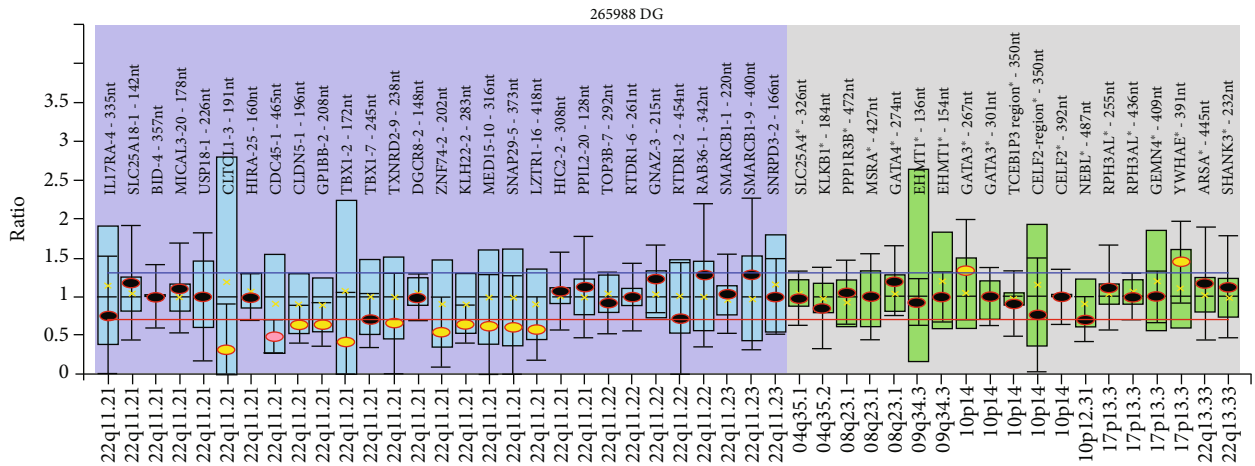


Figure 3: An MLPA ratio chart showing a 3 Mb deletion from *CLTCL1* to *LZTR1* and duplication of *YWHAE*. The black dots display the probe ratios and the error bars with 95% confidence intervals. The blue box plots display genes within the 22q11.2 region whereas the green box plots display other regions associated with 22q11.2DS. The red and blue horizontal lines indicate the arbitrary borders for loss and gains at a ratio of 0.7 and 1.3, respectively. The yellow dots below the red horizontal line indicate duplication of the gene whereas the yellow dots above the blue horizontal lines indicate duplication of the gene. Map view locations are displayed on the *x*-axis while the *y*-axis shows the ratio results.

expression of 22q11.2DS. McDonald-McGinn and colleagues [18] proposed that the mechanism of 22q11.2DS involves a combined effect of multigene deletion and a stochastic phenomenon which includes the sensitivity of individual genes within the 22q11.2 region to gene dosage [19, 20], variants in genes on the intact 22q11.2 [1], and additional "modifying" variants outside the 22q11.2 region [8]. S2 carried a 3 Mb deletion from LCR A and LCR D, and approximately 90% of patients with 22q11.2DS have been reported to harbour this deletion [21]. The result is also in agreement with Carotti and colleagues [22] who reported that up to 40% of patients with MAPCAs have DiGeorge syndrome with chromosome 22q11.2 deletion indicating that the deletion contributes to the occurrence of the disease.

It is noteworthy that in both of the patients, duplication of *YWHAE* was observed. The FISH assay failed to detect this duplication; this is due to the fact that the DiGeorge/VCFC *TUPLE1* probe (Cytocell, Cambridge, UK) used in this study does not contain a probe for *YWHAE* [13]. Studies have reported that individuals with duplications within *YWHAE* were characterised by a mild neurocognitive and pervasive developmental disorder phenotype in the presence of minor craniofacial abnormalities [23, 24]. This is in agreement with previous reports that individuals with 22q11.2DS have high

rates of cognitive and psychiatric problems [25, 26]. In terms of craniofacial abnormalities, only mild but typical facial, skeletal, and dental characteristics, including significant retrusion of the lower part of the face, were observed [27]. However, due to unavailability of phenotypic characteristics of patients in this study, the cognitive and psychiatric characteristics could not be further assessed.

A large proportion (95%) of the recruited subjects in our study did not show deletions and/or duplications, within the 22q11.2DS, despite the use of both MLPA and FISH assays. This occurrence might be explained by the fact that the patients may carry very small deletions or even point mutations, which are below the resolution of the methods used. Alternatively, the patients may have other microdeletion or microduplication syndromes.

In the present study, all recruited patients were examined by two different approaches, enabling first-hand experience in comparing the techniques and the underlying principles of each technique, hence leading to a conclusion that MLPA stands superior to FISH based on the mentioned criteria. Our conclusion is also at par with the findings from Jalali and colleagues [13] who reported that in the near future, MLPA will be able to replace 22q FISH.

The MLPA assay compared to FISH is relatively simple to be used in clinical laboratories of small- or medium-scale dimension with much cheaper reagent cost per assay [28]. A recent report by Sorensen and colleagues [29] even suggested that the MLPA technique is used within paediatric cardiology as a first-tier screen in detecting clinically relevant copy number variants (CNV) and in identifying syndromic patients at an early stage. Another advantage of MLPA is that it does not require cell culturing, which is a tedious technique and often requires trained professional for aseptic handlings, with high contamination issues. The use of DNA as a starting material in MLPA provides additional benefit to a certain group of patients who are reluctant or are unable to provide blood samples, where saliva and hair root samples can be used as alternatives. Furthermore, MLPA can potentially diagnose a broader spectrum of abnormalities [30].

Although the FISH technique is still in routine use in many laboratories, it cannot detect deletions that are either proximal or distal to the particular probe used [21]. Another major downfall of FISH is that both the interphase and metaphase FISH can only detect known genetic aberrations, provided that the specific probe is available [31]. Moreover, owing to limitations in resolution, FISH analysis has been reported to be unable to detect microdeletions or microduplications smaller than 40 kb [32]. Our small data may add to the body of evidence of current findings in determining alternative diagnostic methods for 22q11.2DS screening. A larger cohort to further confirm the concordance of these techniques and provide the prevalence rate of 22q11.2DS in Malaysia is suggested in the future.

5. Conclusions

The incidence of 22q11.2DS in the east coast of Malaysia is estimated as 0.047 with the samples collected in this study. Our study highlighted the scalability, concurrency, and applicability of MLPA as a potential alternative to the FISH assay in detecting 22q11.2DS. Compared to FISH, the MLPA method can be conducted with ease, is less time-consuming, and is less laborious.

Data Availability

The cytogenetic and MLPA data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Research Article

Circulating miRNA-23b and miRNA-143 Are Potential Biomarkers for In-Stent Restenosis

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In-stent restenosis (ISR) is one of the main complications in patients undergoing percutaneous coronary angioplasty, and microRNAs participate in the contractile-to-synthetic phenotypic switch of vascular smooth muscle cells, a hallmark of restenosis development. MicroRNAs (miRNAs) can be released into circulation from injured tissues, enticing a potential role as noninvasive biomarkers. We aimed to evaluate circulating levels of miRNA-23b, miRNA-143, and miRNA-145 as diagnostic markers of ISR. 142 patients with coronary artery disease undergoing successful angioplasty and a follow-up angiography were included. Subjects were classified according to the degree of obstruction at the angioplasty site into cases (≥50%) or controls (<50%). Total RNA was isolated from plasma to quantify circulating miRNAs levels, and the ROC curves were constructed. Among circulating miRNAs assessed, miRNA-23b and miRNA-143 were significantly lower in cases (miRNA-23b: $18.4x10^{-5}$ and miRNA-143: $13.7x10^{-5}$) than controls (miRNA-23b: $5.2x10^{-5}$, $p < 0.0001$; miRNA-143: $4.0x10^{-5}$, $p < 0.0001$). Plasma levels of miRNA-145 showed no significant differences. The analysis of the ROC curves showed an area under the curve for miRNA-23b of 0.71 (95% CI: 0.62-0.80, $p < 0.0001$) and 0.69 for miRNA-143 (95% CI: 0.60-0.78; $p < 0.0001$). Our data suggest that plasma levels of miRNA-23b and miRNA-143 could be useful as noninvasive biomarkers of ISR.

1. Introduction

Restenosis results from a reduced diameter of the lumen of a blood vessel following percutaneous coronary angioplasty (PCA). Angiographically, restenosis is defined dichotomously as a luminal narrowing of more than 50% in the follow-up angiography, which occurs as a consequence of arterial damage with subsequent neointimal tissue proliferation [1]. Although a reduction in the percentage of subjects developing restenosis has been observed at the same time PCA has evolved, in-stent restenosis (ISR) continues to be one of the main complications in patients undergoing this procedure [2]. Moreover, the uptake of percutaneous intervention in larger numbers of patients with increasingly complex lesion characteristics and disease comorbidities means that the number of patients presenting with restenosis remains considerable in absolute terms [3]. Several pathogenic mechanisms have been associated with restenosis development, among which we can find elastic recoil of the vessels, arterial remodeling, and neointimal hyperplasia. The two latter particularly related to ISR though a phenotypic change of the vascular smooth muscle cells (VSMCs)

from a contractile to a proliferative state under the induction of platelet-derived growth factor (PDGF), resulting in accumulation of VSMC in the intimal arterial layer [4–6].

MicroRNAs (miRNAs) are short noncoding RNA molecules that negatively affect gene expression through mRNA cleavage or inhibition of protein translation, resulting in profound and complex regulatory networks [7]. In the cardiovascular system, miRNAs regulate basic functions in almost all cell types including cardiac muscle, endothelial cells, smooth muscle, inflammatory cells, and fibroblasts and, therefore, play a pivotal role in the pathogenesis of several cardiovascular diseases (CVDs). Numerous miRNAs have been implicated in the contractile-to-synthetic phenotypic switch of VSMC, including miRNA-143/145, miRNA-23b, miRNA-22, miRNA-133, miRNA-125a, miRNA-195, miRNA-663, miRNA-21, miRNA-221/222, miRNA-146a, miRNA-206, miRNA-181b, and miRNA-31, among others [8]. For instance, the miRNA-143/145 cluster coordinates the phenotypic modulation of VSMC through a cooperative network of transcription factors, such as Kruppel-Like Factor 5 (KLF5), Kruppel-Like Factor 4 (KLF4), and the potent transcriptional coactivator myocardin (MYOCD), to promote differentiation and suppress VSMCs proliferation [9, 10]. Thereby, deficiency of miRNA-145/143 promotes the synthetic phenotype of VSMCs. Furthermore, adenoviral-mediated gene transfer of miRNA-145/143, which downregulate the expression of this miRNAs after injury, inhibits neointimal lesion formation in injured rat carotid arteries [11]. On the other hand, miRNA-23b plays a critical role in angiogenesis, cardiac ischemia, and homeostasis of endothelial cells [12]. Moreover, the overexpression of miRNA-23b in balloon-injured arteries by Ad-miRNA-23b markedly decreased neointimal hyperplasia in VSMC, *in vitro* [13]. Yet, another relevant and highly attractive feature of miRNAs corresponds to their remarkable extracellular stability, an outcome that has received especial attention due to a promising role as blood-based biomarkers for diagnosis and prognosis of several illnesses [14]. Considering this background, we evaluated levels of circulating miRNA-23b, miRNA-143, and miRNA-145 as potential diagnostic markers of ISR in Chilean subjects with coronary artery disease (CAD).

2. Methods

2.1. Subjects. A diagnostic test study was designed using unpaired incident cases and controls, including 142 patients with CAD who underwent successful coronary artery angioplasty and follow-up coronary angiography after at least 6 months of stent implantation. Participants were classified in two groups: (1) case group, with stenosis $\geq 50\%$ and (2) control group, with stenosis $< 50\%$, a criterion concordant with the degree of obstruction at the angioplasty site. The obstruction was measured in the follow-up and was regarded as the gold standard. Demographic, anthropometric, and cardiovascular risk factors such as diabetes, hypertension, and dyslipidemia were recorded. Patients were invited to participate in the cardiology services of public Chilean hospitals, Dr. Hernán Henríquez Aravena Hospital (Temuco, Chile) and Dr. Guillermo Grant Benavente (Concepción, Chile). All subjects enrolled were included in this study after accepting their participation by signing a written informed consent approved by both, the Ethics Committees of University of La Frontera and Health Service of Concepción.

2.2. Sampling and RNA Extraction. For detection of circulating miRNAs, we obtained a sample of whole blood anticoagulated with EDTA at the time of the angiographic control using standard venipuncture blood extraction. Then, plasma was separated by centrifugation (15 minutes at 3.000 rpm) and transferred into nuclease-free tubes for subsequent storage at -80°C until RNA extraction procedures. Plasma RNA was isolated using the miRNeasy Mini Kit (Qiagen) following the supplemental protocol for RNA purification, including miRNAs. Prior to extraction, 5 μL of the exogenous miRNA-39 (5 nM) of *Caenorhabditis elegans* (cel-miRNA-39) was added to 0.2 mL of plasma, for the normalization of technical variations (sample quality and extraction efficiency) between samples.

2.3. Circulating miRNAs. To quantify circulating miRNA-143, miRNA-145, and miRNA-23b levels from total RNA, a first strand of complementary DNA (cDNA) was synthesized using the TaqMan® Advanced miRNA (Applied Biosystems) synthesis kit, which polyadenylated the $3'$ end and added an adaptor to the $5'$ end of all miRNA sequences contained in the sample. Then, miRNA detection was completed by quantitative polymerase chain reaction (qPCR), using the TaqMan® Advanced miRNA Assays for each selected miRNA (Thermo Fisher Scientific assay IDs 478713_mir, 477915_mir and 477991_mir). These assays were performed in a StepOne™ Real-Time PCR System (Applied Biosystems) using the following thermocycling protocol: 20 seconds at 95°C and 40 cycles of 95°C for 1 second and 60°C for 20 seconds. All qPCR assays were performed in duplicate, and miRNAs quantification was obtained by the $2^{-\Delta Ct}$ method, using cel-miRNA-39 to normalize miRNA expression.

2.4. Statistical Analysis. The statistical analysis was carried out with the software SPSS 25.0 (SPSS, Inc., Chicago, USA). The unpaired two-tailed t-test or its nonparametric equivalent (Mann–Whitney U test) was used to assess differences between demographic and biometric characteristics and circulating miRNA quantification between cases and controls. The comparisons between dichotomous variables were analyzed using the chi-squared test. The receiver operating characteristic (ROC) curves were generated to evaluate the diagnostic capacity of each circulating miRNA, using the area under the curve (AUC) to determine cutoff values that maximizes sensitivity and specificity. A value of $p < 0.05$ was defined as statistically significant.

3. Results

3.1. Clinical Characteristics of Subjects. Clinical and anthropometric characteristics of the participants are shown in Table 1. No significant differences were observed between

Table 1: Clinical and anthropometric characteristics of cases and controls.

Parameter	Controls ($n = 61$)	Cases ($n = 81$)	p value
Age (years)	67.0 ± 9.3	64.1 ± 10.3	0.640
BMI (kg/m^2)	27.6 ± 4.2	28.2 ± 3.9	0.326
SBP (mmHg)	130.7 ± 25.9	130.4 ± 23.6	0.942
DBO (mmHg)	69.5 ± 12.2	73.1 ± 12.1	0.081
HR (bpm)	68.6 ± 11.0	71.0 ± 12.2	0.301
Male (%)	66.7	80.5	0.042
BMS (%)	65.8	86.4	0.002
Diabetes mellitus (%)	28.0	41.0	0.091
Dyslipidemia (%)	69.3	74.4	0.489
Hypertension (%)	84.2	85.9	0.769
Obesity (%)	26.3	27.4	0.870
Lesion vessel (%)			
LMCA	1.1	1.1	—
LAD	47.5	44.8	—
CX	18.3	31.7	—
RCA	33.1	22.4	—
Diameter (mm)	3.1 ± 0.3	2.9 ± 0.1	0.673
Length (mm)	22.3 ± 4.1	22.6 ± 3.4	0.710

Values expressed as mean ± standard deviation. BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; HR: heart rate; ISR: in-stent restenosis; BMS bare-metal stent; LMCA: left main coronary artery; LAD: left anterior descending artery; CX: circumflex artery; RCA: right coronary artery.

age ($p = 0.640$), body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP), heart rate, diabetes, dyslipidemia, hypertension, and obesity. However, a higher percentage of men was observed in the case group ($p = 0.042$), as well as a higher percentage of subjects treated with bare-metal stent (BMS) in the same group ($p = 0.002$).

3.2. Circulating miRNAs and ROC Curves. Plasma levels of circulating miRNA-143 and miRNA-23b were significantly lower in the case group compared to controls ($p < 0.001$). Circulating miRNA-145 levels did not show differences between both groups ($p = 0.099$) (Figure 1). Based on these results, we therefore selected miRNA-23b and miRNA-143 to evaluate their diagnostic potential through the ROC curve analysis. As shown in Figure 2, the ROC curve for miRNA-23b showed an AUC of 0.71 (95% CI, 0.620-0.798, p <0.001), while the ROC curve for miRNA-143 displayed an AUC of 0.692 (95% CI, 0.601-0.782, $p < 0.001$). We then established the most appropriate cutoff point for both tests, considering the best performance for specificity and sensitivity, resulting in 14.3×10^{-5} (miRNA-23b) and 11.4×10^{-5} (miRNA-143). Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) are shown in Table 2. In addition, by combining measures from both miRNAs, the ROC analysis showed superior sensitivity (0.82), whereas specificity reached 0.68 (Table 2).

4. Discussion

In the present study, we evaluated circulating levels of miRNA-23b, miRNA-143, and miRNA-145 in patients undergoing successful coronary arterial angioplasty, with a follow-up coronary angiography after at least 6 months since stent implantation, in order to identify differences in miRNA levels between patients who developed ISR versus controls.

ISR is one of the most relevant complications after stent placement and is characterized by the phenotypic switch of VSMC. Different reports indicate that miRNAs play a significant role in this process, for instance, miRNA-143, miRNA-145, and miRNA-23b maintain a contractile phenotype in VSMCs of healthy blood vessels [9, 10, 15–19]; therefore, circulating miRNAs could reflect cellular processes occurring in the blood vessels after stent implantation [16, 20]. Moreover, due to miRNAs can be found in several body fluids, their potentials to identify different pathologies have been widely explored in CVD such as acute myocardial infarction [21], CAD [22], and stroke [23]. In the case of ISR, we here show that miRNA-23b could be used as a noninvasive biomarker to identify patients with ISR. This miRNA is highly expressed in cell cultures of VSMC, decreasing their proliferation, differentiation, and migration [13]. In addition, *in vitro* studies show that miRNA-23b regulates the expression of the Forkhead Box protein (Fox04) [24], which represses differentiation of smooth muscle cells and activates the migration of VSMC in response to TNF-α and urokinase-type plasminogen activator (uPA) [15, 19] through the MEK/ERK signaling pathway [25] and SMAD3 [19], a protein important for the proliferation of VSMC [26]. These data could explain the decreased levels of miRNA-23b detected in the plasma of patients with ISR.

Similarly, studies have reported AUC values of 0.839 for miRNA-143 and 0.871 for miRNA-145, in addition to high specificity and sensitivity (0.9 and 0.7), respectively [27]. We also observed a repression of miRNA-143 in subjects who develop ISR. Apparently, the most important trigger of miRNA-143 repression is PDGF, a key element in the mediation of vascular response to injury acting through the proto-oncogene tyrosine-protein kinase SRC and phosphoprotein p53, with the net result of promoting both proliferation and migration of VSMC [16]. A significant difference in plasma levels of miRNA-143 in restenosis of vascular occlusive disease of the lower extremities has also been reported, with significantly decreased expression [28].

The availability of noninvasive biomarkers with high resolution for specific biological processes could add significant incremental value to the clinical arsenal currently available for short-term follow-up of patients treated with PCA, which could reduce the number of candidates for invasive angiographic control [29]. Identification of differential miRNAs and their potential use as biomarkers in patients with ISR still requires greater consistency. Moreover, miRNAs are extremely stable in plasma/serum and resistant to some hard conditions including low and high pH, boiling, and long storage and can withstand repetitive freezing and thawing cycles [30]. We also highlight the need for longitudinal studies depicting changes in miRNA levels from the first

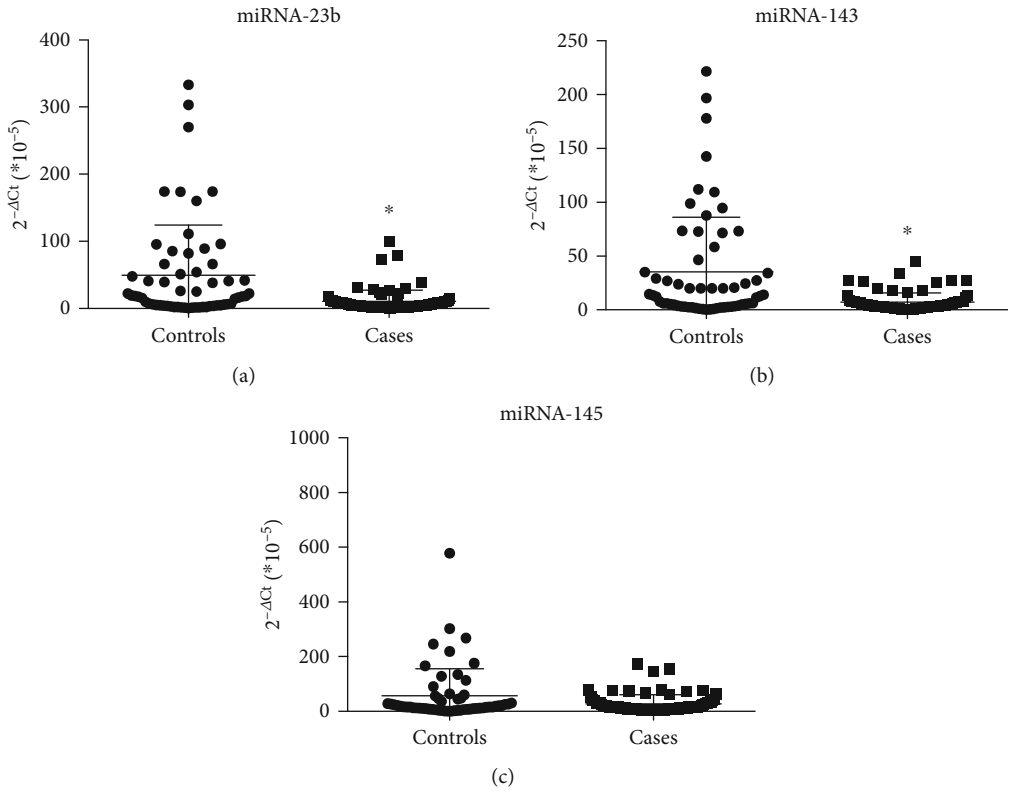

Figure 1: Quantification of miRNAs: (a) miRNA-23b, (b) miRNA-143, and (c) miRNA-145 in plasma samples of cases and controls.

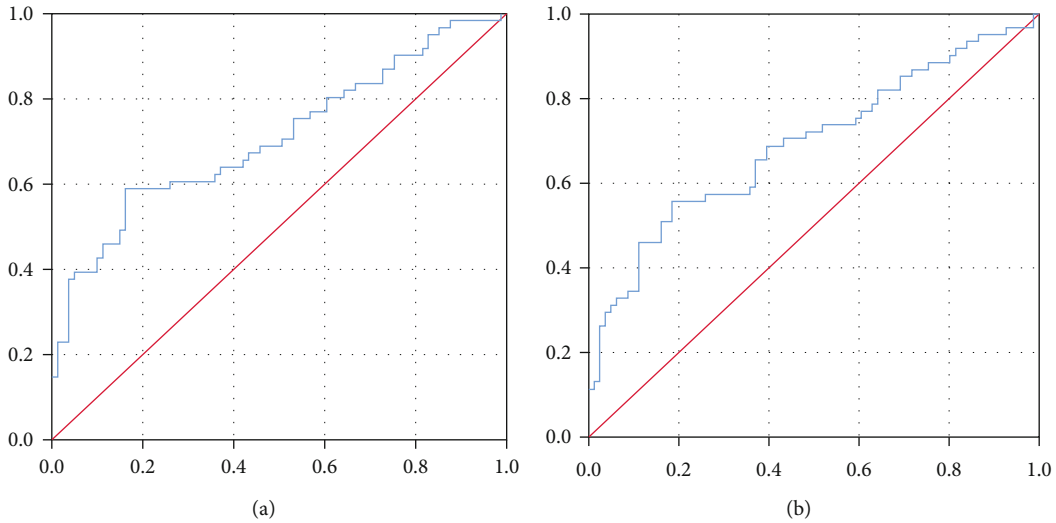

Figure 2: Analysis of the ROC curves showing the sensitivity (Y axis) and 1-specificity (X axis) for (a) miRNA-23b and (b) miRNA-143 as potential diagnostic markers for ISR.

Table 2: Diagnostic characteristics for plasma miRNA-23b and miRNA-143.

miRNAs	Sensitivity	Specificity	PPV	NVP
miRNA-23b	0.59	0.84	0.83	0.61
miRNA-143	0.56	0.82	0.80	0.58
miRNA-23b/miRNA-143	0.82	0.68	0.77	0.73

PPV: positive predictive value; NPV: negative predictive value.

stages of the intervention. Currently, incipient efforts have been reported to a promissory use of miRNAs for stent intervention in animal models, which holds potential for personalized therapy [31, 32].

In conclusion, our data show decreased levels of miRNA-23b and miRNA-143 in the plasma of subjects with ISR versus controls, a differential result that could confer these miRNAs a potential use as noninvasive biomarkers for ISR.

Data Availability

The data and materials in the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

The study design, writing the review, and editing were contributed by N.S., K.S., T.Z., F.L., G.R., and L.S.; the laboratory analysis was done by G.R., J.H., C.R., and J.R.; the clinical analysis was done by B.B., L.P., and F.L.; the data analysis was done by N.S., L.B., F.L., and L.S.

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Research Article

Overexpression of miR-29a-3p Suppresses Proliferation, Migration, and Invasion of Vascular Smooth Muscle Cells in Atherosclerosis via Targeting TNFRSF1A

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Objective. Increasing evidence highlights the significance of microRNAs (miRNAs) in the progression of atherosclerosis (AS). Our aim was to probe out the role and regulatory mechanism of miR-29a-3p in AS. *Methods*. An in vivo model of AS was conducted by high-fat diet ApoE$^{-/-}$ mice. Oxidized low-density lipoprotein- (ox-LDL-) exposed vascular smooth muscle cells (VSMCs) were utilized as an in vitro of AS. Serum levels of total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were detected. Hematoxylin and eosin (H&E) and Masson's staining was presented to investigate the pathological changes. miR-29a-3p and TNFRSF1A expression was detected by RT-qPCR. Proliferative, migrated, and invaded abilities of VSMCs were determined via a series of assays. The interaction between miR-29a-3p and TNFRSF1A was verified through luciferase reporter assay. *Results*. Upregulated miR-29a-3p and downregulated TNFRSF1A were found both in vitro and in vivo models of AS. miR-29a-3p mimic distinctly decreased the serum levels of TC, TG, and LDL-C and increased serum HDL-C levels. Moreover, its overexpression could ameliorate plaque formation of AS mice. In ox-LDL-induced VSMCs, miR-29a-3p overexpression notably decreased cell proliferation, migration, and invasion, which was reversed by TNFRSF1A overexpression. Also, miR-29a-3p could directly target the 3′UTR of TNFRSF1A. *Conclusion*. miR-29a-3p overexpression ameliorated plaque formation of AS and suppressed proliferation, migration, and invasion of ox-LDL-induced VSMCs via TNFRSF1A, which offered novel insights into the progression of AS.

1. Introduction

Atherosclerosis (AS) is a chronic vascular inflammatory disease related with endothelial dysfunction [1]. Despite the progress made in lifestyle management and medication, total mortality of AS exceeds 50% in developed countries. Thus, there is an urgent need to clarify the molecular mechanisms of AS and to determine effective treatment options. AS is triggered by various risk factors, including the aberrant proliferation of VSMCs. The pathological proliferation of VSMCs has been shown to accelerate AS progression and restenosis of arteries [2]. VSMC dysfunction plays a crucial function in the thickening of atherosclerotic intima. During AS, abnormal proliferation of VSMCs participates in the formation of atherosclerotic plaques [3]. Thus, it is essential to fully understand the behaviors of VSMCs in AS for determining therapeutic targets for the prevention and treatment of AS.

Ox-LDL is a common risk factor for AS, which accelerates the formation of atherosclerotic plaques as well as VSMC migration and proliferation. miRNAs are a class of highly conserved noncoding endogenous RNAs, approximately 22 nucleotides in length. They can bind 3′ untranslated region (3′UTR) of a specific-target mRNA sequence, thereby mediating posttranscriptional gene expression [4]. miRNAs may

be crucial in affecting the function of VSMCs, driving atherosclerotic plaque formation and cholesterol homeostasis. Studies have confirmed that the abnormal expression patterns of several miRNAs promote the progression of AS, such as miR-144 [5], miR-155 [6], and miR-148b [7]. The function of miR-29a-3p in the pathogenesis of cardiovascular diseases (like myocardial fibrosis [8], cardiac ischemia-reperfusion [9], and cardiac hypertrophy [10]) has attracted wide attention. Nevertheless, the role of miR-29a-3p in AS remains unclear.

TNF receptor-1 (TNFRSF1A) has been confirmed to be in association with the progression of various cardiac diseases, including OS. It can mediate endothelial cell dysfunction and inflammation [11]. TNFRSF1A could accelerate the exacerbation of AS [12]. Clinical trial results demonstrated that high circulating TNFR2 level in patients with stable coronary heart disease is in association with increased risk of cardiovascular events and death [13]. Nevertheless, the regulatory mechanism of TNFRSF1A is not well uncovered. In this study, we investigated the role of miR-29a-3p and TNFRSF1A in vitro and in vivo models of AS. Furthermore, the interactions between miR-29a-3p and TNFRSF1A were identified for AS in this study.

2. Materials and Methods

2.1. Animal Models. A total of twenty 8-week-old $ApoE^{-/-}$ male mice (SCXK (Su) 2018-0008) were purchased from Jiangsu Jicui Yaokang Biotechnology Co., Ltd. (Jiangsu, China). High-fat feed was composed of 78% basic feed, 10% egg yolk powder, 2% cholesterol, and 10% lard (TP28522; Nantong Trophy Feed Technology Co., Ltd., Jiangsu, China). All mice were randomly allocated into four groups. Three groups of mice were fed free high-fat diet for 12 weeks. At the same time, the remaining group of mice was fed basic diet normally. All mice were fed freely and kept in an environment with a day-night cycle of 12 hours, a temperature of 22-25°C, and a humidity of 50-70%. At the 10th week of modeling, 15 mice freely fed high-fat diet were randomly divided into 3 groups (AS group, AS+NC mimic group, and AS+miR-29a-3p mimic group). Briefly, mice were injected with 50 μg adenovirus vector NC mimic and miR-29a-3p mimic (General Biotechnology Co., Ltd., Anhui, China) through the jugular vein every other day for 12 weeks and continued to be fed a high-fat diet. By week 12, all mice were euthanized. Peripheral blood was collected, and smooth muscle cells were isolated. Our research was approved by the Animal Ethics Committee of The First Affiliated Hospital of Soochow University (2019033).

2.2. RT-qPCR Assay. $1 - 5 \times 10^7$ cells were lysed by 1 ml TRIzol (R1100, Solarbio, Beijing, China) at room temperature for 5 min. After centrifugation, the precipitate was treated by 0.2 ml chloroform for 2 min. After centrifugation at 12,000g for 15 min at 4°C, the supernatant was harvested. The concentration of RNA extract was then determined according to the OD260/OD280 ratio. RNA was reverse transcribed into cDNA using the reverse transcription kit (11123ES60, YEASEN, Shanghai, China). qPCR was presented using the qPCR kit (11201ES03, YEASEN). The primer sequences were as follows: mmu-miR-29a-3p, 5′-CGTAGCACCATCTGAAATCG-3′ (forward), 5′-GTGCAGGGTCCGAGGT-3′ (reverse); mmu-U6, 5′-CGCAAGGATGACACGCAAAT-3′ (forward), 5′-GCAGGGTCCGAGGTATTC-3′ (reverse); human-miR-29a-3p, 5′-AGCACCAUCUGAAAUCGGUUA-3′ (forward), 5′-GTGCAGGGTCCGAGGT-3′ (reverse); human-U6, 5′-CTCGCTTCGGCAGCACA-3′ (forward), 5′-AACGCTTCACGAATTTGCGT-3′ (reverse); mmu-TNFRSF1A, 5′-GGTCTTTGCCTTCTATCCTTTATC-3′ (forward), 5′-CTTTCCAGCCTTCTCCTCTTTG-3′ (reverse); human-TNFRSF1A, 5′-GAGAATGTTAAGGGCACTGAG-3′ (forward), 5′-CCCACAAACAATGGAGTAGA-3′ (reverse); mmu-actin, 5′-ATGTGCGACGAAGACGAGAC-3′ (forward), 5′-CCTTCTGACCCATACCTACCAT-3′ (reverse); and human-actin, 5′-AATCGTGCGTGACATTAAGGAG-3′ (forward), 5′-ACGTGTTGGCGTAACAGGTCTT-3′ (reverse). The relative expression was calculated with the $2^{-\Delta\Delta Ct}$ method.

2.3. Hematoxylin and Eosin (H&E) and Masson's Staining. Fresh tissue was fixed in 4% paraformaldehyde for over 24 h. After dehydration and paraffin embedding, the tissue was cut to a thickness of 4 μm. After paraffin sections were dewaxed, H&E staining was presented. The sections were stained with hematoxylin (G1004, Servicebio, Wuhan, China) for 5 min and 0.5% eosin solution (G1001, Servicebio) for 2 min. For Masson's staining, the sections were soaked in iron hematoxylin staining solution (G1006-2/3, Servicebio) for 3 min and Lichun acid magenta staining solution (G1006-4, Servicebio) for 5 min. After rinsing with tap water, the sections were stained with phosphomolybdic acid (G1006-5, Servicebio) for 3 min, aniline blue (G1006-6, Servicebio) for 5 min, and differentiated with 1% glacial acetic acid for 1 min. Following dehydration, the sections were mounted with neutral gum (10004160, Sinopharm, Shanghai, China) and placed under an optimal microscope (Nikon Eclipse CI, Nikon, Japan) for observation.

2.4. Biochemical Test. Serum levels of total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were determined using mouse the TG kit (AUTEC), mouse LDL-C kit (LABO, Germany), and HDL-C kit (LABO, Germany), respectively.

2.5. Cell Culture and Treatment. Human VSMCs from umbilical artery from American-type culture collection (ATCC; Rockville, Maryland) were cultured in DMEM (11965092, Gibco, New York, USA) plus 10% fetal bovine serum (10270-106, Gibco) at 37°C with 5% CO_2. VSMCs were treated with different concentrations of ox-LDL (0, 25 μg/ml, 50 μg/ml, and 100 μg/ml). miR-29a-3p mimic, pcDNA3.1-TNFRSF1A, and their controls were purchased from the GenePharma (Shanghai, China). Cells were transfected with 30 nM oligonucleotides via Lipofectamine 2000 (11668019, Thermo, Shanghai, China).

2.6. Cell Counting Kit-8 (CCK-8). Cells were seeded onto a 96-well plate (TCP011096, Guangzhou, China), at the density of 5×10^3 cells/well for 24 h. After discarding the cell culture medium, 100 μl fresh medium containing 0.5% FBS as well as and 10 μl CCK-8 kit (C0038, Beyotime, Beijing, China) was added to each well to each well and incubated at 37°C for 2 h. Optical density value was determined using a microplate reader (RT-6000, KAYTO, USA) at the 450 nm.

2.7. Western Blot. Tissues or cells were lysed with 200 μl of RIPA lysate (BR0002, BEST, Xian, China) plus 1 mM PMSF at 4°C for 30 min. After centrifugation at 12,000 rpm for 10 min at 4°C, the supernatant was harvested and stored at -80°C. The BCA method was used to determine protein concentration using the BCA quantitative kit (P0012, Beyotime, Shanghai, China). Protein samples were subjected to polyacrylamide gel electrophoresis, which were then transferred onto PVDF membrane (IPVH00010, Millipore, Massachusetts, USA). The membrane was blocked by 5% skimmed milk powder at room temperature for 2 h. Then, the membrane was incubated with primary antibodies including Ki67 (ab92742, Abcam), proliferating cell nuclear antigen (PCNA; 10205-2-AP, ProteinTech, Chicago, USA), β-actin (ab8227, Abcam, Cambridge, United Kingdom), matrix metalloproteinase- (MMP-) 2 (10373-2-AP, ProteinTech), MMP9 (ab38898, Abcam), and TNFRSF1A (ab90463, Abcam) at 4°C overnight, followed by goat anti-rabbit IgG-HRP (BK0027, BEST) and goat anti-mouse anti-rabbit IgG-HRP (BK0023, BEST) secondary antibodies at room temperature for 1.5 h. The blots were visualized using the ECL Plus Luminous Kit (S17851, Yeasen, Shanghai, China).

2.8. Wound Healing Assay. Cells were seeded onto a 6-well plate. When the cells were fused 60%-80%, 1% double antibody serum-free medium was replaced. After 12-18 h, a straight line was scratched in the center of each well. After washing twice with phosphate-buffered solution (PBS), DMEM was replaced. The plate was cultured in a 5% CO_2 incubator at 37°C. At 0 h and 48 h, the images were observed and taken.

2.9. Transwell Assay. Cell migration and invasion were determined using the Transwell assay. 600 μl 12% FBS was added to the 24-well plate, followed by Transwell chamber (3422, Thermo, Shanghai, China). Then, 600 μl FBS was added to the lower layer of Transwell chamber, and 4×10^4 cells were added to the upper layer. For cell invasion assay, 100 μg/ml Matrigel (356234, Franklin Lake, New Jersey, USA) was added to the upper layer of chamber. After 24 h of cultivation, the cells in the upper chamber were removed. The sample was fixed with 600 μl fixative solution for 10 min. The chamber was stained with 600 μl crystal violet (G1014, Servicebio, Wuhan, China) for 15 min. Finally, the results were photographed and counted under an inverted microscope (IX51, Olympus, Japan).

2.10. Dual-Luciferase Report. The construction of TNFRSF1A's wild-type (WT) and mutation-type (Mut) vectors was completed by General Bio. The experimental groups were as follows: TNFRSF1A-3′UTR-WT + NC mimic; TNFRSF1A-3′UTR-WT + miR-29a-3p mimic; TNFRSF1A-3′UTR-Mut and NC mimic; and TNFRSF1A-3′UTR-WT + miR-29a-3p mimic. Four to six hours before transfection, the complete medium was replaced with serum-free basic medium, and the cells were starved. 0.8 μg plasmid, 2 μl Lipofectamine 2000 (11668019, Thermo, Shanghai, China), and 20 pmol of miR-29a-3p mimic were cocultured with 50 μl OPTI-MEM (31985088, Gibco, California, United States) for 5 min, respectively. The above solution was mixed and cultured at room temperature for 20 min. Following the removal of the cell culture medium, 450 μl of fresh medium was added to the cells. After 48 h of culture, the cells were lysed by100 μl passive lysis buffer at room temperature for 20 min. In line with the instructions of the Promega dual fluorescence reporter (E1910, Promega, Madison, Wisconsin, USA), the total amount of LAR II and Stop & GLo Reagent reagents was calculated. 10 μl cell lysate was added into the microtiter plate, followed by 50 μl LAR II. The firefly luciferase value was read using GLoMax 20/20 detector (Promega). 50 μl Stop & GLo Reagent was then added, and the Renilla luciferase value was determined. The ratio firefly/Renilla luciferase value was calculated as the dual-luciferase activity.

3. Results

3.1. Overexpression of miR-29a-3p Distinctly Reduces Aorta Plaque Formation for AS Mice. In this study, we conducted an AS model using $ApoE^{-/-}$ male mice. In Figures 1(a) and 1(b), miR-29a-3p expression was distinctly reduced in the serum and aorta vascular tissues of the AS model. As expected, miR-29a-3p mimic notably increased its expression in the AS model. We also found that there was an increased mRNA expression level of TNFRSF1A in the AS model compared to controls (Figure 1(c)). However, for AS mice injected with miR-29a-3p mimic, TNFRSF1A mRNA expression was significantly reduced (Figure 1(c)). Similarly, TNFRSF1A protein expression was significantly elevated in the AS model, which was reversed by miR-29a-3p mimic (Figures 1(d) and 1(e)). Biochemical test results demonstrated that there were higher serum levels of TC (Figure 1(f)), TG (Figure 1(g)), and LDL-C (Figure 1(h)) and lower HDL-C (Figure 1(i)) in the AS model compared to controls. However, after treatment with miR-29a-3p mimic, the serum levels of TC (Figure 1(f)), TG (Figure 1(g)), LDL-C (Figure 1(h)), and HDL-C (Figure 1(i)) were prominently reversed in the AS model. As shown in H&E results, the aorta vascular wall was smooth, and there was no atheromatous plaque in the vascular intima for mice in the control group (Figure 1(j)). In converse, there were obvious plaques in the AS mice. For AS mice treated with miR-29a-3p mimic, plaques were distinctly suppressed compared to those with NC mimic. Masson's staining results demonstrated that there was no atheromatous plaque formation in the vascular intima of the control mice (Figure 1(k)). In the AS model, the intima of the arteries was significantly thickened, and there were fibrous and lipid plaques. Furthermore, the surface of the plaque was covered with collagen fibers, and there were thin fiber cap and few collagen

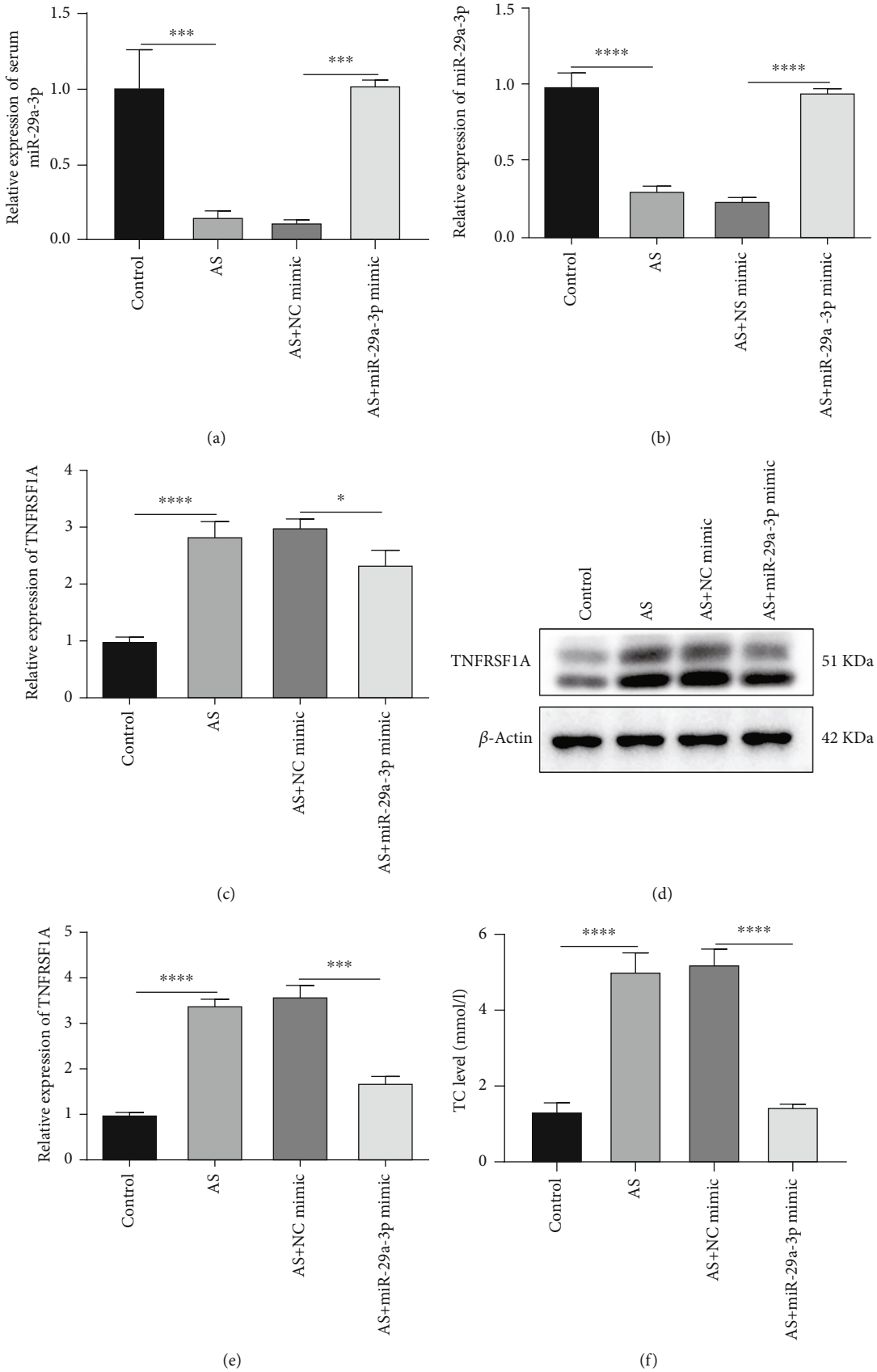


Figure 1: Continued.

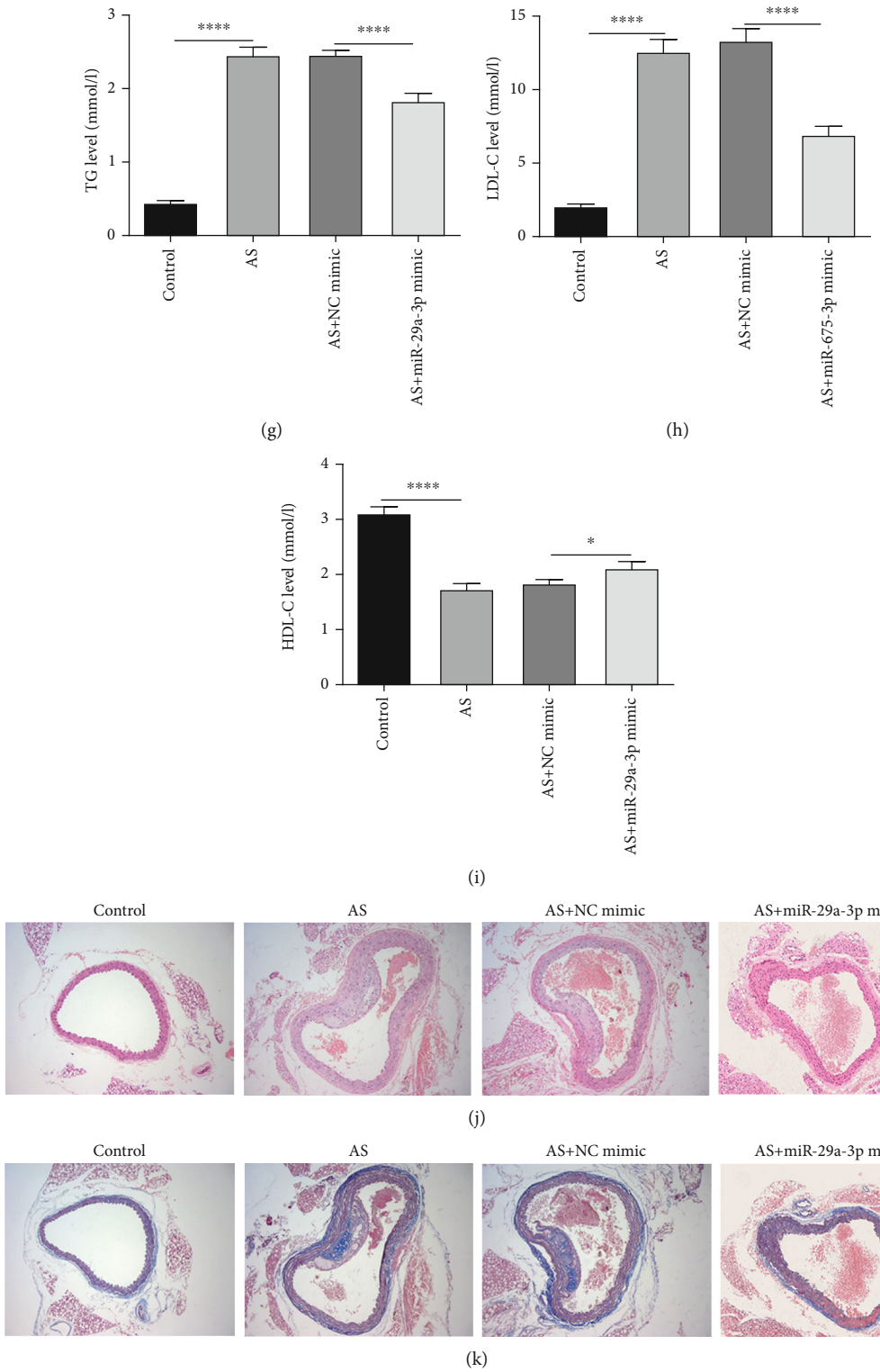


Figure 1: Overexpression of miR-29a-3p distinctly reduces aorta plaque formation for atherosclerosis (AS) mice. (a) Serum miR-29a-3p levels were examined in AS mice with or without miR-29a-3p mimic injection using RT-qPCR. (b, c) RT-qPCR was presented to detect miR-29a-3p as well as TNFRSF1A expression in AS mice with or without miR-29a-3p mimic injection. (d, e) TNFRSF1A protein expression was tested in AS mice with or without miR-29a-3p overexpression via western blot. (f–i) Biochemical test was performed to test the serum levels of total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) in AS mice with or without miR-29a-3p mimic injection. (j) Representative images of H&E staining for aorta of AS mice. (k) Representative images of Masson's staining results for aorta of AS mice. $*p < 0.05$; $***p < 0.001$; $****p < 0.0001$.

fibers for AS mice. Intriguingly, miR-29a-3p mimic injection prominently reduced plaque formation of AS mice.

3.2. Overexpression miR-29a-3p Suppresses Proliferation of ox-LDL-Induced VSMCs in AS. VSMCs were exposed by different concentrations of ox-LDL to induce AS. CCK-8 was utilized the appropriate concentration of ox-LDL. In Figure 2(a), cell viability of VSMCs was notably increased with a concentration manner. Moreover, the expression of miR-29a-3p was significantly reduced with a concentration-dependent manner (Figure 2(b)). When the concentration of ox-LDL was 100 μg/ml, VSMCs had the highest cell viability, and miR-29a-3p expression had the lowest expression level. Thus, 100 μg/ml ox-LDL was determined for further analysis. When induced by 100 μg/ml ox-LDL, the cell viability of VSMCs was distinctly increased, with a time-dependent manner (Figure 2(c)). Also, as time went by, miR-29a-3p expression was gradually reduced (Figure 2(d)). In ox-LDL-induced VSMCs, its expression was elevated after transfection by miR-29a-3p mimic (Figure 2(e)). CCK-8 results suggested that miR-29a-3p mimic prominently suppressed the viability of VSMCs induced by ox-LDL (Figure 2(e)). Furthermore, colony formation assay results showed that the proliferative ability of VSMCs was induced by ox-LDL, which was reversed by miR-29a-3p mimic (Figures 2(g) and 2(h)). We also detected the expression of proliferation-related proteins including Ki67 and PCNA in VSMCs. As expected, the expression of Ki67 and PCNA was significantly increased in VSMCs exposed by ox-LDL, which was distinctly reduced by miR-29a-3p mimic (Figures 2(i) and 2(j)).

3.3. Overexpression of miR-29a-3p Reduces Migration as Well as Invasion of ox-LDL-Induced VSMCs in AS. Wound healing assay results demonstrated that ox-LDL-induced VSMCs had significantly higher migrated ability compared to control (Figures 3(a) and 3(b)). However, miR-29a-3p mimic distinctly suppressed the migrated abilities of VSMCs induced by ox-LDL (Figures 3(a) and 3(b)). As shown in Transwell assay results, ox-LDL exposure notably elevated the migrated and invaded abilities of VSMCs, which were reversed by miR-29a-3p overexpression (Figures 3(c)–3(f)). The expression of MMP9 and MMP2 proteins was examined in VSMCs using western blot. In Figures 3(g) and 3(h), their expression was elevated in VSMCs induced by ox-LDL, which was decreased by miR-29a-3p overexpression. The above findings suggested that miR-29a-3p could inhibit migration and invasion of ox-LDL-induced VSMCs in AS.

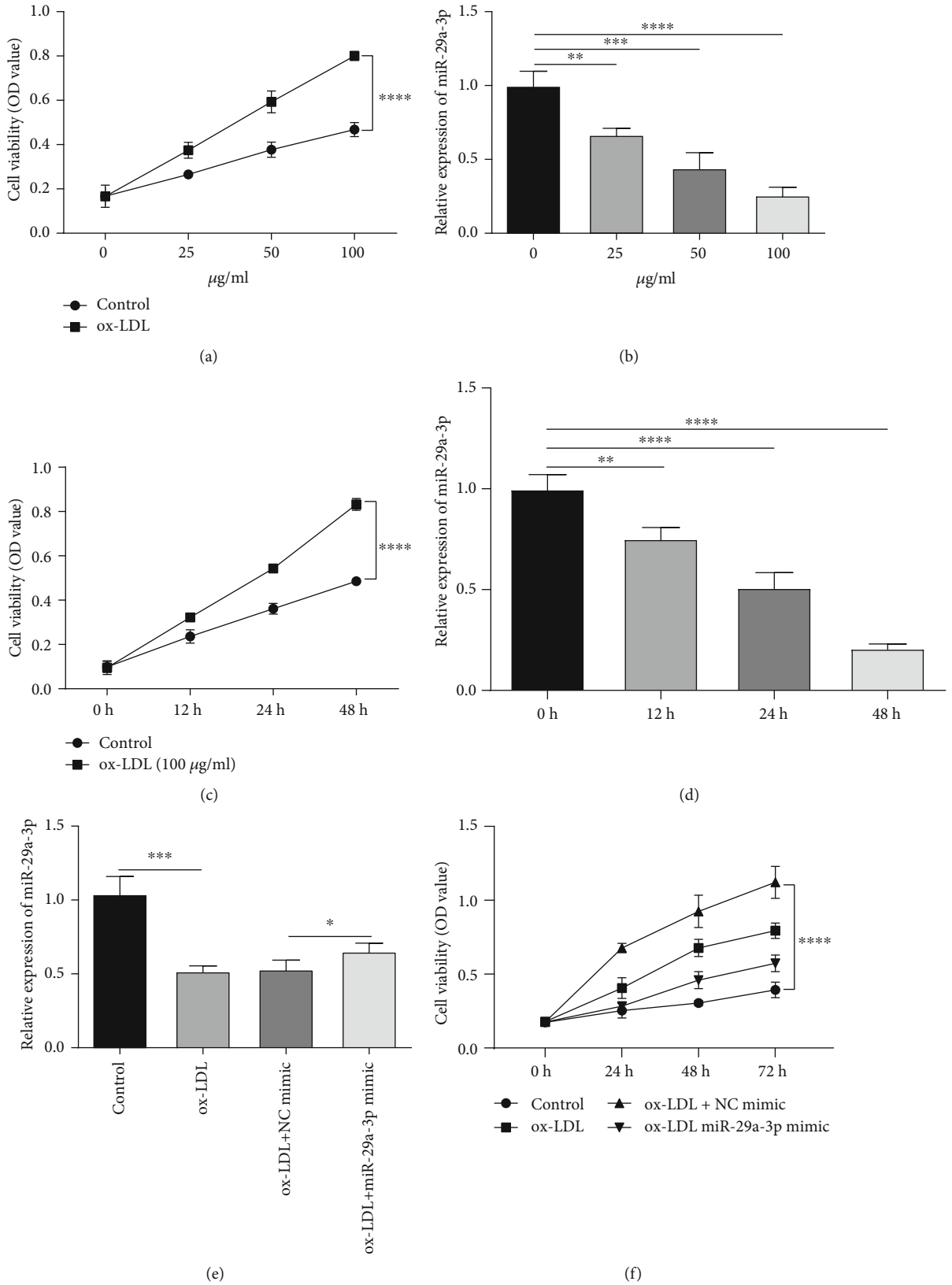
3.4. Overexpression of miR-29a-3p Suppresses Proliferation of ox-LDL-Induced VSMCs by Directly Targeting TNFRSF1A in AS. The mRNA and protein expression levels of TNFRSF1A were remarkably increased in ox-LDL-induced VSMCs compared to controls, which were decreased following miR-29a-3p overexpression (Figures 4(a)–4(c)). The dual-luciferase report confirmed that miR-29a-3p could target the 3′UTR of wide-type TNFRSF1A (Figure 4(d)). miR-29a-3p mimic conspicuously decreased the wide-type TNFRSF1A expression. To further probe out the interaction between miR-29a-3p and TNFRSF1A, TNFRSF1A was successfully overexpressed according to RT-qPCR (Figure 4(e)). CCK-8 results showed that TNFRSF1A overexpression could reverse the reduction of cell viability caused by miR-29a-3p overexpression in ox-LDL-induced VSMCs (Figure 4(f)). Furthermore, colony formation assay results showed that TNFRSF1A overexpression could remarkably changeover the decrease in proliferative abilities by miR-29a-3p overexpression in ox-LDL-induced VSMCs (Figures 4(g) and 4(h)). As expected, miR-29a-3p mimic notably decreased Ki67 and PCNA expression, which was reversed following TNFRSF1A overexpression in ox-LDL-induced VSMCs (Figures 4(i) and 4(j)).

3.5. Upregulation of miR-29a-3p Inhibits Migration as well as Invasion of ox-LDL-Induced VSMCs via Directly Targeting TNFRSF1A in AS. In Figures 5(a) and 5(b), miR-29a-3p overexpression obviously lessened the number of migrated ox-LDL-induced VSMCs, which was reversed following pcDNA3.1-TNFRSF1A. Moreover, TNFRSF1A overexpression could reverse the reduction of the number of invaded ox-LDL-induced VSMCs caused by miR-29a-3p overexpression (Figures 5(c) and 5(d)). The expression of MMP9 and MMP2 proteins was reduced in ox-LDL-induced VSMCs following miR-29a-3p overexpression, which was reversed following pcDNA3.1-TNFRSF1A (Figures 5(e) and 5(f)). These findings revealed that miR-29a-3p overexpression could cut down migration and invasion of ox-LDL-induced VSMCs via directly targeting TNFRSF1A in AS.

4. Discussion

In our study, ApoE$^{-/-}$ mice was used to conduct the AS model using high-fat diet [14, 15]. miR-29a-3p was prominently lowly expressed in the AS mouse model. Increasing evidence suggests that abnormal expression miR-29a-3p is in association with various heart diseases. For example, its expression is upregulated in the right ventricular outflow tract of congenital heart disease patients [16]. Moreover, it suppresses cardiomyocyte proliferation. It has been found that miR-29a-3p could possess the protective function against cardiac fibrosis via inhibition of macrophage migration [17]. Also, upregulation of miR-29a-3p may protect cardiomyocytes from damage induced by hypoxia [18]. Our biochemical test results suggested that serum levels of TC, TG, and LDL-C were distinctly increased, and serum HDL-C levels were prominently decreased in the AS model. Nevertheless, miR-29a-3p mimic treatment improved serum levels of TC, TG, LDL-C, and HDL-C for the AS model. It has been acknowledged that TC, TG, and LDL-C are risk factors and HDL-C is a protective factor for AS [2]. More importantly, H&E and Masson's staining results confirmed that miR-29a-3p overexpression could prominently reduce arterial wall thickening as well as plaque formation of AS mice.

ox-LDL is a key risk factor for AS progression, which can affect the proliferation, migration, and invasion of VSMCs [19]. Thus, in this study, ox-LDL-induced VSMCs were utilized to construct an in vitro AS model. As expected, miR-29a-3p expression was prominently decreased with a concentration and time-dependent manner. Previous studies have shown that proliferative [20], migrated [21], and



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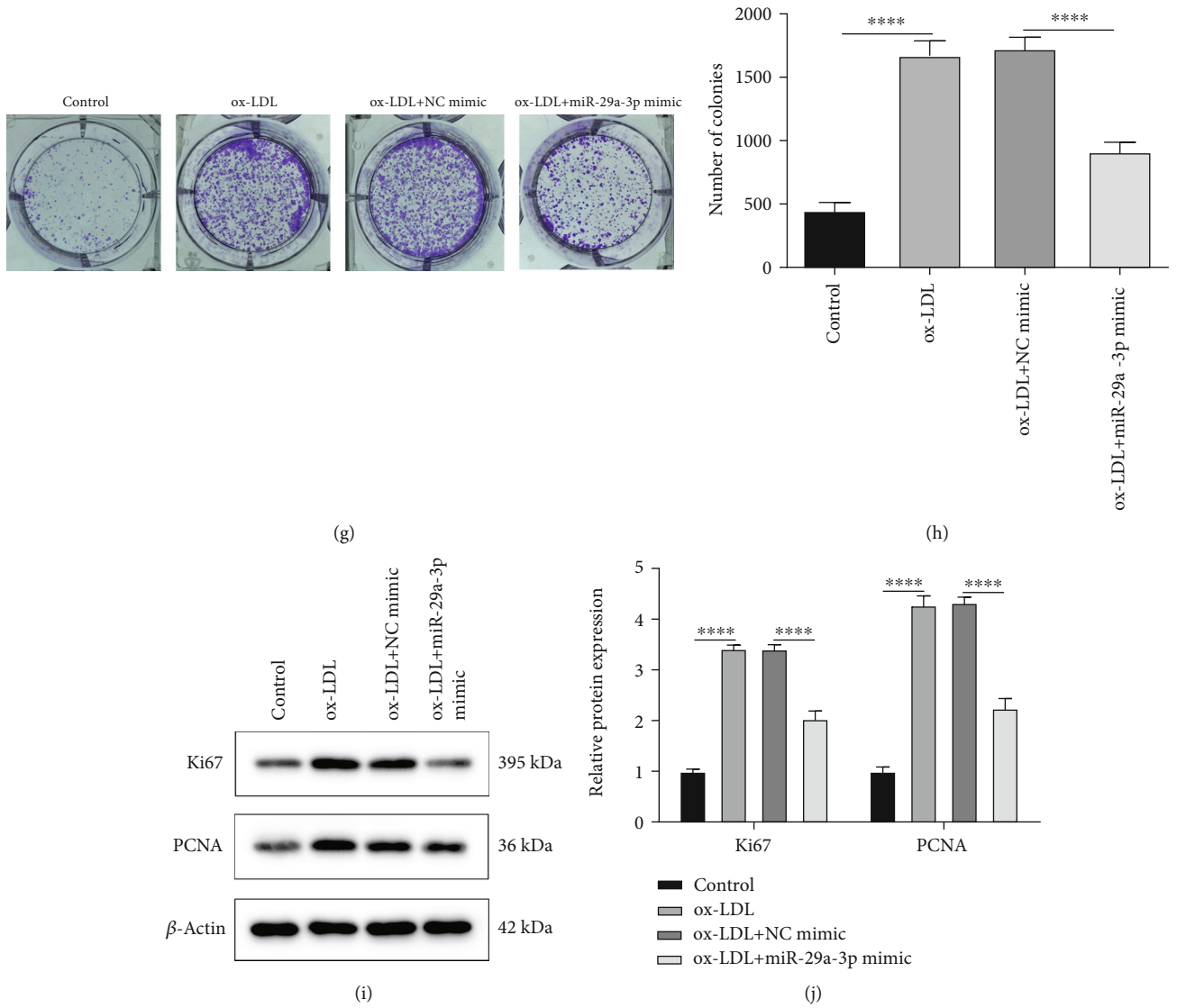
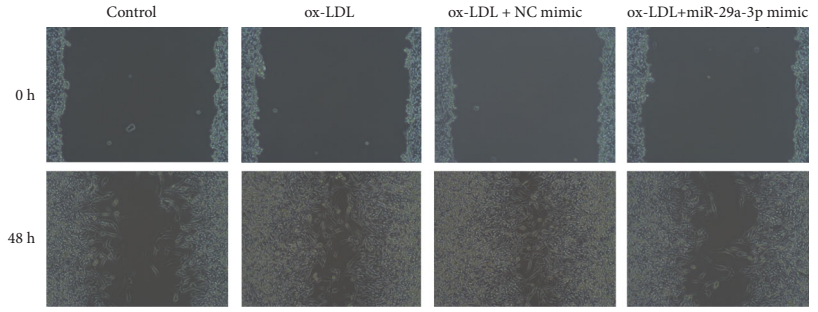


Figure 2: Overexpression miR-29a-3p suppresses proliferation of ox-LDL-induced vascular smooth muscle cells (VSMCs) in AS. (a) CCK-8 assay was used to detect the cell viability of VSMCs under different concentrations of ox-LDL. (b) miR-29a-3p expression was determined in VSMCs induced by different concentrations of ox-LDL by RT-qPCR. (c) The cell viability of VSMCs induced by 100 μg/ml ox-LDL was detected using CCK-8 assay in different time periods. (d) RT-qPCR was presented to examine the expression of miR-29a-3p in VSMCs induced by100 μg/ml ox-LDL in different time periods. (e) miR-29a-3p expression was tested in ox-LDL-induced VSMCs with or without miR-29a-3p mimic transfection by RT-qPCR. (f) CCK-8 assay was presented in ox-LDL-induced VSMCs with or without miR-29a-3p mimic transfection. (g, h) Colony formation assay was used to examine the proliferative ability of ox-LDL-induced VSMCs after transfection with miR-29a-3p mimic or not. (i, j) The expression of Ki67 and PCNA proteins was examined in ox-LDL-induced VSMCs with or without miR-29a-3p mimic transfection. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $***p < 0.0001$.

invaded [22] abilities of VSMCs are closely related to AS progression. After treatment with miR-29a-3p mimic, we found that the cell proliferative ability of VSMCs was suppressed according to CCK-8 and colony formation assays. Also, miR-29a-3p mimic transfection notably decreased the expression of Ki67 and PCNA induced by ox-LDL in VSMCs. Ki67 and PCNA are signs of cell proliferation and mediate DNA replication [23]. Our wound healing and Transwell assay results showed that miR-29a-3p mimic distinctly inhibited the migrated and invaded abilities of VSMCs induced by ox-LDL. Also, miR-29a-3p overexpression suppressed the expression of MMP9 and MMP2 proteins in VSMCs induced by ox-LDL. During the formation of atherosclerotic plaques, VSMCs migrate from the medium to the intima. VSMCs secrete MMPs, a family of zinc-dependent endopeptidases in the intima [24]. MMPs can promote the degradation of the extracellular matrix (ECM) as well as migration of VSMCs [25].

TNFRSF1A mRNA and protein expression was notably elevated both in the AS mouse model and ox-LDL-induced VSMCs, consistent with a previous study [26]. Intriguingly, after treatment with miR-29a-3p mimic, TNFRSF1A expression was remarkably inhibited. Emerging evidence suggests that miRNAs function via mediating the translation or



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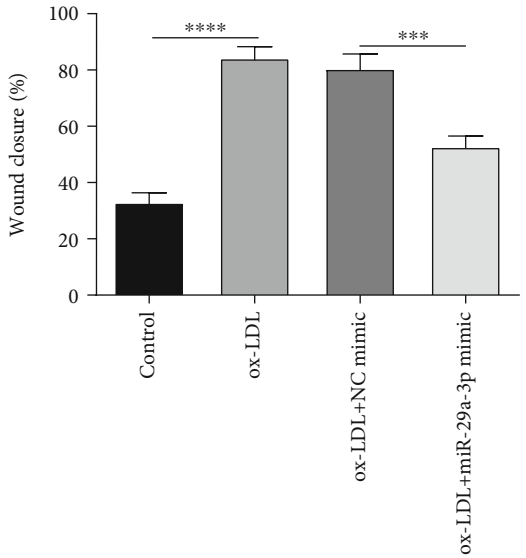

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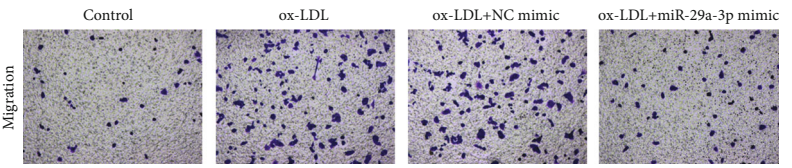

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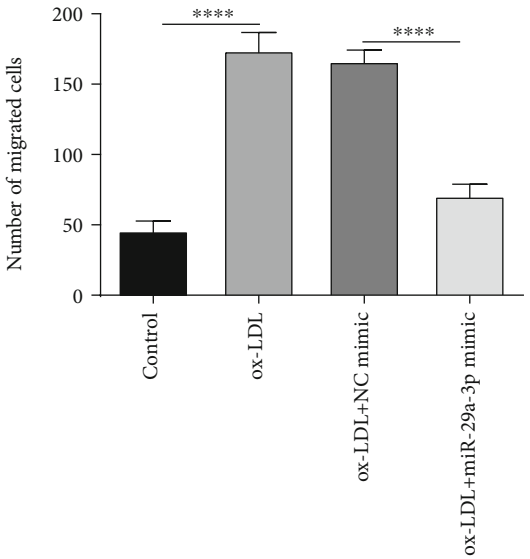

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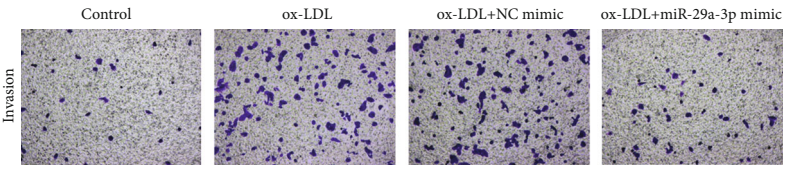

(e)

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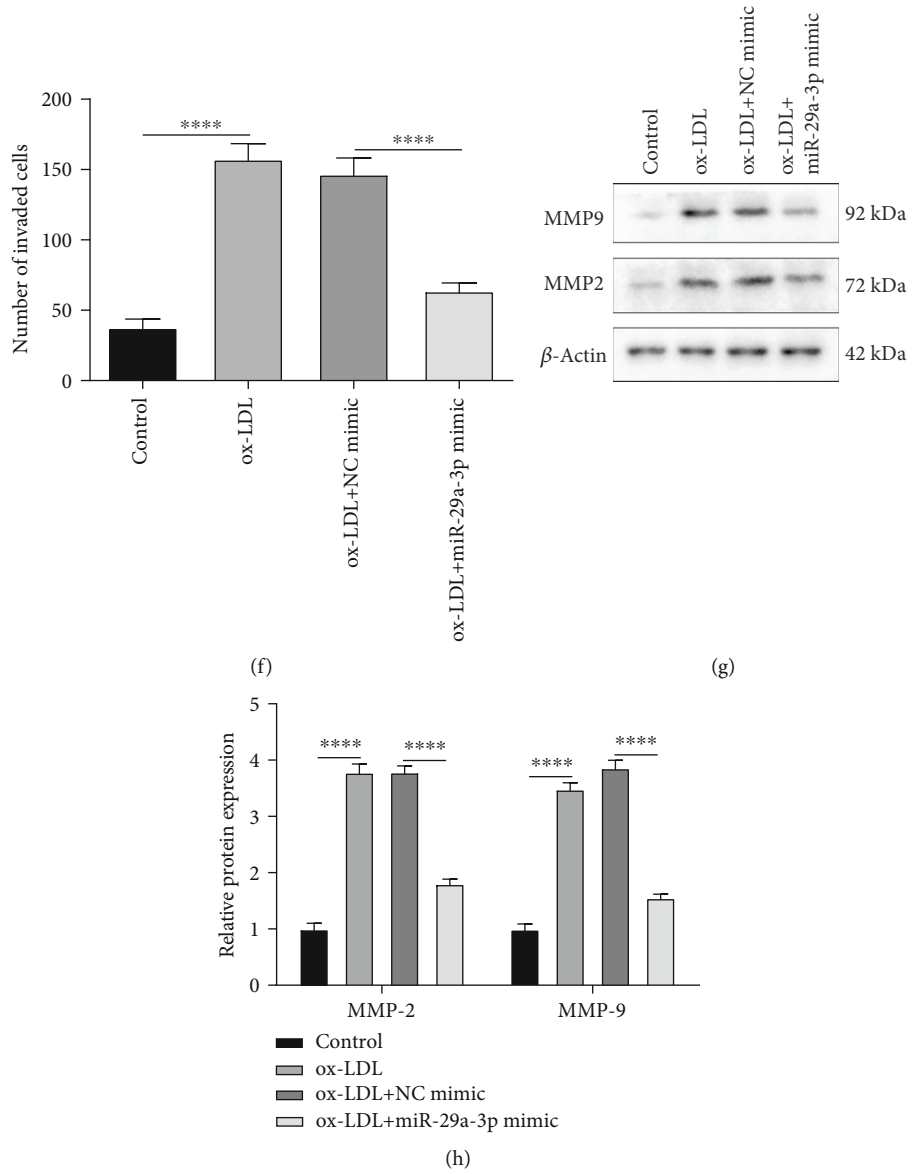
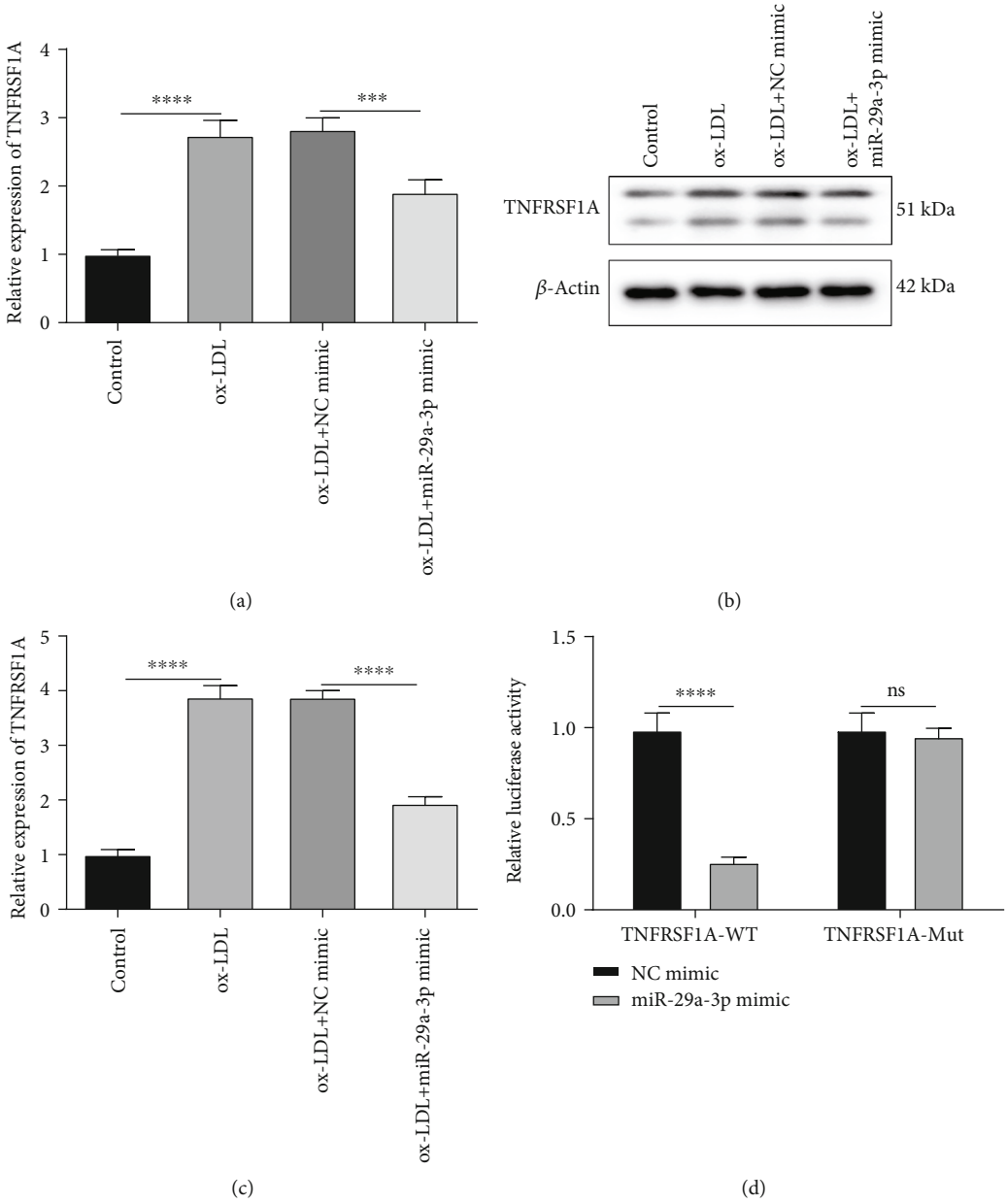


Figure 3: Overexpression of miR-29a-3p inhibits migration and invasion of ox-LDL-induced vascular smooth muscle cells (VSMCs) in AS. (a, b) Wound healing assay was utilized to examine the migrated ability of ox-LDL-induced VSMCs with or without miR-29a-3p mimic transfection. (c–f) The migrated and invaded abilities of VSMCs were determined using Transwell assays. (g, h) Western blot was presented to investigate the expression of MMP9 and MMP2 proteins in ox-LDL-induced VSMCs with or without miR-29a-3p mimic. $***\ p < 0.001$; $***\ *p < 0.0001$.

stability of target mRNA [27–29]. As previous studies, TNFRSF1A could be mediated by microR-29c, thereby reducing poststroke depression [30]. Furthermore, TNFRSF1A regulated by miR-29a promotes AR42J cell apoptosis [31]. Our dual-luciferase report confirmed that TNFRSF1A could be targeted by miR-29a-3p. Further analysis found that TNFRSF1A overexpression could reverse the reduction of cell proliferative, migrated, and invaded abilities by miR-29a-3p overexpression in ox-LDL-induced VSMCs. Also, its upregulation distinctly inhibited the expression of Ki67, PCNA, MMP9, and MMP2 proteins, which were reversed by TNFRSF1A overexpression in ox-LDL-induced VSMCs.

However, several limitations of this study need to be pointed out. AS is a complex disease. Plaque progression involves different stages, from early atherosclerotic lesions to advanced lesions. During this process, the molecular players involved change continuously; the same can be true for potential target genes of miRNAs. Thus, further large-scale studies are needed to in-depth clarify the specific treatment mechanism of AS and transfer these findings to clinical research. Taken together, we conducted an in vivo model of AS by high-fat diet ApoE$^{-/-}$ mice and an in vitro model by ox-LDL-exposed VSMCs. miR-29a-3p overexpression could improve plaque formation of AS and inhibit proliferation, migration,



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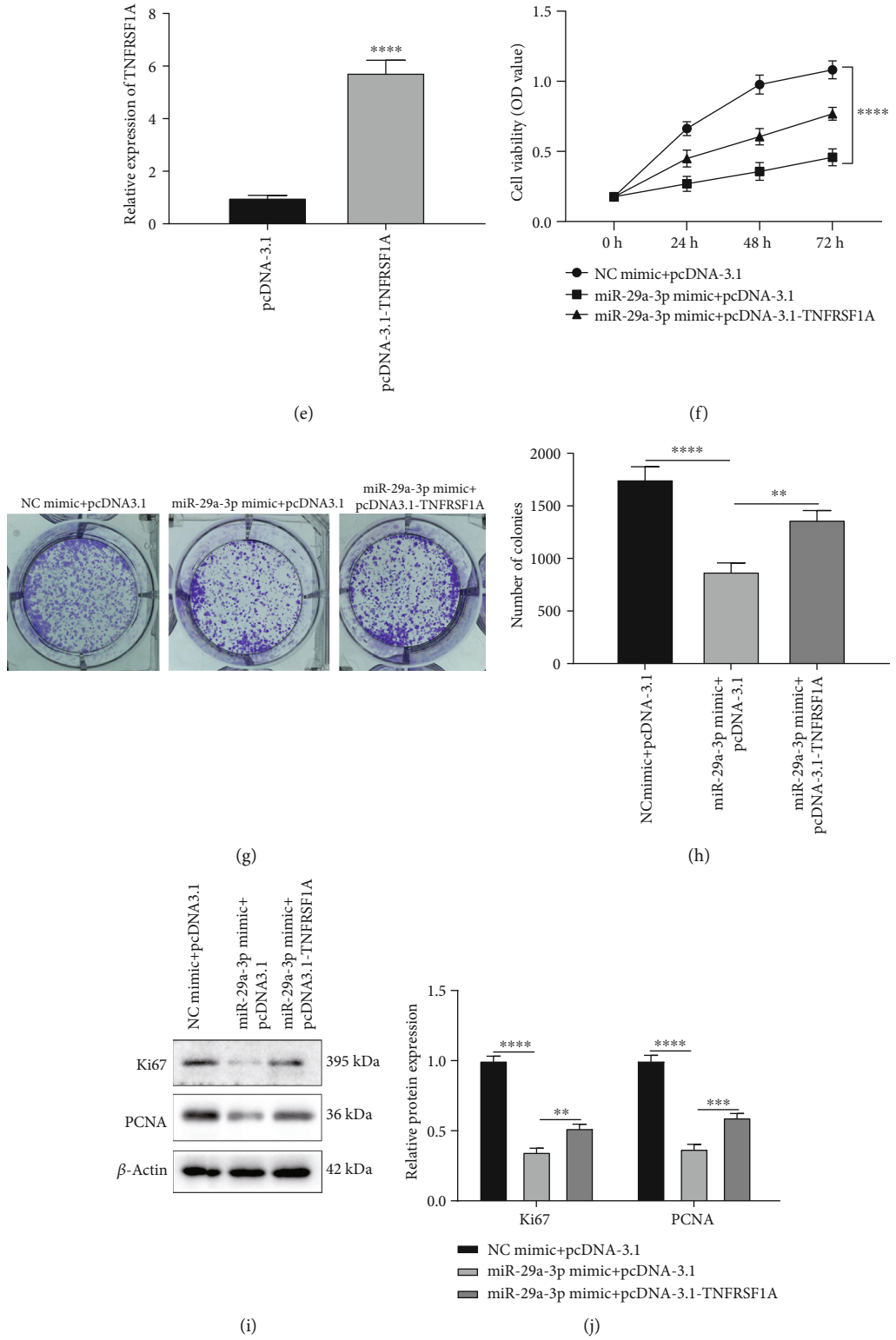

Figure 4: Overexpression of miR-29a-3p decreases proliferation of ox-LDL-induced vascular smooth muscle cells (VSMCs) by directly targeting TNFRSF1A in AS. (a–c) TNFRSF1A mRNA and protein expression was detected in ox-LDL-induced VSMCs with or without miR-29a-3p mimic injection using RT-qPCR and western blot. (d) Dual-luciferase report verified the direct interaction between miR-29a-3p and TNFRSF1A. (e) RT-qPCR was utilized to validate the transfection effect of pcDNA3.1-TNFRSF1A in ox-LDL-induced VSMCs. (f) CCK-8 assay was presented to examine the cell viability of ox-LDL-induced VSMCs injected with miR-29a-3p mimic and/or pcDNA3.1-TNFRSF1A. (g, h) Cell proliferation of ox-LDL-induced VSMCs was detected following transfection with miR-29a-3p mimic and/or pcDNA3.1-TNFRSF1A through colony formation assay. (i, j) Ki67 and PCNA expression in ox-LDL-induced VSMCs transfected with miR-29a-3p mimic and/or pcDNA3.1-TNFRSF1A. $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$; ns: no statistical significance.

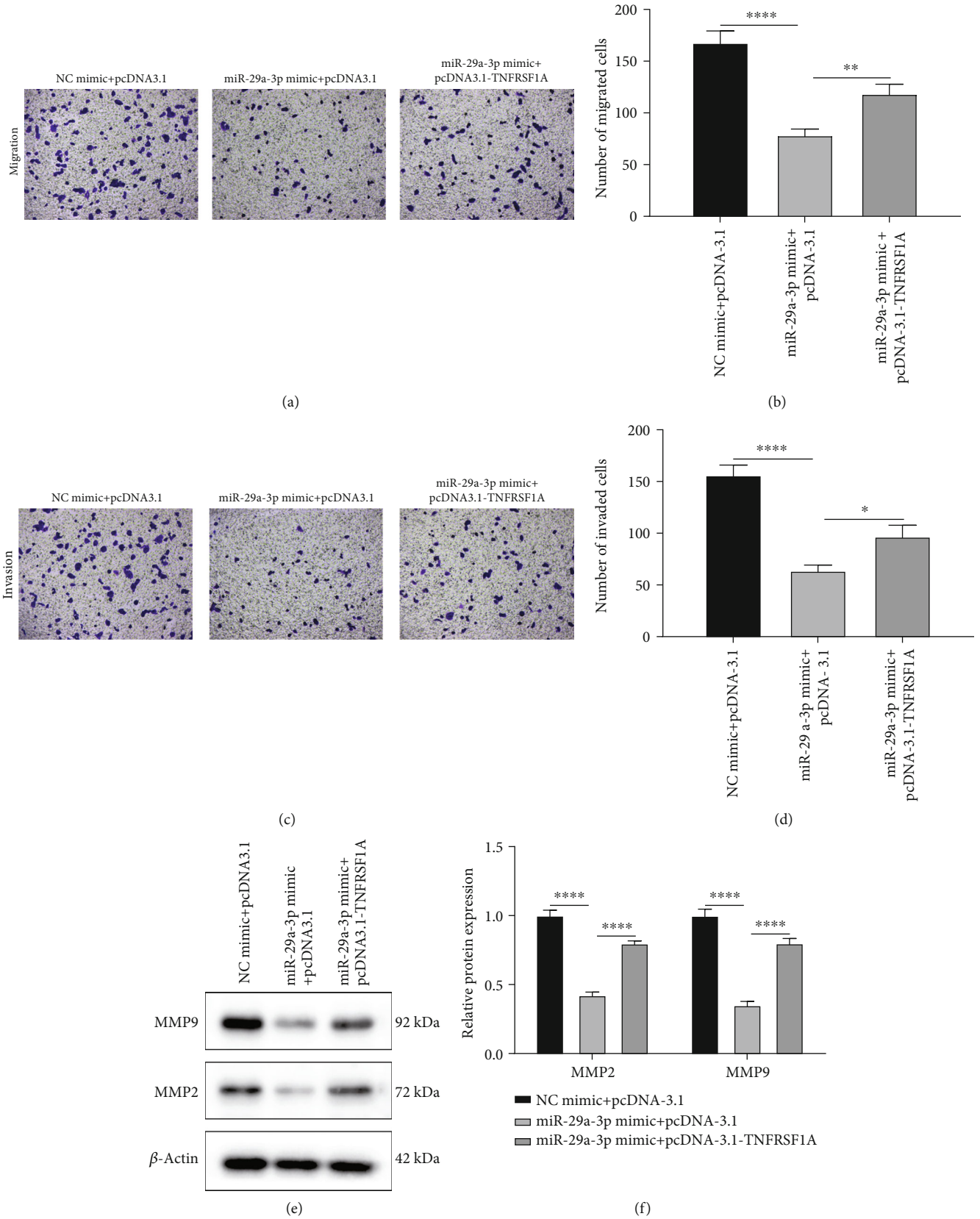

FIGURE 5: Upregulated miR-29a-3p inhibits migration as well as invasion of ox-LDL-induced vascular smooth muscle cells (VSMCs) via directly targeting TNFRSF1A in AS. (a–d) Transwell assays were carried out to examine the number of migrated and invaded ox-LDL-induced VSMCs in ox-LDL-induced VSMCs transfected with miR-29a-3p mimic and/or pcDNA3.1-TNFRSF1A. (e, f) MMP9 and MMP2 protein expression in ox-LDL-induced VSMCs. $*p < 0.05$; $**p < 0.01$; $*** *p < 0.0001$.

and invasion of ox-LDL-induced VSMCs via TNFRSF1A. The function of miR-29a-3p in AS is worthy of further analysis.

5. Conclusion

In this study, miR-29a-3p was downregulated and TNFRSF1A was upregulated both in the AS mouse model and ox-LDL-induced VSMCs. miR-29a-3p overexpression reduced plaque formation of AS mice. It could directly target the 3′UTR of TNFRSF1A. Moreover, miR-29a-3p overexpression suppressed cell proliferation, migration, and invasion of ox-LDL-induced VSMCs, which was reversed by TNFRSF1A overexpression. Thus, our findings could deepen the understanding about the molecular mechanisms of OS.

Abbreviations

miRNAs:	MicroRNAs
AS:	Atherosclerosis
ox-LDL:	Oxidized low-density lipoprotein
VSMCs:	Vascular smooth muscle cells
H&E:	Hematoxylin and eosin
3′UTR:	3′ untranslated region
TNFRSF1A:	TNF receptor-1
TG:	Triglyceride
LDL-C:	Low-density lipoprotein cholesterol
HDL-C:	High-density lipoprotein cholesterol
CCK-8:	Cell counting kit-8.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflicts of interest.

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Research Article

The Association between Hyperhomocysteinemia and Thoracoabdominal Aortic Aneurysms in Chinese Population

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Objective. To shed light on the association between hyperhomocysteinemia (HHcy) and thoracoabdominal aortic aneurysms (TAAAs). *Methods.* From July 2013 to March 2017, we conducted a matched case–control study involving individuals who presented to the Chinese People's Liberation Army General Hospital and underwent thoracoabdominal magnetic resonance angiography or computed tomography angiography. A total of 73 patients with TAAAs were enrolled in the case group, and 219 sex-matched subjects without TAAAs were included in the control group. We then examined the relationship between HHcy and TAAAs by logistic regression models and subgroup as well as interaction analyses. *Results.* Serum total homocysteine (tHcy) concentrations and the proportion of HHcy were significantly higher in the patients with TAAAs than in those without TAAAs ($P < 0.001$). Furthermore, the multivariate logistic regression models indicated that participants with HHcy had a 2.14-fold higher risk of TAAAs than those with a normal serum tHcy level (adjusted odds ratio (OR), 2.14; 95% confidence interval, 1.00–4.56). Similarly, each 1 μmol/L increase in the serum tHcy concentration was associated with a 4% higher risk of TAAAs (adjusted OR, 1.04; 95% confidence interval, 1.00–1.07). Subgroup analyses indicated that HHcy tended to be associated with a greater risk of TAAAs in all stratified subgroups (adjusted ORs > 1). Furthermore, the interaction analyses revealed no interactive role in the association between HHcy and TAAAs. *Conclusions.* The present case–control study suggests that HHcy is an independent risk factor for TAAAs. Larger prospective cohort studies are warranted to validate these findings.

1. Introduction

A thoracoabdominal aortic aneurysm (TAAA) is defined as a permanent and continuous dilatation of the descending thoracic aorta and abdominal aorta [1]. Although TAAAs reportedly have a low incidence of 5.9 cases per 100,000 inhabitants annually [2], surgical repair of TAAAs is associated with a perioperative mortality rate ranging from 7.5% to 46.1% [3, 4]. In addition, one study showed that the 5- and 10-year survival rates of patients with TAAAs undergoing open repair were 63.6% and 36.8%, respectively [3], indicating that the prognosis of patients with TAAAs is far from satisfactory. Consequently, it is essential to decrease the incidence of TAAAs by discovering and controlling their risk factors.

Many patients with TAAAs are asymptomatic, and these TAAAs may remain undiagnosticated for years [5]. The risk of rupture increases with the aortic diameter, and the risk dramatically increases at diameters > 7 cm [1, 5]. Most ruptured TAAAs are catastrophic. Among patients with ruptured TAAAs who are able to undergo surgical repair, the operative mortality rate is reportedly as high as 46.1%, which is significantly higher than that of unruptured TAAA repair (15.9%) [4]. Therefore, the natural history of TAAAs causes many patients to easily miss the optimal timing of intervention. Identifying the biomarkers that indicate the presence

of TAAAs might benefit early detection and intervention and thus improve the prognosis.

The interaction between media degeneration of the aortic wall and hemodynamic tension leads to the majority of TAAAs [1]. During the last few decades, hypertension, hyperlipidemia, obesity, smoking, a family history, and connective tissue diseases have been described as risk factors for TAAAs [1, 6], indicating that many risk factors for TAAAs are similar to those for atherosclerosis. Hyperhomocysteinemia (HHcy), defined as an elevated serum total homocysteine (tHcy) level, is associated with atherosclerotic diseases and intracranial aneurysms as well as abdominal aortic aneurysms (AAAs) [7–13]. However, no study has been performed to investigate the association between HHcy and TAAAs. Therefore, we performed a matched case–control study to explore the relationship between the serum tHcy level and TAAAs. We also performed subgroup and interaction analyses to detect the interactions of HHcy with other conventional factors in the association with TAAAs.

2. Patients and Methods

2.1. Study Design and Population. From July 2013 to March 2017, we conducted a matched case–control study involving individuals who presented to the Chinese People's Liberation Army General Hospital and underwent thoracoabdominal magnetic resonance angiography or computed tomography angiography. Consecutive patients who were newly diagnosed with TAAAs in the Department of Vascular and Endovascular Surgery were enrolled in the case group. These patients were matched 1 : 3 by sex with controls without TAAAs in the Health Management Center and were recruited during the same period (within 3 months). Patients with TAAAs caused by aortic dissection, trauma, or infection were excluded from the case group. The other exclusion criteria for all subjects were a history of vitamin B, folate, or antiepileptic medication use; a history of contraception and/or hormone replacement therapy; a chronic history of connective tissue disease (such as the Marfan syndrome, Loeys–Dietz syndrome, or Ehlers–Danlos syndrome), Takayasu arteritis, or aortitis; a history of serum tHcy-associated disease such as a malignant tumor or hypothyroidism; a history of mental disorders or diseases; and pregnancy. Our study was approved by the Ethics Committee of the Chinese People's Liberation Army General Hospital. We obtained a written informed consent from all participants, and all procedures in our study adhered to the principles of the Declaration of Helsinki.

Finally, 292 participants met the criteria (73 in the case group and 219 in the control group) during the study period and were recruited in our study.

2.2. Data Collection. All subjects underwent a medical consultation, standard physical examination. A uniform questionnaire was administered to record the participants' demographic characteristics and medical history, including age, sex, body mass index (BMI), smoking and drinking habit, hypertension, diabetes mellitus, hyperlipidemia, ischemic stroke, and coronary artery disease (CAD). The subjects filled out the questionnaire according to the guidance provided by trained doctors. A smoking habit was defined as consuming ≥100 cigarettes throughout life, and a drinking habit was defined as drinking ≥50 mL of alcohol per week for 6 months [8]. Hypertension was defined as receiving antihypertensive treatment or having a systolic blood pressure of ≥140 mmHg or diastolic blood pressure of ≥90 mmHg. Diabetes mellitus was defined as a glycated hemoglobin level of ≥6.5% or current treatment with medication for diabetes. Moreover, hyperlipidemia was defined as either the use of hypolipidemic agents or a serum total cholesterol concentration of >5.7 mmol/L, triglyceride concentration of >1.7 mmol/L, low-density lipoprotein cholesterol (LDL) concentration of >3.4 mmol/L, and high-density lipoprotein cholesterol (HDL) concentration of <1.0 mmol/L [8]. The medical histories of CAD and ischemic stroke were self-reported and confirmed by the subjects' families.

All subjects underwent blood biochemical tests for either a preoperative evaluation or health check-up. We retrieved the value of the serum tHcy, glucose, creatine, uric acid, total cholesterol, triglyceride, HDL, and LDL concentrations from the medical records. The blood biochemical test was performed as follows. After an 8-hour overnight fast, blood samples were withdrawn from the subjects and placed in a refrigerator at 4°C. The serum was centrifuged for separation within 1 hour and then stored at −80°C until analysis. All biochemical parameters were measured using a Roche Modular chemistry analyzer (Roche Diagnostics, Basel, Switzerland) at the clinical laboratory of our hospital. The intra- and interassay coefficients of variation were <5% for all of the assays performed. The estimated glomerular filtration rate (eGFR) was calculated by the Chronic Kidney Disease Epidemiology Collaboration equation based on the serum creatinine concentration [14].

2.3. Statistical Analysis. To minimize selection bias that might occur if we had excluded participants with missing data on BMI, LDL, HDL, glucose, uric acid, or creatine (<1%), we used multivariate imputation by chained equations to impute missing values. Five datasets were generated, and the results were pooled according to Rubin's rules. Categorical variables are summarized as frequency and percentage, and continuous variables are presented as mean and standard deviation. The Chi-squared test or Mann–Whitney test was used to compare the distributions of demographic and clinical characteristics between the TAAAs and control groups. Next, we performed a univariable logistic regression analysis to detect the relationship between TAAAs and conventional risk factors including age, sex, smoking and drinking habit, diabetes, hypertension, CAD, ischemic stroke, hyperlipidemia, eGFR, and BMI. Continuous parameters including age, eGFR, and BMI were transformed into categorical variables based on the recognized clinical cutoff points or laboratory reference ranges in the logistic regression models. HHcy was defined as a circulating tHcy level ≥ 15 μmol/L [15]. Next, the independent association between the serum tHcy level (as either a categorical variable or a nontransformed continuous variable as well as a log(e)-transformed continuous variable because the distribution of

the tHcy level was found to be skewed toward the left) and TAAAs was determined by two different multivariable logistic regression models. Adjusted odds ratios (ORs) with corresponding 95% confidence intervals (CIs) were estimated. The minimally logistic regression model was adjusted for sex and age. The fully adjusted model was adjusted for sex, age, smoking habit, hypertension, BMI, and eGFR (variable changing the coefficient of the continuous tHcy level by ≥10% when adding it into the basic logistical model including only the serum tHcy level or when deleting it from the complete logistical model comprising all variables in the abovementioned univariable logistic regression analysis). Finally, interaction and subgroup analyses were performed, including sex, age, smoking habit, hypertension, BMI, and eGFR.

All statistical analyses were performed with the software packages R (http://www.R-project.org, The R Foundation, Vienna, Austria) and EmpowerStats (http://www.empowerstats.com, X&Y Solutions, Inc., Boston, MA, USA). A two-tailed P value of < 0.05 was considered statistically significant.

3. Results

3.1. Participants' Baseline Characteristics. The subjects' baseline characteristics are summarized in Table 1. Generally, patients with TAAAs were slightly older (64.59 ± 12.73 years vs. 59.29 ± 7.92 years, $P < 0.001$), and there were higher proportions of smokers (57.53% vs. 19.18%, $P < 0.001$), drinkers (34.25% vs. 18.72%, $P < 0.01$), those with hypertension (68.49% vs. 32.42%, $P < 0.001$), and those with a chronic medical history of CAD(23.29% vs. 9.59%, $P < 0.01$) compared with individuals in the control group. The rates of patients with a chronic medical history of diabetes, hyperlipidemia, and ischemic stroke were not significantly different between the two groups. Additionally, a significantly lower BMI ($24.22 \pm 3.78\,kg/m^2$ vs. $26.07 \pm 3.33\,kg/m^2$, $P < 0.001$), serum HDL concentration (1.12 ± 0.32 mmol/L vs. 1.21 ± 0.32 mmol/L, $P < 0.05$), and eGFR (97.40 ± 30.21 mL/min per $1.73\,m^2$ vs. 112.05 ± 22.53 mL/min per $1.73\,m^2$, $P < 0.001$) were observed in patients with TAAAs, whereas there were no statistically significant differences in the blood pressure, serum concentrations of cholesterol, triglycerides, LDL, glucose, or uric acid between the two groups.

3.2. HHcy and tHcy Levels. HHcy was found in 57 of 73 patients with TAAAs (78.08%) and 116 of 219 participants without TAAAs (52.97%), as is shown in Figure 1(a). The proportion of HHcy was significantly greater in the case than the control group ($P < 0.001$). Likewise, the serum tHcy level was significantly higher in the patients with TAAAs (median, 19.40; interquartile range, 16.50–23.10) than in those without TAAAs (median, 15.20; interquartile range, 11.60–19.90) with a P value of < 0.001 (Figure 1(b)).

3.3. Relationship between tHcy Level and TAAAs. The univariate logistic regression analyses indicated that age, hypertension, CAD, smoking and drinking habit, BMI, and eGFR were significantly associated with the presence of TAAAs (Table 2). The results of the multivariate logistic regression models are shown in Table 3. After adjustment for confounders, the serum tHcy level was independently associated with the risk of TAAAs in different multivariate logistic regression models (as either a categorical variable or continuous variable). Subjects with HHcy had a 2.14-fold higher risk of TAAAs than those with a normal serum tHcy level (adjusted OR, 2.14; 95% CI, 1.00–4.56). Similarly, each 1 μmol/L increase in the serum tHcy concentration was associated with a 4% higher risk of TAAAs (adjusted OR, 1.04; 95% CI, 1.00–1.07). The relationship remained significant when the log(e)-transformed tHcy level was used in the multivariate logistic regression model (adjusted OR, 2.98; 95% CI, 1.36–6.53).

The results of the stratified and interaction analyses are presented in Table 4. The stratified analysis indicated no statistically significant association between HHcy and TAAAs in all stratified subgroups. Nevertheless, HHcy tended to be associated with a greater risk of TAAAs in all stratified subgroups (adjusted ORs > 1). Furthermore, the interaction analysis revealed no interactive role in the association between HHcy and TAAAs.

4. Discussion

4.1. Brief Discussion of Main Results and Implications. This paper describes the first case–control study to address the association between the serum tHcy level and risk of TAAAs. Our main finding is that an elevated serum tHcy level is independently associated with a higher risk of TAAAs. This association was robust when the serum tHcy concentration was included as either a nontransformed or log(e)-transformed continuous variable or a categorical variable in the same multivariate logistic regression model and when different potential confounders were adjusted in two multivariate logistic regressions. The stratified analyses revealed that HHcy was associated with a greater risk of TAAAs in all stratified subgroups (adjusted ORs > 1), although not statistically significant, which may have been a result of the limited sample size after stratification. Furthermore, no interactive role was found in the association between HHcy and TAAAs. Our main finding might have certain implications in identifying individuals at higher risk of developing TAAAs and tailoring more aggressive prevention. In addition, our results may help to detect higher risk among patients who already have TAAAs to provide earlier diagnostic strategies and treatments that can improve their prognosis.

4.2. Mechanisms. Homocysteine is a thiol-containing nonessential amino acid derived from the metabolism of the essential amino acid methionine [13]. Homocysteine can be degraded via the remethylation pathway in which 5-methyltetrahydrofolate is the substrate, employing vitamin B_{12} and folate as cofactors. It can also be converted into cysteine via the transsulfuration pathway catalyzed by cystathionine β synthase and vitamin B_6 [16]. Consequently, the homocysteine level is affected by the folate and vitamin status. Previous studies have proven that an elevated serum tHcy level is associated with atherosclerotic diseases and intracranial aneurysms as well as AAAs [7–10, 13,

Table 1: Baseline characteristics of the study population.

Variables	All subjects	Control	TAAA	*P* value
N	292	219	73	
Age (years)	60.62 ± 9.61	59.29 ± 7.92	64.59 ± 12.73	<0.001
Sex				1.00
Female	36 (12.33%)	27 (12.33%)	9 (12.33%)	
Male	256 (87.67%)	192 (87.67%)	64 (87.67%)	
Smoking habits				<0.001
Yes	84 (28.77%)	42 (19.18%)	42 (57.53%)	
No	208 (71.23%)	177 (80.82%)	31 (42.47%)	
Drinking habits				0.006
Yes	66 (22.60%)	41 (18.72%)	25 (34.25%)	
No	226 (77.40%)	178 (81.28%)	48 (65.75%)	
Hypertension				<0.001
Yes	121 (41.44%)	71 (32.42%)	50 (68.49%)	
No	171 (58.56%)	148 (67.58%)	23 (31.51%)	
Diabetes				1.00
Yes	21 (7.19%)	16 (7.31%)	5 (6.85%)	
No	271 (92.81%)	203 (92.69%)	68 (93.15%)	
Hyperlipidemia				0.95
Yes	149 (51.03%)	112 (51.14%)	36 (49.32%)	
No	143 (48.97%)	107 (48.86%)	37 (50.68%)	
Ischemic stroke				0.098
Yes	19 (6.51%)	11 (5.02%)	8 (10.96%)	
No	273 (93.49%)	208 (94.98%)	65 (89.04%)	
CAD				0.004
Yes	38 (13.01%)	21 (9.59%)	17 (23.29%)	
No	254 (86.99%)	198 (90.41%)	56 (76.71%)	0.12
DBP (mmHg)	135.39 ± 17.90	134.15 ± 16.36	139.12 ± 21.57	
SBP (mmHg)	81.39 ± 9.58	81.42 ± 8.60	81.33 ± 12.11	0.58
Glucose (mmol/L)	5.18 ± 1.37	5.20 ± 1.47	5.11 ± 1.05	0.37
BMI (kg/m^2)	25.61 ± 3.53	26.07 ± 3.33	24.22 ± 3.78	<0.001
eGFR (mL/min per 1.73 m^2)	108.39 ± 25.45	112.05 ± 22.53	97.40 ± 30.21	<0.001
Urid acid (*u*mol/L)	338.81 ± 86.81	333.58 ± 81.67	354.49 ± 99.64	0.35
Cholesterol (mmol/L)	4.50 ± 0.94	4.52 ± 1.00	4.42 ± 0.75	0.87
Triglyceride (mmol/L)	1.45 ± 1.06	1.41 ± 0.94	1.56 ± 1.36	0.84
HDL (mmol/L)	1.19 ± 0.32	1.21 ± 0.32	1.12 ± 0.32	0.03
LDL (mmol/L)	2.79 ± 0.76	2.81 ± 0.80	2.74 ± 0.64	0.70

Abbreviations: TAAA: thoracoabdominal aortic aneurysm; CAD: coronary heart disease; DBP: diastolic blood pressure; SBP: systolic blood pressure; BMI: body mass index; eGFR: estimated glomerular filtration rate; HDL: high-density lipoprotein cholesterol; LDL: low-density lipoprotein cholesterol. For the continuous variables, the *P* value was calculated using the Mann–Whitney test. For the count variable, if there is a theoretical number < 10, the *P* value was calculated using the Fisher exact probability test. Otherwise, the Chi-squared test was applied.

17]. In the present study, we showed a similar association between the tHcy level and TAAAs. The rarity of TAAAs has made the investigation of the molecular mechanisms underlying the association between HHcy and TAAAs difficult. However, basic research exploring the mechanisms underlying the association between HHcy and AAAs (the more common and closely related aortic aneurysms) has been more comprehensively conducted. These studies have provided important clues for us to explain and understand the association mechanically. From a molecular perspective, TAAAs might be associated with the same pathological features as AAAs, including degradation of the extracellular matrix, accumulation of reactive oxygen species, dedifferentiation or apoptosis of smooth muscle cells, and activation of various inflammatory cells [5, 18, 19]. HHcy may also mediate the formation of TAAAs through some

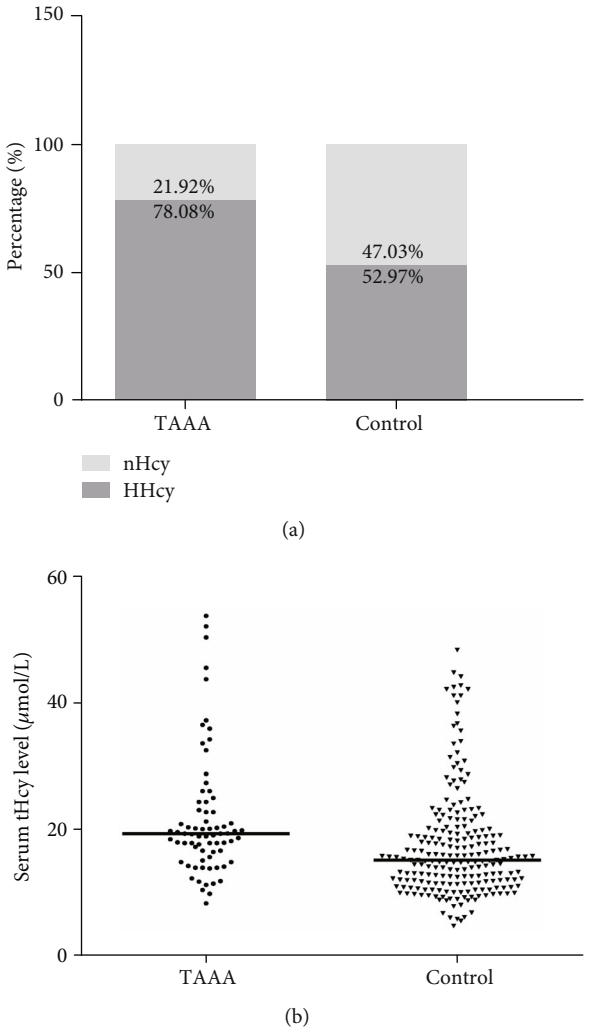


Figure 1: (a) Percentage of patients with HHcy in the two groups (nHcy: normal serum tHcy level; HHcy: hyperhomocysteinemia). (b) Distribution of serum tHcy levels in the two groups.

of these pathogenetic pathways. Previous *in vitro* studies have shown that HHcy can increase serine elastase synthesis in aortic smooth muscle cells by 5- to 6-fold. Elastase leads to fragmentation of elastin, which provides the aorta's elasticity [20]. Elevated levels of tHcy also reportedly increase the secretion of elastolytic metalloproteinase-2 and metalloproteinase-9 in human endothelial cells [21]. These phenomena account for, at least in part, the degeneration of extracellular matrix in TAAAs. Previous studies have also shown that HHcy mediates formation of AAAs through recruitment of monocytes and macrophages to the aortic wall and activation or polarization of macrophages into inflammatory M1 cells [18, 22, 23], with subsequently aggravation of the aortic inflammation. Furthermore, homocysteine also directly interacts and activates the angiotensin II type I receptor to aggravate the vascular injury and then the development of AAAs [24]. It is reasonable to consider that the abovementioned mechanisms are also implicated in the association between HHcy and TAAAs. However, the pathological features of TAAAs and AAAs might be different

Table 2: Univariable logistic regression models evaluating the association between the variables and TAAA.

Variables	Statistics	OR (95% CI)	P value
Sex			1.00
Female	36 (12.33%)	Ref.	
Male	256 (87.67%)	1.00 (0.45, 2.24)	
Age, years			<0.001
<60	143 (48.97%)	Ref.	
≥60	149 (51.03%)	2.64 (1.50, 4.62)	
Hypertension			<0.001
No	171 (58.56%)	Ref.	
Yes	121 (41.44%)	4.53 (2.56, 8.01)	
Diabetes			0.90
No	271 (92.81%)	Ref.	
Yes	21 (7.19%)	0.93 (0.33, 2.64)	
CAD			<0.01
No	254 (86.99%)	Ref.	
Yes	38 (13.01%)	2.86 (1.41, 5.79)	
Ischemic stroke			0.10
No	273 (93.49%)	Ref.	
Yes	19 (6.51%)	2.17 (0.86, 5.46)	
Smoking habits			<0.001
No	208 (71.23%)	Ref.	
Yes	84 (28.77%)	5.71 (3.22, 10.13)	
Drinking habits			<0.01
No	226 (77.40%)	Ref.	
Yes	66 (22.60%)	2.26 (1.25, 4.08)	
Dyslipidemia			0.95
No	143 (48.97%)	Ref.	
Yes	149 (51.03%)	0.98 (0.58, 1.67)	
BMI (kg/m^2)			<0.01
<24	86 (29.45%)	Ref.	
≥24	206 (70.55%)	0.42 (0.24, 0.73)	
eGFR (mL/min per 1.73 m^2)			<0.001
<90	39 (13.36%)	Ref.	
≥90	253 (86.64%)	0.11 (0.05, 0.24)	

Abbreviations: TAAA: thoracoabdominal aortic aneurysm; CAD: coronary heart disease; BMI: body mass index; eGFR: estimated glomerular filtration rate; Ref: reference.

to a certain extent [1]. Basic researches should be carried out to identify the specific mechanisms underlying the association between HHcy and TAAAs.

Additionally, longstanding atherosclerosis is thought to be the most frequent cause of degenerative aneurysms [6]. HHcy promotes atherosclerosis by causing vascular injury and adversely affecting several cellular functions, including lipid dysregulation, programmed cell death, and inflammation [25]. HHcy might also lead to TAAAs by aggravating atherosclerosis.

It might be effective to lower the serum tHcy level by folic acid and vitamin B_{12} or B_6 supplementation to decrease the risk of TAAA formation in patients with HHcy. Future

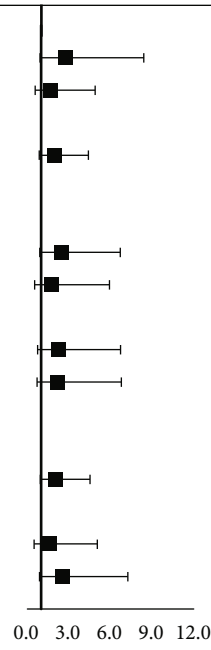
Table 3: Logistic regression models evaluating the association between tHcy and TAAA.

Variable	No. (%) of participants		OR (95% CI)		
	Control	TAAA	Crude[a]	Minimally adjusted model[b]	Fully-adjusted model[c]
tHcy (μmol/L)	219	73	1.04 (1.02, 1.07)	1.05 (1.02, 1.08)	1.04 (1.00, 1.07)
ln (tHcy)			3.47 (1.85, 6.49)	3.75 (1.90, 7.39)	2.98 (1.36, 6.53)
tHcy level (μmol/L)					
<15	103 (47.03%)	16(21.92%)	Ref.	Ref.	Ref.
≥15	116 (52.97%)	57(78.08%)	3.16 (1.71, 5.85)	3.10 (1.60, 6.01)	2.14 (1.00, 4.56)

Values in the table are OR (95% CI). Abbreviations: TAAA: thoracoabdominal aortic aneurysm; Ref: reference. [a]Crude model adjust for: none. [b]Minimally adjust model: adjust for sex, age (years). [c]Fully adjust model: adjusted for sex, age (years), smoking, hypertension, BMI, and Cr-based eGFR (variables changing the coefficient of categorical tHcy level by >10%).

Table 4: The subgroup analyses and interaction analyses of association between HHcy and the risk of TAAA.

Variables	TAAA		Control		Crude[a]	Adjusted[b]	P for interaction
	HHcy	nHcy	HHcy	nHcy	(OR 95% CI)	(OR 95% CI)	
Age, years							0.51
<60	16	7	56	64	2.61 (1.00, 6.81)	2.77 (0.91, 8.40)	
>=60	41	9	60	39	2.96 (1.30, 6.77)	1.65 (0.56, 4.88)	
Sex							0.22
Male	22	9	111	81	3.52 (1.73, 7.15)	1.94 (0.86, 4.40)	
Female	35	7	5	22	3.52 (0.69, 18.05)	NA[c]	
Smoking habit							0.68
No	17	6	90	87	2.36 (1.03, 5.42)	2.45 (0.90, 6.69)	
Yes	40	10	26	16	3.08 (1.11, 8.56)	1.75 (0.52, 5.91)	
Hypertension							0.97
No	27	9	74	74	2.83 (1.06, 7.59)	2.23 (0.74, 6.71)	
Yes	30	7	42	29	2.76 (1.19, 6.39)	2.17 (0.70, 6.78)	
Cr-based eGFR							0.63
<90	43	13	11	101	4.55 (0.37, 55.54)	NA[c]	
>=90	14	3	105	2	2.05 (1.05, 4.02)	2.05 (0.93, 4.52)	
BMI, kg/m^2							0.55
<24	22	10	23	31	2.97 (1.18, 7.45)	1.57 (0.49, 5.05)	
>=24	35	6	93	72	4.52 (1.80, 11.32)	2.52 (0.88, 7.24)	



Abbreviations: TAAA: thoracoabdominal aortic aneurysm; BMI: body mass index; eGFR: estimated glomerular filtration rate. In each item, the model is not adjusted for the stratification variable. [a]Crude model adjusted for none variable. [b]Adjusted model adjusted for: sex, age (years), smoking, hypertension, categorical BMI, and categorical Cr-based eGFR. [c]The model failed because of the small sample size.

prospective studies are warranted to address this issue. However, a previous randomized controlled trial with a large population was performed to investigate the effect of tHcy-lowering therapy on the risk of major cardiovascular events in patients with vascular disease. The results showed that the tHcy-lowering treatments did not decrease the incidence of myocardial infarction, death of any cause, or adverse events compared with placebo. Only the incidence of stroke was reduced by tHcy-lowering interventions [26]. One possible reason for the failure to demonstrate certain clinical benefits of the tHcy-lowering interventions is that the study included patients who already had chronic tHcy elevation with vascular alterations that could not be reversed by vitamin supplementation [25]. Another reason may be that individuals with HHcy have an "Hcy memory effect" as a result of epigenetic alterations, which could continue to promote progression of cardiovascular complications even after the tHcy level is lowered [13]. Therefore, therapies targeting the epigenetic alterations should be developed to lower the risk of TAAA formation and atherosclerosis together with folic acid and vitamin B_{12} or B_6 supplementation.

5. Limitations

This study has several limitations. First, our study was retrospective in nature, and recall bias cannot be totally avoided. For example, the participants' self-reported histories of chronic disease might have affected the accuracy of estimation of the disease prevalence. Second, the serum tHcy level was measured after the diagnosis of TAAAs; therefore, the causality of the association between an elevated tHcy level and TAAAs cannot be determined. Third, our study had a

limited sample size, and only a few patients were included in each subgroup after stratifying by conventional risk factors, yielding limited statistical power. This limitation especially concealed some meaningful results in the stratified and interaction analyses. Fourth, this study mostly focused on the association between the tHcy level and TAAA occurrence, while further analysis involving the type, length, or maximum diameter of TAAAs was not performed. Fifth, the C677T polymorphism in methylenetetrahydrofolate reductase (MTHFR) closely affects the enzymatic activity of homocysteine metabolism. However, data on that genetic background was lacking, precluding assessment of the role of MTHFR C677T polymorphism in the association between serum tHcy concentration and TAAA formation. Finally, the serum levels of vitamin B_6, vitamin B_{12}, and folic acid were not measured, preventing us from assessing their interaction with the serum tHcy level.

Despite the aforementioned limitations, this is the first attempt to explore the association between the serum tHcy level and TAAAs and suggests that HHcy is independently associated with a higher risk of TAAA formation. We believe that our study can provide a foundation for subsequent larger prospective cohort studies.

Abbreviations

HHcy: Hyperhomocysteinaemia
TAAAs: Thoracoabdominal aortic aneurysms
CAD: Coronary artery disease
AAAs: Abdominal aortic aneurysms
HDL: High-density lipoprotein cholesterol
LDL: Low-density lipoprotein cholesterol
eGFR: Estimated glomerular filtration rate
BMI: Body mass index.

Data Availability

All of the data supporting the findings in this study are available from the corresponding author upon request.

Conflicts of Interest

The authors have no competing interests.

Authors' Contributions

W.G., H.Z., and X.L. designed the study. J.L., L.C., and Q.W. collected the data. J.D. and J. L analyzed the data. J.D., H.Z., and X.L. analyzed and interpreted the results. J.D. and J.L. wrote the article. All the authors have revised and reviewed this manuscript. Jianqing Deng and Jie Liu contributed equally to this work.

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Clinical Study

Blood Monocyte Phenotype Fingerprint of Stable Coronary Artery Disease: A Cross-Sectional Substudy of SMARTool Clinical Trial

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Background and Aims. Atherosclerosis is an inflammatory disease with long-lasting activation of innate immunity and monocytes are the main blood cellular effectors. We aimed to investigate monocyte phenotype (subset fraction and marker expression) at different stages of coronary atherosclerosis in stable coronary artery disease (CAD) patients. *Methods*. 73 patients with chronic coronary syndrome were evaluated by CT coronary angiography (CTCA) and classified by maximal diameter stenosis of major vessels into three groups of CAD severity: CAD1 (no CAD/minimal CAD, $n^\circ = 30$), CAD2 (non-obstructive CAD, $n^\circ = 21$), and CAD3 (obstructive CAD, $n^\circ = 22$). Flow cytometry for CD14, CD16, and CCR2 was used to quantify Mon1, Mon2, and Mon3 subsets. Expression of CD14, CD16, CD18, CD11b, HLA-DR, CD163, CCR2, CCR5, CX3CR1, and CXCR4 was also measured. Adhesion molecules and cytokines were quantified by ELISA. *Results*. Total cell count and fraction of Mon2 were higher in CAD2 and CAD3 compared to CAD1. By multivariate regression analysis, Mon2 cell fraction and Mon2 expression of CX3CR1, CD18, and CD16 showed a statistically significant and independent increase, parallel to stenosis severity, from CAD1 to CAD2 and CAD3 groups. A similar trend was also present for CX3CR1 and HLA-DR expressions on total monocyte population. A less calcified plaque composition was associated to a higher Mon2 expression of CD16 and higher TNF-α levels. IL-10 levels were lower at greater stenosis severity, while the IFN-γ/IL-10 ratio, a marker of a systemic pro-inflammatory imbalance, was directly correlated to stenosis degree and number of noncalcified plaques. *Conclusions*. The results of this study suggest that a specific pattern of inflammation-correlated monocyte marker expression is associated to higher stenosis severity and less calcified lesions in stable CAD. The clinical trial Identifier is NCT04448691.

1. Introduction

Atherosclerosis is a chronic non-resolving inflammatory disease of the vessel wall, characterized by the long-term activation of the innate immune system [1, 2].

Blood monocytes and tissue-resident macrophages are the main cellular effectors of innate immunity. Monocyte accumulation within the vascular wall, in particular, contributes to atherogenesis and plaque growth through the generation of "foam" macrophages and the promotion of further leukocyte recruitment into complicated plaques [3, 4]. Following the discovery of blood monocyte subsets, CD14 and CD16 have emerged as standard markers of their definition [5]. The 2010 nomenclature document, in accordance with

the recommendation of the Nomenclature Committee of the International Union of Immunological Societies, acknowledged the existence of three main circulating subsets and standardized their definitions as $CD14^{++}CD16^{-}$ or "classical," $CD14^{++}CD16^{+}$or "intermediate," and $CD14^{+}CD16^{++}$ or "non-classical" [6].

It has been demonstrated that innate immune cells can adopt a persistent pro-inflammatory functional phenotype after exposure to a variety of activating stimuli, defined as "trained innate immunity" [7]. The idea that trained innate immunity contributes to the development and progression of atherosclerosis is progressively emerging, both in the setting of traditional cardiovascular risk factors and in the setting of non-traditional risk factors, such as acute and chronic infections, as well as non-infectious chronic inflammatory disorders [8–10]. A pathophysiological relation between atherosclerosis extent, plaque composition, and the circulating mononuclear phagocyte system profile is therefore hypothesized. Previous studies, performed in large prospective cohorts, revealed that an increase of intermediate CD14++/CD16+ monocytes in the blood is an independent predictor of acute adverse cardiovascular outcomes in subjects with chronic kidney disease, presence of many cardiovascular risk factors, and after percutaneous transluminal coronary angioplasty (PTCA) [11–13]. However, few studies have investigated the relationship between the phenotype of blood monocytes and the stenosis severity of coronary artery disease (CAD), assessed by non-invasive CTCA coronary imaging in stable patients with chronic coronary syndrome according to ESC definition [14].

Accordingly, this study is aimed at assessing blood monocyte subset distribution and functional phenotype marker expression at different stages of CAD—classified by guidelines recommended for the standard criteria, based on the degree of maximal diameter stenosis in major vessels—as a putative fingerprint of the presence of an innate immunity training process.

For this purpose, in an observational cross-sectional substudy of 73 patients with chronic coronary syndrome, prospectively included in the clinical study of the EU Project SMARTool (GA number: 689068) to receive a computed tomography coronary angiography (CTCA) scan [15], we analyzed blood monocyte phenotypic profile in three groups of patients, classified according to the standard stenosis criteria, from minimal to non-obstructive and obstructive diseases.

2. Materials and Methods

2.1. Patients. The study was approved by the Ethical Committee of the "Area Vasta Nord-Ovest" (CEAVNO Prot. number 23534) of Tuscany Region (Italy) and was conducted in accordance with the Declaration of Helsinki, and all patients provided written informed consent. Patients ($n^{\circ} = 73$) were recruited at the Cardiology and Cardiovascular Medicine Division of Fondazione Toscana "G. Monasterio" (Pisa–Italy) from September 2016 to November 2017, within the H2020 EU Project SMARTool. Patients in stable clinical conditions and referred to CTCA for suspected stable CAD were included according to the study criteria reported in Supplementary Materials (see Table S1).

2.2. Computed Tomography Coronary Angiography (CTCA) Image Acquisition Protocol. Subjects underwent ECG-triggered cardiac CTCA scan using 64-slice scanners or higher, according to the predefined standard operating procedure of SMARTool Project Clinical Study, in order to ensure optimal image quality. The main points of the standard operating procedure (SOP) for CT scan acquisition, relevant for accurate analysis, can be summarized as follows:

(i) Absence of motion or other artifacts in acquired images

(ii) Heart rate < 65 beats/min.

(iii) Nitroglycerin administration prior to acquisition.

(iv) Optimized reconstruction of the most suitable cardiac cycle (diastole at 70%-80% RR interval).

(v) Upload of multiple cardiac phases as to identify all coronary segments if needed.

(vi) Reconstructed field of view of 200-250 mm for CTCA.

All coronary CTCA images were analyzed blinded to clinical data in a Core Laboratory (Leiden University Medical Center), and coronary arteries were visually analyzed according to the modified 17-segment American Heart Association classification [16].

CAD was assessed by semi-quantitative visual assessment of two expert cardioradiologists and classified by maximal diameter stenosis percentage in at least 1 of major epicardial artery segments into the following severity classes, in accordance with computed tomography (CT) CAD-RADS guidelines [17, 18]: Class 0: no plaque nor stenosis in any major vessel ($n^{\circ} = 14$); Class 1: stenosis < 25% (minimal CAD) ($n^{\circ} = 16$); Class 2: 25% ≥ stenosis < 50% (non-obstructive CAD) ($n^{\circ} = 21$); Class 3: 50% ≥ stenosis < 70% (sub-obstructive CAD) ($n^{\circ} = 14$); and Class 4: stenosis ≥ 70% (obstructive CAD) ($n^{\circ} = 8$). The five CAD-RADS classes have been merged into three main categories of stenosis severity, in agreement with the clinically oriented approach proposed by others [19]: CAD-RADS 0 or 1, CAD-RADS 2, and CAD-RADS 3 or 4, thus originating three subsets of patients: (a) no CAD/-minimal CAD (CAD1), (b) non-obstructive CAD (CAD2), and (c) obstructive CAD (CAD3).

In each coronary artery segment, an atherosclerotic plaque is defined as a tissue structure above 1 mm^2 on the cross-sectional view of the vessel wall. Mean plaque composition was qualitatively assessed as noncalcified, mixed, or calcified by using both fixed (-30 to 130, 131 to 350 and ≥351 HU density value, respectively) and adaptive, luminal contrast density-corrected, thresholds, as reported [20–22].

CAD staging by maximal stenosis degree is also representative, in our study population, of the extension of the atherosclerotic disease throughout the coronary tree, expressed as the total number of visible plaques at CTCA scan (see Table S2 in Supplementary Materials).

2.3. Biochemical Analyses. Peripheral blood samples were collected in Vacutainer® tubes for plasma and serum separation. EDTA-anticoagulated blood was centrifuged at 1000xg, for 10 minutes at 4°C in order to obtain plasma aliquots. Whole blood samples, collected in serum separator tubes, were kept at room temperature for 30 minutes to allow sample coagulation before centrifugation at 1000xg, for 20 minutes at 4°C. The supernatants (serum and plasma) were transferred into polypropylene tubes and stored in aliquots at -80°C. Blood cell count (n° of cells/μl) was carried out by using the automated cell analyzer SF-3000 (Sysmex, Kobe, Japan). Serum lipid profile (total cholesterol, LDL and HDL cholesterol, and triglycerides) and general biochemical parameters (creatinine, uric acid, glucose, Hs-CRP, and fibrinogen) were measured according to routine clinical protocols. Antigenic immunoassay procedure based on enzyme-linked immunosorbent assay (ELISA) was instead used for the quantification of plasma adhesion molecules (VCAM-1, ICAM-1) and serum cytokines (IL-6, IFN-γ, TNF-α, and IL-10). The following commercial ELISA kits were used: DCD540 and DVC00 (R&D Systems, Inc.) for human ICAM-1 and VCAM-1 determinations; 950.035 (Diaclone) for high sensitivity IL-6 assessment; E-EL-H0108, E-EL-H0109 and E-EL-H0103 (Elabscience® Biotechnology) for IFN-γ, TNF-α, and IL-10 evaluations, respectively. All the biomarkers were determined in duplicates.

2.4. Flow Cytometry Analysis. Flow cytometry was performed with a FACScan instrument interfaced with CellQuest software (Becton Dickinson, San Jose, CA, USA). Overtime monitoring of instrument alignment was carried out with CaliBRITE beads (Becton Dickinson). Flow cytometric identification and quantification of monocyte markers was performed on EDTA anticoagulated whole blood samples (50 μl), within 1 hour after collection, by using a lyse-no-wash staining procedure, as described [23]. The following combinations of PC5- (Beckman Coulter), FITC- (BD Pharmingen) and PE-conjugated (R&D) mouse anti-human monoclonal antibodies were employed: (a) CD14/CD16/CCR2, (b) CD14/CD16/CCR5, (c) CD14/CD16/CX3CR1, (d) CD14/CD16/CXCR4, (e) CD14/CD16/CD18, (f) CD14/CD16/CD11b, (g) CD14/CD16/HLA-DR, and (h) CD14/CD16/CD163. Appropriate mouse isotype controls were carried out in parallel. In brief, after antibody incubation for 20 min at room temperature (RT) in the dark, erythrocytes were lysed with 750 μl lysing buffer (BD Biosciences) for 10 min at RT before quenching with an equal volume of ice-cold PBS. The tubes were then maintained on ice until FACS analysis. The sequence of analysis, conducted according to the minimal requirement suggested by the joint consensus document of the European Society of Cardiology (ESC) Working Groups [24], is described below and represented in Figure 1: stringent monocyte morphological cluster identification based on its forward and side scatter characteristics [Figure 1(a)]; selection of CD14++/+ (bright/low) events and preliminary monocyte subsets quantification (as percentage) initially based on their differential expression of surface markers CD14 and CD16 (CD14++/CD16-, CD14++/CD16+, CD14+/CD16++) [Figure 1(b)]; measurement of the actual circulating fraction (percentage) of subsets Mon1(CD14++/CD16-/CCR2+) and Mon2(CD14++/CD16+/CCR2+) obtained by multiplying, and then dividing by one hundred, the percentages of subsets derived at the point b with their corresponding percentages of positivity for the distinctive marker CCR2 [Figure 1(c)]. These last are calculated by using an isotype median-based overlaid histogram subtraction analysis, according to the Overton subtraction technique [Figure 1(c)] [25]. For calculation of the actual percentage of subset Mon3 (CD14+/CD16++/CCR2-), the value of its CCR2 negative fraction was used.

The use of CCR2 is essential for determining the absolute count of the three monocyte subsets. The two markers CD14 and CD16 are enough for subset identification and characterization of their surface marker profile [23]. The expression of surface markers has been also quantified on the whole CD14++/+ monocyte population. The Overton electronic analysis allows the quantification of marker expression either in terms of percentage of positivity (%+) or relative fluorescence intensity (RFI) (median of the positive events distribution minus median of the isotype control histogram), as reported in our previous works [26–28].

2.5. Statistical Analysis. Continuous data are presented as mean ± mean standard error (SEM) and categorical variables as number of patients and percentage. The comparison between groups has been performed by ANOVA (with Bonferroni's correction) for continuous data and by Chi-Square (χ^2) test for categorical data. At univariate and multivariate multinomial logistic regression analyses, the three categories of CAD stenosis severity have been considered the dependent variable and analyzed on the basis of the following grouping of variations: CAD1 vs. CAD2 (CAD1/CAD2) and CAD1 vs. CAD3 (CAD1/CAD3). Monocyte data significantly higher in higher stenosis severity groups by univariate logistic regression have been subsequently tested by multivariate logistic regression. Only monocyte data showing statistical significance in both groups of variation at multivariate logistic regression analysis have been considered. The variables used for the adjustment in multivariate logistic regression were continuous and categorical and were chosen by their known pathophysiological relevance in the development and progression of atherosclerotic disease. They included Framingham Risk Score, metabolic syndrome and diabetes, systemic pro-/anti-inflammatory environment (plasma levels of Hs-CRP and IL-6, IFN-γ, TNF-α, and IL-10), endothelial activation (plasma levels of ICAM-1 and VCAM-1), presence and dosage (mg/die) of statin therapy, and use of oral hypoglycemic drugs. Linear regression analyses have been performed to correlate monocyte activation markers HLA-DR on CD14++/+ population with monocyte subset frequencies. All statistical analyses were performed by StatView 5.0 software program (SAS Institute, Cary, NC, USA). A P value < 0.05 was considered statistically significant.

3. Results

3.1. Patients Characteristics. Patient demographic, clinical, and laboratory characteristics are reported in Table 1, as a whole and by the three groups of CAD.

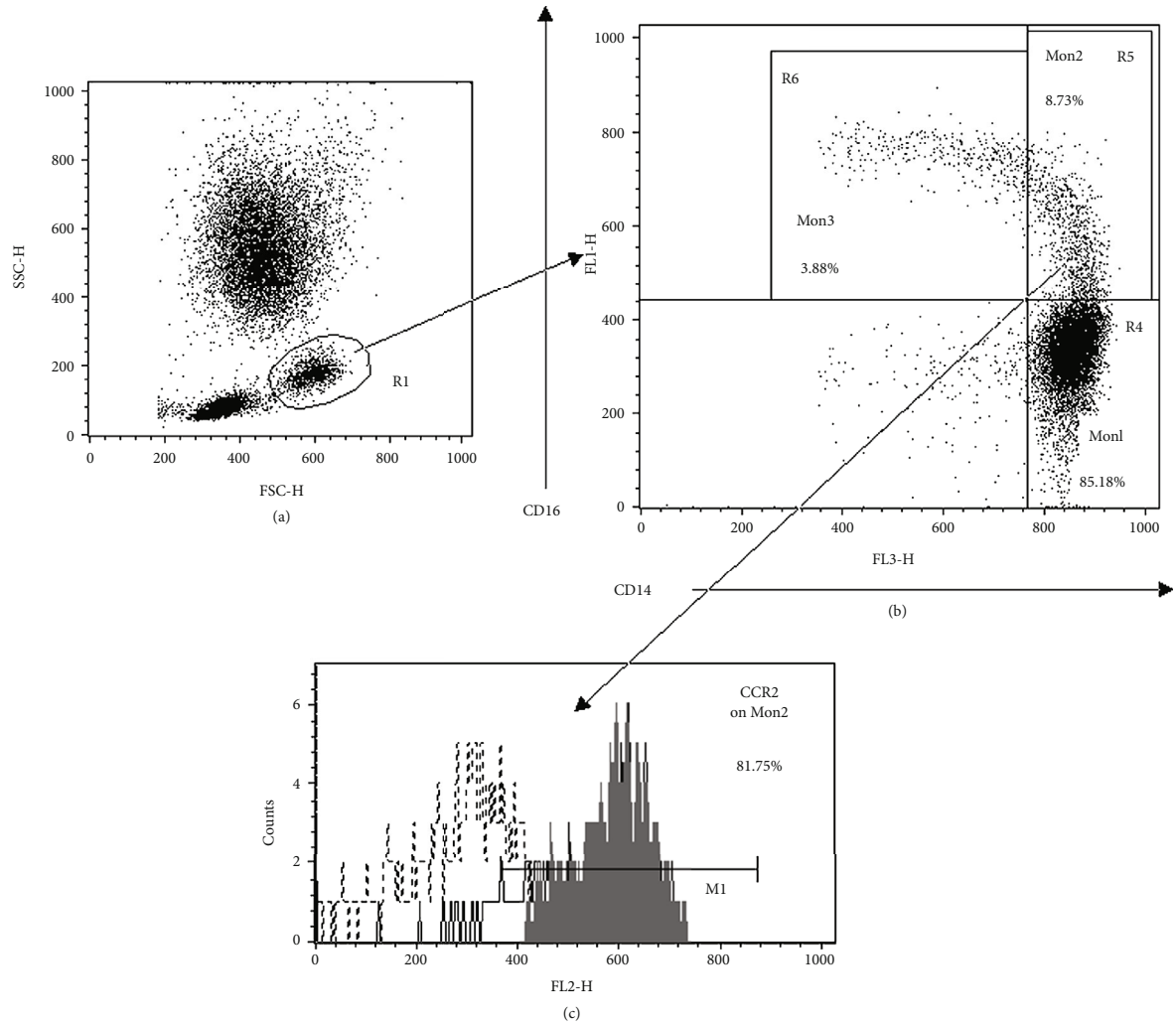

Figure 1: Representative example of the flow cytometry analysis for monocyte subset quantification. (a) Total monocyte cluster identification based on its forward and side scatter morphological characteristics (region R1). (b) Selection of CD14++/+ events and preliminary monocyte subset quantification (as percentage) [(CD14++/CD16- (Mon1, R4), CD14++/CD16+ (Mon2, R5), and CD14+/CD16++ (Mon3, R6)] initially based on their differential expression of markers CD14 and CD16. (c) The measurement of the actual circulating fraction of subsets Mon1 and Mon2 is obtained by multiplying, and then dividing by one hundred, the percentages of events measured at the point *b* (regions R4 and R5) with their corresponding percentages of positivity of the distinctive marker CCR2 (grey subtraction histogram by the Overton technique). For the final quantification of the Mon3 fraction (region R6), the value of its CCR2 negative fraction is used.

Among CAD groups, males were more numerous in CAD3 (86.3%), when compared to CAD1 (56.7%) and CAD2 (57.1%). The frequency of diabetic patients increased progressively from CAD1 to CAD3. Total and LDL cholesterol concentrations were significantly lower in CAD3 patients compared to CAD1 and CAD2, probably as a consequence of the higher frequency of statin-treated subjects in this group (95.4%). Not only the use of statin therapy but also its daily dosage was significantly higher both in CAD2 and CAD3 compared to CAD1 patients. CAD2 patients had lower plasmatic levels of VCAM-1 when compared to other two, while no differences were observed in ICAM-1 concentrations among groups.

3.2. Cytokines. The ANOVA post-hoc test showed significant lower levels of IL-10 in CAD2 and CAD3 groups than in CAD1 [Figure 2(a)], together with a parallel increase of the IFN-γ/IL-10 ratio [Figure 2(b)], suggestive of a systemic pro-inflammatory unbalance. On the other hand, no significant differences were found in the plasma concentration of the other cytokines tested (VCAM-1, ICAM-1, IL-6, and TNF-α) relative to stenosis severity.

3.3. Monocyte Phenotype Analysis. The total cell count of CD14++/+ monocyte population was not significantly different in the three groups of CAD. However, by multivariate logistic regression, significantly higher RFI values were found in CAD2 and CAD3 groups for membrane receptors CX3CR1 ($P = 0.0118$, OR = 1.087, *CAD1*/*CAD2*; $P = 0.0478$, OR = 1.066, *CAD1*/*CAD3*) and HLA-DR ($P = 0.0353$, OR = 1.030, *CAD1*/*CAD2*; $P = 0.0170$, OR = 1.038, *CAD1*/*CAD3*).

Subset analysis evidenced higher values of Mon2 subset fraction in CAD2 (6.42 ± 0.85) and CAD3 (7.02 ± 0.94) compared to CAD1 (4.75 ± 0.47). Moreover, a significantly

Table 1: Demographic, laboratory and clinical characteristics of patients.

	All patients ($n^\circ = 73$)	CAD1 ($n^\circ = 30$)	CAD2 ($n^\circ = 21$)	CAD3 ($n^\circ = 22$)	ANOVA P
Demographics					
Age (years)	68.2 ± 0.9	65.48 ± 1.4	70.2 ± 1.7	69.7 ± 1.5	Ns
Sex (M/F, n°)	48/25	17/13	12/9	19/3	Ns
BMI (kg/m^2)	27.36 ± 0.44	27.84 ± 0.63	26.77 ± 0.75	27.30 ± 0.98	Ns
Risk factors					
Framingham Risk Score (FRS)	15.32 ± 0.38	14.83 ± 0.76	16.10 ± 0.56	15.27 ± 0.62	Ns
Metabolic syndrome, n° (%)	2 (2.7)	1 (3.3)	0 (0.0)	1 (4.5)	Ns
Obesity, n° (%)	15 (20.5)	7 (23.3)	4 (19.0)	4 (18.1)	Ns
Hypertension, n° (%)	58 (79.4)	22 (73.3)	20 (95.2)	16 (72.7)	Ns
Active smoking, n° (%)	8 (10.9)	2 (6.6)	3 (14.2)	3 (13.6)	Ns
Dyslipidemia, n° (%)	65 (89.0)	24 (80.0)	20 (95.2)	21 (95.4)	Ns
Diabetes, n° (%)	21 (28.7)	4 (13.3)	6 (28.5)	11 (50.0)	0.0155
Family history of CAD, n° (%)	38 (52.0)	17 (56.6)	10 (47.6)	11 (50.0)	Ns
Laboratory exams					
WBC (10^9/l)	7.91 ± 0.23	7.47 ± 0.35	8.57 ± 0.44	7.90 ± 0.43	Ns
Neutrophil (10^9/l)	4.73 ± 0.19	4.48 ± 0.28	5.03 ± 0.38	4.79 ± 0.39	Ns
Monocyte (10^9/l)	0.62 ± 0.02	0.58 ± 0.03	0.67 ± 0.04	0.63 ± 0.03	Ns
Lymphocyte (10^9/l)	2.35 ± 0.08	2.19 ± 0.09	2.65 ± 0.16	2.29 ± 0.17	Ns
Platelet (10^9/l)	226.13 ± 5.48	220.03 ± 7.55	237.09 ± 10.36	224.00 ± 11.34	Ns
Glucose (mg/dl)	102.54 ± 3.52	97.93 ± 2.93	105.67 ± 8.28	105.44 ± 7.44	Ns
Creatinine (mg/dl)	0.85 ± 0.02	0.87 ± 0.03	0.78 ± 0.04	0.90 ± 0.04	Ns
Acid uric (mg/dl)	5.34 ± 0.12	5.43 ± 0.20	5.31 ± 0.25	5.25 ± 0.20	Ns
Total cholesterol (mg/dl)	187.99 ± 4.46	197.57 ± 7.22	192.00 ± 8.94	171.09 ± 5.93	0.0373*
HDL-cholesterol (mg/dl)	56.93 ± 1.98	60.40 ± 3.93	56.48 ± 2.41	52.64 ± 2.88	Ns
LDL-cholesterol (mg/dl)	104.78 ± 4.10	112.83 ± 6.11	109.55 ± 8.71	89.45 ± 6.09	0.0417*
Triglycerides (mg/dl)	134.14 ± 7.38	121.50 ± 10.08	140.38 ± 12.00	145.41 ± 16.70	Ns
Hs-CRP (mg/dl)	0.38 ± 0.08	0.38 ± 0.10	0.33 ± 0.07	0.44 ± 0.20	Ns
Fibrinogen (mg/dl)	324.01 ± 8.96	324.18 ± 11.41	325.57 ± 19.51	322.29 ± 17.93	Ns
ICAM-1 (ng/ml)	214.35 ± 10.97	210.85 ± 15.15	222.04 ± 21.22	211.80 ± 22.86	Ns
VCAM-1 (ng/ml)	625.16 ± 17.95	652.58 ± 35.75	547.93 ± 17.11	661.48 ± 24.69	0.0210§^
Medications					
Statin therapy, n° (%)	50 (68.5)	15 (50.0)	14 (66.6)	21 (95.4)	0.0022
Statin therapy (dosage, mg/die)	11.5 ± 1.2	7.3 ± 1.5	13.1 ± 2.7	15.9 ± 2.1	0.0104§*
Antihypertensive therapy, n° (%)	59 (80.8)	22 (73.3)	18 (85.7)	19 (86.3)	Ns
Insulin therapy, n° (%)	4 (5.4)	1 (3.3)	3 (14.2)	0 (0.0)	Ns
Oral hypoglycemic therapy, n° (%)	19 (26.0)	4 (21.0)	5 (26.3)	10 (52.6)	0.0321

Data are presented as mean ± SEM or as number (n°) and percentage (%), when appropriate. The Bonferroni post-hoc: *CAD1/CAD3, §*CAD1*/*CAD2*, and ^*CAD2*/*CAD3*. Ns: not significant.

higher Mon2 cell count (n°. of cells/μl) was also found in CAD2 (39.89 ± 5.98) and CAD3 (42.44 ± 6.26) compared to CAD1 (24.88 ± 2.58). Cumulative data, including statistical differences, of Mon2 circulating cell fraction and count from CAD1 to CAD3 are shown in Figures 3(a) and 3(b), respectively. The Mon3 subset cell count shows a statistically not significant trend to increase from CAD1 (38.02 ± 2.61) to CAD2 (46.33 ± 3.75) and CAD3 (48.18 ± 5.81).

Monocyte subset frequencies and counts, compared by univariate logistic regression and subsequently processed by multivariate logistic regression analysis in the three groups of CAD stenosis severity, are summarized in Table 2.

Significant relationships were found between the circulating fractions of monocyte subsets and the expression level (as RFI) of the monocyte activation marker HLA-DR on all CD14++/+ cells. In particular, HLA-DR expression

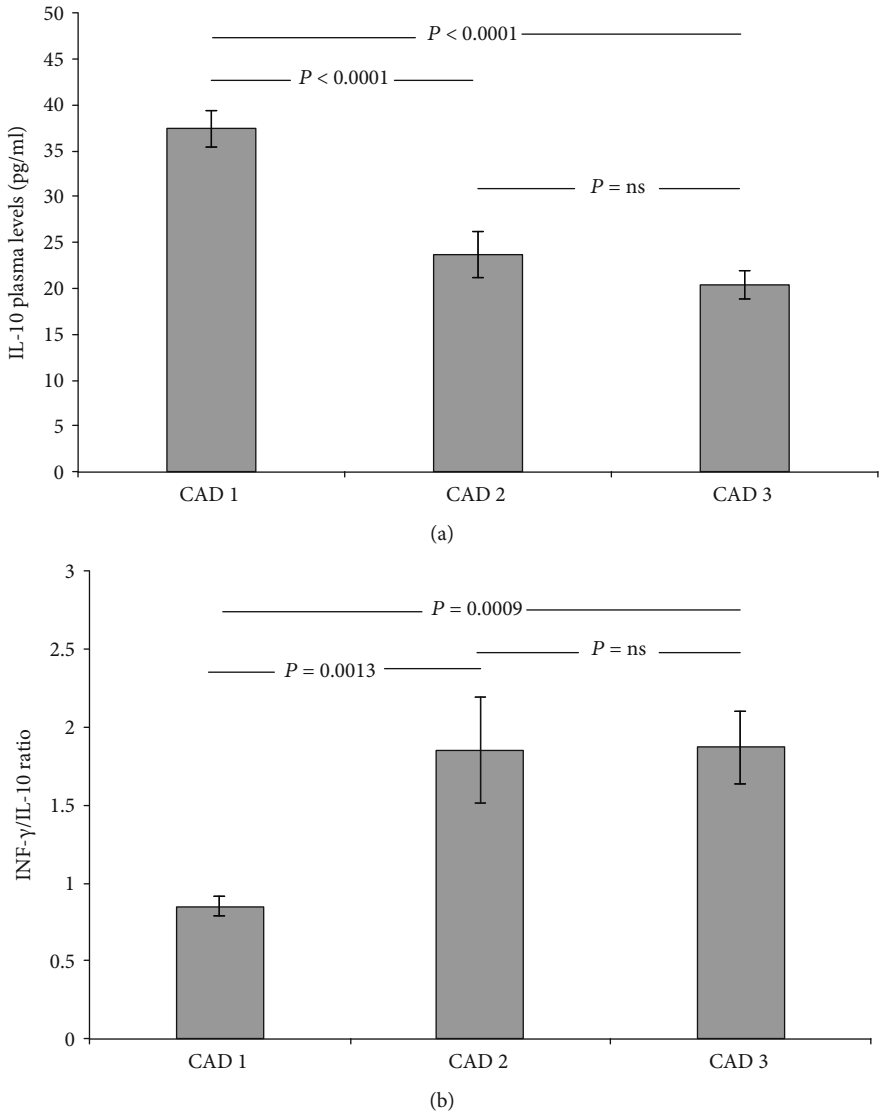

Figure 2: Cumulative graphic representation (mean ± SEM) of IL-10 plasma levels (pg/ml) [(a); ANOVA $P < 0.0001$] and IFN-γ/IL-10 ratio [(b); ANOVA $P = 0.0006$] in the three CAD severity groups. CAD1: no CAD/minimal CAD; CAD2: non-obstructive CAD; CAD3: obstructive CAD.

correlated positively with Mon2 [Figure 4(b)] and Mon3 [Figure 4(c)] frequencies, and negatively with Mon1 frequency [Figure 4(a))]

Following multivariate logistic regression, Mon2 cell fraction was the only subset showing a significant progressive increase from CAD1 to CAD3 (Table 2), and the same significant trend was present for Mon2 markers CX3CR1 and CD18, expressed as RFI, and CD16, evaluated as percentage of positivity (Table 3). No changes in Mon3 markers among the 3 groups were found, while in subset Mon1, the RFI expression level of HLA-DR was also higher in the CAD2 and CAD3 groups ($P = 0.0425$, OR = 1.031, *CAD1/CAD2*; $P = 0.0281$, OR =1.035, *CAD1/CAD3*).

3.4. Associations between Immunological Features and Plaque Composition. RFI values of CD16 on all circulating CD14++/+ monocytes showed a significant positive correlation with number of mixed plaques ($P = 0.0024$, $R = 0.348$) in the whole CAD population (73 patients). Mon2 RFI values of CD16 correlated both with the number of mixed plaques ($P = 0.0023$, $R = 0.349$) and with the cumulative number of noncalcified and mixed plaques ($P = 0.0085$, $R = 0.304$); on the opposite, Mon2 CXCR4 expression (RFI) was significantly correlated with the number of calcified plaques ($P = 0.0026$, $R = 0.345$). TNF-α plasma levels exhibited positive correlations with number of mixed plus noncalcified lesions ($P = 0.0362$, $R = 0.246$) and alike IFN-γ/IL-10 cytokine ratio with that of mixed plaques ($P = 0.0186$, $R = 0.275$).

4. Discussion

The main findings of the present study are (1) a specific proinflammatory circulating monocyte phenotype is independently and significantly associated to more advanced CAD

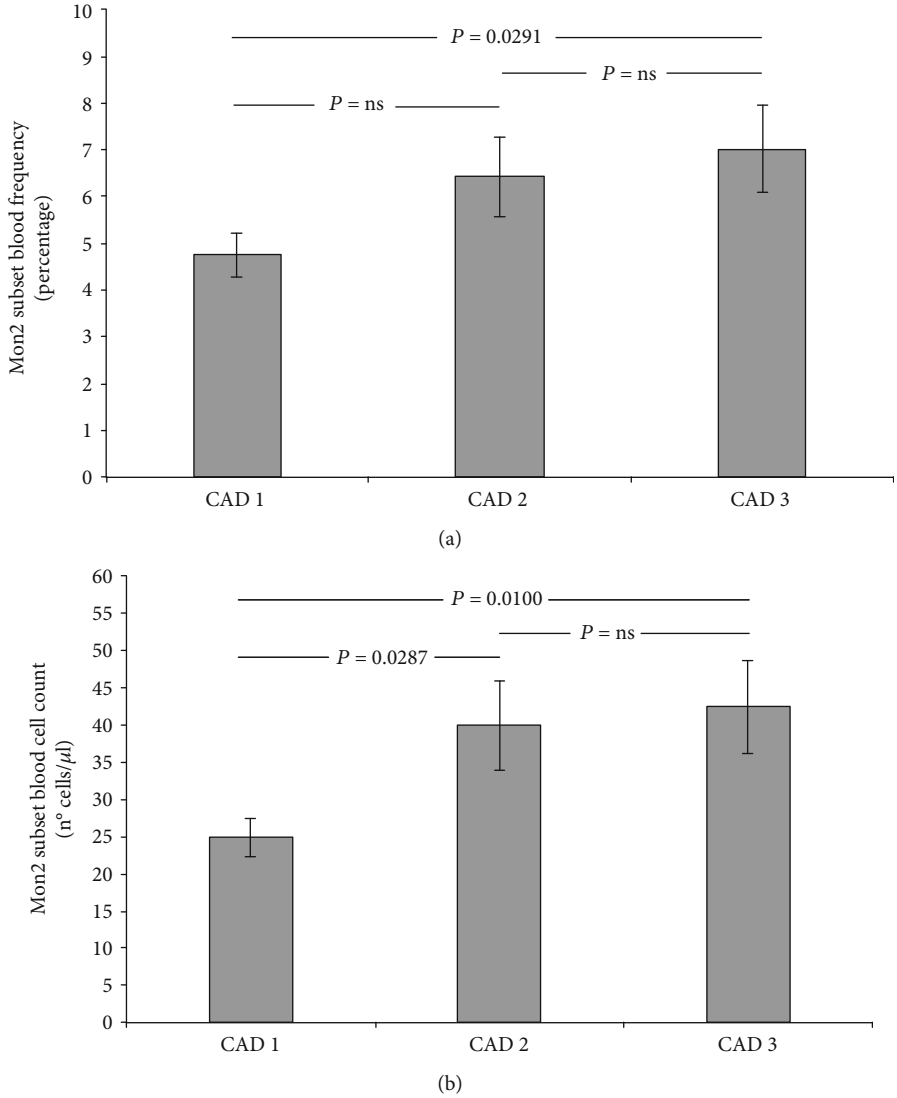

Figure 3: Cumulative graphic representation (*mean* ± *SEM*) of Mon2 subset circulating fractions [(a); ANOVA $P = 0.0695$] and counts [(b); ANOVA $P = 0.0177$] in the three CAD severity groups. CAD1: no CAD/minimal CAD; CAD2: non-obstructive CAD; CAD3: obstructive CAD.

Table 2: Univariate and multivariate multinomial logistic regression analyses of blood monocyte subset frequency (%) and count (n° of cells/μl).

Subset frequency and count	Comparison between groups	Univariate regression coefficient	Unadjusted OR	95% CI	*P*	Adjusted OR[a]	95% CI	*P*
Mon2 (%)	*CAD1/CAD2*	0.156	1.169	0.978-1.398	Ns 0.0314	1.421	1.043-1.936	0.0260
	CAD1/CAD3	0.193	1.213	1.017-1.447		1.550	1.051-2.285	0.0269
Mon3 (%)	*CAD1/CAD2*	0.047	1.048	0.862-1.274	Ns	1.351	0.885-2.061	Ns
	CAD1/CAD3	0.125	1.134	0.936-1.372	Ns	1.355	0.865-2.125	Ns
Mon1 (n°/μl)	*CAD1/CAD2*	0.003	1.003	0.999-1.006	Ns	0.999	0.995-1.004	Ns
	CAD1/CAD3	0.001	1.001	0.996-1.005	Ns	0.996	0.988-1.003	Ns
Mon2 (n°/μl)	*CAD1/CAD2*	0.036	1.036	1.005-1.069	0.0237	1.050	0.998-1.105	Ns
	CAD1/CAD3	0.039	1.040	1.008-1.073	0.0126	1.059	0.998-1.125	Ns
Mon3 (n°/μl)	*CAD1/CAD2*	0.025	1.025	0.993-1.058	Ns	1.031	0.974-1.091	Ns
	CAD1/CAD3	0.029	1.029	0.998-1.062	Ns	1.014	0.961-1.070	Ns

CAD: coronary artery disease; OR: odds ratio; CI: confidence interval; Ns: not significant. [a]Adjusted for Framingham Risk Score (FRS), metabolic syndrome, diabetes, Hs-CRP, IL-6, IFN-γ, TNF-α, IL-10, VCAM-1, ICAM-1, use and dosage of statin therapy (mg/die), and use of oral hypoglycemic drugs.

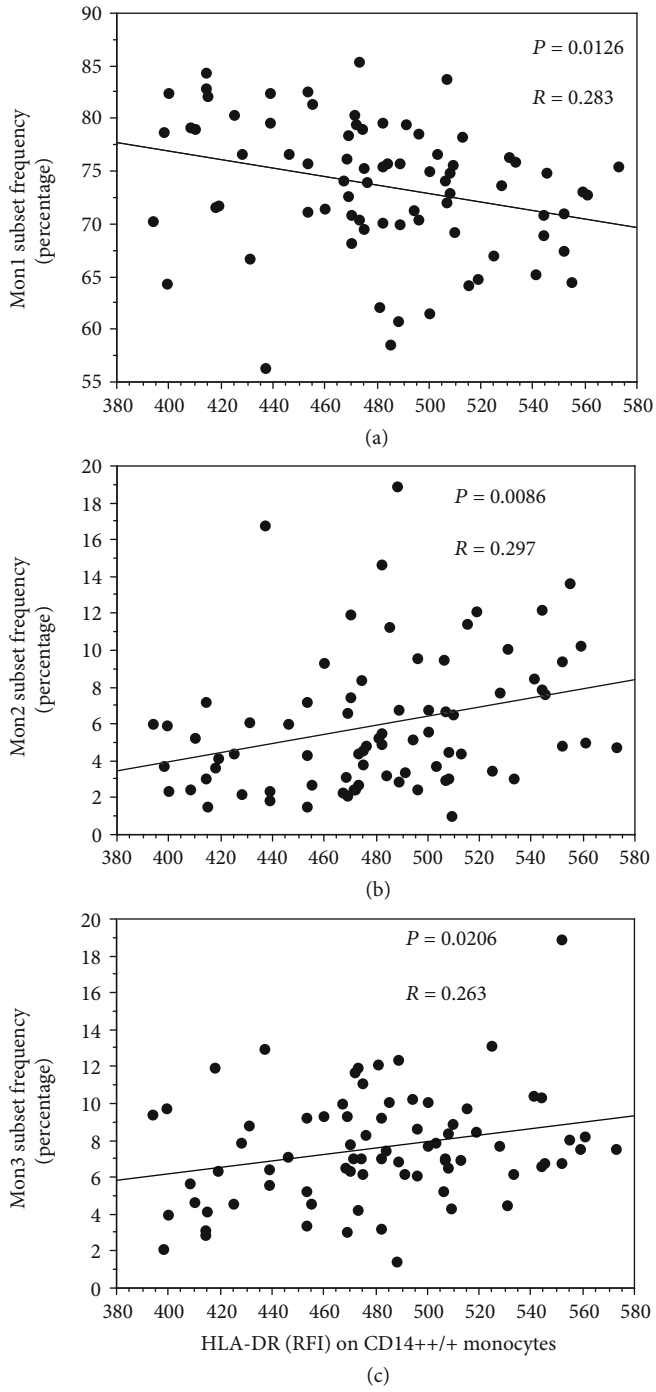

Figure 4: Linear correlations between the HLA-DR expression level (as RFI units) of all CD14++/+ monocytes and the circulating fractions (as percentages) of the Mon1 (a), Mon2 (b), and Mon3 (c) monocyte subsets.

stages, from minimal to non-obstructive and to obstructive CAD and (2) this profile is partly related to a less calcified pattern of CTCA-assessed coronary plaques.

A previous retrospective cross-sectional study, performed in asymptomatic CAD patients, showed that only the count (n° of cells/μl) of the intermediate CD14++/CD16 + monocyte subset was significantly associated with mixed and calcified plaque numbers and with stenosis severity [29]. In our study, we extended the multivariate regression adjustment to several clinical and immunological features known to influence phenotype and function of circulating monocytes, as well as their ability to adhere to activated endothelium and migrate into inflammatory sites and atherosclerotic plaques [1]. As a result, we have observed that not only a higher Mon2 subset circulating fraction but also a higher expression level of its activation markers (CD16), adhesion molecules (CD18), and chemokine receptors (CX3CR1) are independently correlated with more advanced CAD stage. The extended multivariate regression adjustment, as well as the CCR2-based flow cytometry

Table 3: Univariate and multivariate multinomial logistic regression analyses of Mon2 markers (% positivity% and RFI).

Mon2 markers	Comparison between groups	Univariate regression coefficient	Unadjusted OR	95% CI	*P*	Adjusted OR[a]	95% CI	*P*
CX3CR1 (RFI)	*CAD1*/*CAD2*	0.014	1.014	0.996-1.032	Ns	1.055	1.009-1.103	0.0187
	CAD1/*CAD3*	0.015	1.015	0.998-1.033	Ns	1.057	1.008-1.108	0.0215
CD18 (RFI)	*CAD1*/*CAD2*	0.008	1.008	0.997-1.020	Ns	1.030	1.005-1.055	0.0197
	CAD1/*CAD3*	0.005	1.005	0.994-1.016	Ns	1.028	1.002-1.054	0.0338
HLA-DR (RFI)	*CAD1*/*CAD2*	0.011	1.012	0.997-1.026	Ns	1.028	0.998-1.059	Ns
	CAD1/*CAD3*	0.006	1.006	0.993-1.019	Ns	1.029	0.999-1.060	Ns
CXCR4 (RFI)	*CAD1*/*CAD2*	0.011	1.011	0.997-1.025	Ns	1.002	0.980-1.023	Ns
	CAD1/*CAD3*	0.013	1.013	0.999-1.027	Ns	1.016	0.993-1.040	Ns
CCR2 (RFI)	*CAD1*/*CAD2*	0.014	1.014	1.001-1.027	0.0402	1.036	1.011-1.061	0.0042
	CAD1/*CAD3*	0.005	1.005	0.992-1.017	Ns	1.026	0.997-1.056	Ns
CD163 (RFI)	*CAD1*/*CAD2*	0.025	1.025	1.005-1.045	0.0126	1.037	1.004-1.071	0.0297
	CAD1/*CAD3*	0.009	1.009	0.992-1.026	Ns	1.031	0.993-1.069	Ns
CD16 (%+)	*CAD1*/*CAD2*	0.116	1.123	0.972-1.298	Ns	1.341	1.021-1.761	0.0352
	CAD1/*CAD3*	0.171	1.187	1.030-1.367	0.0179	1.463	1.051-2.037	0.0240

CAD: coronary artery disease; OR: odds ratio; CI: confidence interval; Ns: not significant; %+: percentage of positivity; RFI: relative fluorescence intensity. [a] Adjusted for same variables of Table 3.

quantification of circulating monocyte subsets performed in our study, could explain the differences observed with the previously cited study [29] regarding the associations between plaque composition and subset frequency and count. Interestingly, we also found that the increase of Mon2 subset frequency is related to the activation state (HLA-DR expression) of CD14++/+ cells, suggesting an accelerated activation-induced maturational transition of circulating mononuclear cells in patients with more severe disease. This hypothesis is also supported by the observation of independent correlations between stenosis severity and the expression level (as RFI) of monocyte activation markers HLA-DR and CX3CR1 in all CD14++/+ cell population. The inverse relationship of IL-10 plasma levels, as well as the direct relationship between increasing stenosis severity and IFN-γ/IL-10 cytokine ratio, further reinforce this hypothesis. Notably, stenosis severity and the expression level of fractalkine receptor CX3CR1, both on Mon2 subset and on all CD14++/+ mononuclear cells, are also significantly associated. This activation-induced chemokine receptor, in addition to playing a key role during monocyte migration, also regulates their survival and accumulation within inflamed tissues [30, 31].

Finally, our results indicate that the monocyte phenotypic pattern of more advanced CAD (higher Mon2 subset frequency and higher expression of some of its cell surface molecules, especially of the activation marker CD16) is also associated to a less calcified plaque pattern of the disease. In particular, the correlations found between the number of less calcified plaques and the CD16 RFI values, both on Mon2 subset and on all CD14++/+ monocyte, suggest that the expression level of this marker may represent a useful blood signature of a more unstable and inflammation-associated evolving atherosclerotic disease, albeit in the context of stable clinical conditions, as supported by the parallel increase of TNF-α plasma levels and IFN-γ/IL-10 cytokine ratio. On this basis, our findings confirm, and at the same time expand, previous acquisitions on the clinical utility of the Mon2 subset blood frequency in predicting plaque vulnerability and clinical outcome in asymptomatic patients [32, 33].

In summary, the results of our study indicate that, in stable CAD, the combination of a more advanced atherosclerotic disease by stenosis severity and a higher number of less calcified plaque lesions is reflected by specific changes of functional phenotype markers of the entire blood monocyte population and of its subsets, consistent with the existence of a pro-inflammatory systemic environment. In particular, deep phenotyping of circulating monocytes by flow cytometry assessment of Mon2 subset cell number and receptor expression emerges as a promising approach for monitoring CAD evolution in stable patients with chronic coronary syndrome.

The main purpose of this substudy of SMARTool clinical trial was to characterize the blood monocyte phenotype in stable CAD. The major limit is the relatively low number of patients included: the results of monocyte phenotypic association with CAD anatomical stage should be interpreted with caution. The need of processing immediately fresh blood for flow cytometry analysis, soon after coronary CT scan and inflammatory biomarkers assay is one of the main reasons for the low number of recruitments and for its limited use in clinical studies. For a clinical exploitation, observations on larger patient populations are needed to confirm pathophysiological hypotheses and associations between immunological features and the stenosis severity of CAD.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

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

Authors' Contributions

Silverio Sbrana was responsible for flow cytometry data acquisition and analysis, conceptualization, and writing and original draft preparation of the manuscript. Jonica Campolo, Antonella Cecchettini, Elisa Ceccherini, and Chiara Caselli cooperated in the execution of ELISA tests and in the preparation of the final draft of the manuscript. Luca Bastiani supported the statistical analysis. Alberto Clemente, Dante Chiappino, Jeff M. Smit, and Arthur J. Scholte were responsible for the Computed Tomography Coronary Angiography (CTCA) image collection and analysis. Gualtiero Pelosi and Silvia Rocchiccioli conceived the study, supervised, and revised the manuscript. Danilo Neglia and Oberdan Parodi enrolled the patients for the interview and informed consent and revised the writing of the manuscript.

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Supplementary Materials

Supplemental Table S1: full list of inclusion, exclusion, and exit criteria of SMARTool clinical study. Supplemental Table S2: relationships between maximum stenosis classes and coronary artery plaque number. *(Supplementary Materials)*

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Research Article

Ankle-Brachial Index as the Best Predictor of First Acute Coronary Syndrome in Patients with Treated Systemic Hypertension

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Objective. The objective of our study was to evaluate the incidence of target organ damages (TOD) in patients with arterial hypertension and the first ever episode of myocardial infarction (N-STEMI or STEMI) and to determine which of the analyzed kinds of TOD had the highest predictive value for the assessment of the likelihood of acute coronary syndrome (ACS). *Material and Methods*. The study group consisted of 51 patients with treated systemic hypertension, suffering from the first episode of myocardial infarction (N-STEMI or STEMI), confirmed by coronary angiography and elevation of troponin. The control group consisted of 30 subjects with treated hypertension and no history of myocardial ischaemia. In all subjects' measurements of blood lipids, hsCRP and eGFR were measured. TOD, such as intima-media thickness (IMT), presence of atherosclerotic plaques, ankle-brachial index (ABI), and left ventricular hypertrophy, were assessed. *Results*. Age, BMI, blood pressure, and time since diagnosis of hypertension did not differ between the study groups. There were no differences regarding blood lipids and eGFR, while hsCRP was significantly increased in the study group. The left ventricular mass index was similar in both groups. Patients with myocardial infarction had significantly increased IMT and decreased ABI. The statistical analysis revealed that only ABI was the most significant predictor of ACS in the study group. *Conclusion*. Among several TOD, ABI seems to be the most valuable parameter in the prediction of ACS.

1. Introduction

Arterial hypertension is one of the most important modifiable risk factors for cardiovascular complications [1]. In clinical practice, we often find that the extent of cardiovascular damage implies that the actual duration of arterial hypertension is longer than that declared by the patient, possibly due to the long-term asymptomatic course of the disease as well as the cooccurrence of other risk factors for cardiovascular diseases. Therefore, in addition to the presence of risk factors, an important role in global cardiovascular risk assessment is played by identification of subclinical target organ damage (TOD) [2]. TOD such as left ventricular hypertrophy (LVH), atherosclerotic plaque, carotid intima-media thickness (IMT), ankle-brachial index (ABI), pulse wave velocity (PWV), and renal injury features are the best determinants of the condition of the cardiovascular system [3]. In the ESC/ESH guidelines, each TOD has equivalent weight, meaning that slight thickening of the intima-media complex has the same score as advanced left ventricular hypertrophy or hemodynamically significant stenosis within the carotid or lower limb arteries [1].

The objective of our study was to evaluate the incidence of TOD in patients with arterial hypertension and the first ever episode of myocardial infarction (N-STEMI or STEMI) and to determine which of the analyzed kinds of TOD had

Table 1: General characteristics of the study group and the control group subjects.

	Study group ($n = 51$)	Control group ($n = 30$)	p
Mean age (years)	64.84 ± 9.83	64.2 ± 9.34	NS
Mean age, females (years)	69.6 ± 8.73	68.5 ± 6.02	NS
Mean age, males (years)	61.9 ± 9.33	60.4 ± 10.23	NS
BMI (kg/m^2)	27.73 ± 5.06	28.47 ± 5.04	NS
Diabetes	14 (27.45%)	11 (36.6%)	NS
Time since the diagnosis of hypertension (years)	8.54 ± 6.82	8.37 ± 6.9	NS
Smokers	13 (25.49%)	9 (30%)	NS
Systolic blood pressure (mmHg)	129.3 ± 8.2	127.1 ± 7.8	NS
Diastolic blood pressure (mmHg)	77.7 ± 6.1	79.2 ± 6.4	NS

NS: nonsignificant.

the highest predictive value for the assessment of the likelihood of acute coronary syndrome.

2. Material and Methods

2.1. Study Subjects. The study was conducted in a group of 51 patients aged 47 to 80 years, including 32 men and 19 women with arterial hypertension who had experienced their first acute coronary episode. Study group inclusion criteria included the age of ≤80 years, typical anginal pain or changes in ECG records, increased troponin I or T levels, and no history of previous acute coronary syndrome (ACS) episodes. Coronary angiography was performed in all patients confirming the presence of coronary lesions; the time elapsed since the ACS was not longer than 4 days. All patients had been diagnosed with arterial hypertension. The diagnosis of arterial hypertension was based on the use of 1 or more antihypertensive drugs. The average age in the study group was 64.8 years, including 69.6 years in women and 61.9 years in men. The mean BMI was 27.7 kg/m^2; overweight or obesity was diagnosed in 34 (66.6%) patients, including 16 (31.37%) patients diagnosed with obesity. Thirteen (25.49%) patients smoked, and 14 (27.45%) received antidiabetic treatment.

The control group consisted of 30 subjects aged 45 to 78, including 16 men and 14 women treated for arterial hypertension with no history of episodes of myocardial infarction. Just as in the study group, the diagnosis of arterial hypertension was based on the use of 1 or more antihypertensive drugs. The average age in the study group was 64.2 years, including 68.5 years in women and 60.4 years in men. The mean BMI was 28.4 kg/m^2; overweight or obesity was diagnosed in 22 (73.3%) patients, including 12 (40.0%) patients with BMI of 30 kg/m^2 or higher. There were 9 (30%) smokers in the control group. Antidiabetic treatment was received by 11 (36.6%) subjects.

As it was not possible to unambiguously determine the duration of arterial hypertension, time since the diagnosis of arterial hypertension was taken into account and was similar in both groups.

All subjects in the study and the control group received statins as part of primary or secondary prevention.

Qualified patients were informed of the study objectives and expressed their consent to participate. The study was approved by the Bioethics Committee at the Medical University of Lublin.

The characteristics of the study groups are given in Table 1.

2.2. Biochemical Analysis

2.2.1. Laboratory Parameters. Blood samples of all the subjects were collected in the morning before the first meal. Samples of whole blood (5 ml) were collected from the basilic vein into tubes containing ethylenediaminetetraacetic acid tripotassium salt (Sarstedt, S-Monovette with 1.6 mg/ml EDTA-K_3) and into tubes with a clot activator (Sarstedt, S-Monovette).

Total cholesterol, HDL cholesterol, and LDL cholesterol (T-Chol, HDL-Chol, and LDL-Chol) and triglyceride (TG) concentrations in serum were estimated using routine techniques (COBAS INTEGRA 400 plus analyzer, Roche Diagnostics, Mannheim, Germany). Concentrations were expressed in mg/dl. The inter- and intra-assay coefficients of variations (CV) were, respectively, 2.8% and 5.4% for T-Chol, 3.2% and 5.4% for HDL-Chol, 2.6% and 6.5% for LDL-Chol, and 2.5% and 7.6% for TG. Estimated GFR (eGFR) and hsCRP levels were also determined in all patients.

2.3. Estimation of the Ankle-Brachial Index. A Vivid 4 ultrasound system equipped with a 7–10 MHz adjustable frequency vascular transducer was used for ABI assessments. The ABI values were measured after a 5-minute rest in a recumbent position. At the first stage, a color Doppler technique was used to visualize blood flow within the dorsal artery of the foot or, in cases of visualization problems, within the posterior tibial artery. Next, the cuff of a mercury sphygmomanometer previously placed above the ankle of the right lower limb was inflated until the blood flow in the visualized artery stopped. Upon slow deflation of the sphygmomanometer cuff, the value of systolic blood pressure at which the return of blood flow was observed in the examined artery was recorded. In order to verify the measured systolic pressure value, a second measurement was made using the pulsed-wave Doppler method to assess the return of blood flow. Immediately after the color Doppler and pulsed-wave Doppler measurements were completed within the lower

limb, the value of systolic blood pressure at which blood flow returned to the right brachial artery was also recorded. Similar measurements were made on the arteries of the left lower limb and the left upper limb. ABIs were calculated as ratios of the systolic pressure values for ipsilateral lower and upper limb arteries. Separate ABI values were determined for color Doppler and pulsed-wave Doppler measurements. Lower ABI values were used for statistical purposes, with subjects presenting with ABI > 1.3 not being included in statistical analysis [4].

2.4. Echocardiography. Echo scans were acquired on a Vivid 4 ultrasound system with a 2 MHz transducer. During the scan, patients were lying on their left sides. The transducer was placed above the 4th intercostal space near the left edge of the sternum to produce a 2D image of the heart in the parasternal longitudinal view. The following cardiac chamber size and wall thickness measurements were made in M-mode to evaluate the left ventricular mass:

LVEDD: left ventricular end diastolic dimension (mm),

IVSD: interventricular septal thickness at end diastole (mm),

PWD: posterior left ventricular wall thickness at end diastole (mm).

The left ventricular mass (LVM) was calculated using the following formula[5, 6]:

$$\mathrm{LVM} = 1.04 \times [(\mathrm{LVEDD} + \mathrm{IVSD} + \mathrm{PWD})3 - \mathrm{LVEDD}3] - 13.6 \quad (1)$$

The left ventricular mass index (LVMI) was calculated by dividing the left ventricular mass (in grams) by the body surface area (in square meters).

2.5. Estimation of Intima-Media Thickness. The final stage of the study consisted of ultrasound measurements of the carotid intima-media thickness (IMT). Measurements were made using a Vivid 4 ultrasound system with a 7–10 MHz vascular transducer. During the examination, patients were lying on their backs with their heads facing backwards and away from the examination side.

IMT was assessed at the common carotid artery, carotid bulb, and internal carotid artery on the right and the left. Due to better repeatability, measurements were made on distal arterial walls. First, a measurement was made within the common carotid artery about 2 cm distally from the bifurcation site; then, measurements were made at bifurcation and within the internal carotid artery 2 cm proximally from the carotid bulb. The average IMT was calculated separately for the left and the right side from all the above measurements. The presence of atherosclerotic plaque was also assessed, with 1.5 mm being taken as the minimum atherosclerotic plaque thickness.

2.6. Statistical Analysis. Statistical analysis was carried out using the SPSS 14 software package for the MS Windows operating system.

The chi-square compliance test was performed to verify any statistically significant differences in the numbers of subjects within the study groups. The same test was used to verify whether the compared groups differed in terms of the incidence of comorbidities, smoking, and atherosclerotic plaque being present in the carotid arteries.

The Mann-Whitney U test (unequal sample sizes) was performed to assess whether subjects from the study group differed from those in the control group in terms of measurement variables.

At the next stage of the statistical analysis, potential significant correlations between the study variables were verified using Pearson's correlation coefficient.

The final step consisted in a logistic regression analysis with myocardial infarction status as the dependent variable and the measurement variables as predictors (quantitative scale).

Differences at the significance level of $p < 0.05$ were considered statistically significant.

3. Results

The Mann-Whitney U test was performed in order to verify whether the patients with the history of ACS differed from those with no history of coronary incidents in terms of measurement variables.

Patients with the history of ACS were found to present with increased hsCRP levels, decreased ABI values, and carotid intima-media complex thickness. The results of the statistical analysis of the results are presented in Table 2.

A statistically significant difference in the incidence of atherosclerotic plaque was observed. In the study group, the presence of one or more atherosclerotic plaque(s) was observed in 47 patients (92.15%) as compared to 21 patients (70%) in the control group (Figure 1).

The mean LVMI value in the study group was $120.49 \pm 32.21\ \mathrm{g/m^2}$ as compared to $113.53 \pm 25.19\ \mathrm{g/m^2}$ in the control group; the difference was not statistically significant. Significant gender-specific LVMI differences were also sought as different normal LVMI values had been adopted for men and women. No statistically significant differences were observed for the examined variables between the study groups. Results of echocardiographic LVH assessments are presented in Table 3.

Pearson's correlation analysis was used to check whether there were any statistically significant relationships between the examined variables.

The analysis of Pearson's correlation coefficients for the study group revealed a negative correlation between ABI and IMT ($r = -0.40, p < 0.05$). Data are presented in Figure 2.

In order to determine potential correlations between the study variables and the occurrence of myocardial infarction, the Logistic Regression Variable Selection Method was used with myocardial infarction status as the dependent variable and the measurement variables as predictors. Due to the small number of subjects, predictors were entered into the model using the forward selection (Wald) method.

They proved to match the data well: $\chi^2 = 9.77$ and $p = 0.282$. It explained approximately 27.8% of the observed variance of the dependent variable. The model introduced two predictors, CRP and ABI, in two steps (Table 4).

Table 2: Results of measured laboratory and ultrasonography variables.

Parameters	Study group ($n = 51$)	Control group ($n = 30$)	Mean range (study group)	Mean range (control group)	p
hsCRP (mg/l)	14.70 ± 23.81	5.78 ± 9.26	47.19	30.48	0.002
Total cholesterol (mg/dl)	186.39 ± 40.65	174.23 ± 42.75	40.65	42.75	0.231
LDL cholesterol (mg/dl)	111.57 ± 29.5	116.13 ± 61.6	41.74	39.75	0.714
Triglycerides (mg/dl)	141.96 ± 80.96	131.23 ± 111.14	43.82	36.20	0.159
T-Chol/HDL cholesterol	4.20 ± 1.15	3.75 ± 1.41	44.75	34.62	0.061
eGFR (ml/min)	83.22 ± 31.91	78.74 ± 30.39	42.12	39.10	0.577
ABI	0.89 ± 0.25	1.05 ± 0.13	34.68	51.75	0.002
IMT (mm)	1.11 ± 0.13	1.00 ± 0.18	46.31	31.97	0.008
IMT/ABI	1.45 ± 0.87	0.96 ± 0.2	47.44	30.05	0.001
Mean plaque thickness (mm)	2.27 ± 0.99	1.79 ± 1.34	43.33	37.03	0.243

The average thickness of plaque or plaques (if more than one is present) was taken into account for calculation purposes.

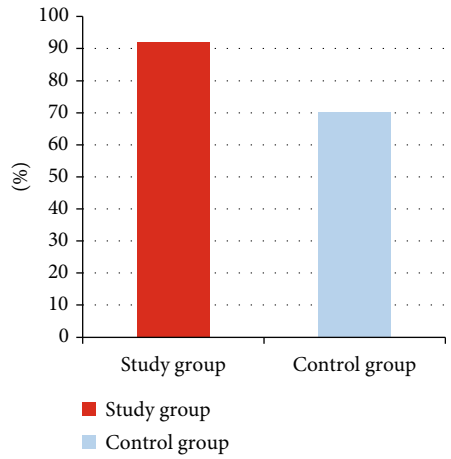

Figure 1: Prevalence of atherosclerotic plaque in carotid arteries ($p = 0.021$).

Table 3: Mean LVMI values in the study and control groups according to gender.

	LVMI (study group) (g/m^2)	LVMI (control group) (g/m^2)	p
Females	112.63 ± 26.15	106.79 ± 22.32	0.689
Males	125.16 ± 34.87	119.44 ± 26.75	0.562

The statistical analysis revealed that only ABI was an important predictor of ACS in the study group. The Exp(B) factor indicates that higher ABI values reduce the likelihood of ACS.

4. Discussion

Along with strokes, acute coronary syndromes belong to the most dramatic episodes in the long-term process of atherosclerosis. The risk factors and organ-related complications associated with higher cardiovascular risk are well defined in the current guidelines of European and American cardiac and hypertension societies [1]. Cardiovascular risk assessment scales included in these guidelines are based on population-based studies and facilitate the classification of patients into different risk categories. Organ-related complications of arterial hypertension included in the global cardiovascular risk assessment, i.e., left ventricular hypertrophy, carotid intima-media thickness, ankle-brachial index, and renal injury features, are taken into account as equivalent to one another. This means that a patient with extensive atherosclerotic lesions within the carotid arteries and reduced ABI is categorized into the same group as a patient with mild left ventricular hypertrophy and eGFR of 50 ml/min/1.73 m^2. The intuitive evaluation of both patients, however, suggests that particular attention should be paid to the patient presenting with features of the extensive atherosclerotic process in the imaging studies. Therefore, it is interesting to answer the question of which kinds of target organ damage due to arterial hypertension can be best used to differentiate the group of patients who have experienced an ACS episode from the group of patients who have hitherto not experienced such an episode.

The main objective of the study was to answer the question of whether any differences can be found in the intensity of asymptomatic target organ damage between the two study groups otherwise homogeneous in terms of anthropometric variables and the incidence of risk factors and comorbidities. Another objective of the study was to identify a potential target organ damage parameter characterized by the highest ACS predictive strength. The selected parameters of target organ damage, highly valued in clinical practice mainly due to their ease of use as well as to the availability of diagnostic tools such as echocardiography and vascular ultrasound, were assessed.

As confirmed by the results, the analysis of generally accepted cardiovascular risk factors such as diabetes, smoking, lipid disorders, overweight, or obesity may by itself not be sufficient for a reliable evaluation of ACS risk level. Therefore, the current risk assessment scales have been expanded to include modules that take into account the presence of target organ damage and comorbidities [7].

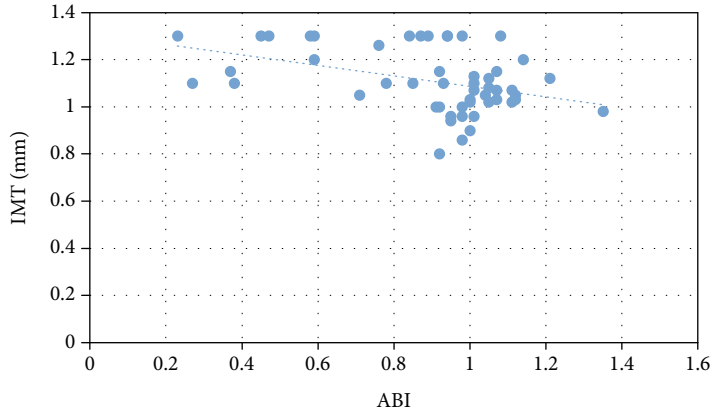

Figure 2: Pearson's correlation between IMT and ABI ($r = -0.40$, $p < 0.05$).

Table 4: Predictors of acute coronary syndrome.

ACS predictors (study group)	B	Wald	p	Exp(B)
hsCRP	0.05	3.14	0.076	1.055
ABI	-5.89	7.15	0.007	0.003

As shown by the analysis of the study results, the study and the control groups differed in a statistically significant manner in terms of ABI, carotid IMT, and IMT/ABI ratio values. A statistically significant difference in the incidence of atherosclerotic plaque within the carotid arteries was identified; the difference, however, did not apply to the average thickness of atherosclerotic plaque. Another finding which emerged following the analysis of the measurement variables was that subjects with a history of myocardial infarction presented with significantly higher levels of C-reactive protein compared to subjects within the control group, suggesting the involvement of inflammation within the vascular wall and atherosclerotic plaque. However, no statistically significant intergroup differences were observed concerning the LVMI values.

In order to determine the factor(s) most strongly associated with ACS, a logistic regression analysis was performed with myocardial infarction status as the dependent variable and measurement variables as predictors. As shown by the statistical method used for that purpose, ABI values of <0.9 are associated with high risk of ACS. The second predictor, albeit of a low prognostic value, was the elevation of C-reactive protein levels.

Numerous studies showed that reduced ABI is associated with the presence of atherosclerotic lesions in the carotid and coronary arteries. According to Criqui and Denenberg, as well as to Dormandy et al., the incidence of coronary artery disease in patients with ABI < 0.9 ranges from 20 to 60% if the diagnosis was based on physical examination, history, and electrocardiogram to as much as 90% in patients subjected to angiographic diagnostics [8–10]. Unfortunately, the sensitivity of ABI is too low for the presence of, e.g., coronary disease to be excluded on the basis of its correct value alone. On the other hand, ABI is specific enough to indicate elevated cardiovascular risk at low ABI values. Therefore, the presence of atherosclerotic lesions within the lower extremity arterial bed expressed as ABI of <0.9 should not be considered an isolated disease but rather an indicator of similar lesions being present in other parts of the circulatory system [8]. The relationships between peripheral arterial disease and cardiovascular mortality are independent of age, BMI, smoking, LDL and HDL cholesterol levels, blood pressure, glycemia, or the history of symptoms of angina pectoris, myocardial infarction, and stroke [11, 12].

However, no explanation is provided by the above data as to why the low ABI values rather than, for example, carotid intima-media thickening had the highest correlation with the incidence of ACS in our study group. In addition, the mean ABI in the study group was 0.89 and thus was only slightly below the value decisive for confirmation or rejection of PAD diagnosis. Perhaps the answer to this question can be provided by demands made by some researchers regarding the arbitrary character of the ABI threshold for the diagnosis of peripheral arterial disease. In normal conditions, systolic blood pressure at the ankle level is 8–15% higher than that at the shoulder level, and therefore, ABI values are considered to be normal when greater than 1.0. In the MESA, SHS, and CHS studies where ABI values in ranges 0.9–0.99 and 1.0–1.09 were considered, respectively, as borderline and low normal, these values were also shown to be associated with an increased risk of death for cardiac reasons [12–14]. The results of these studies suggest that despite the absence of the diagnosis of peripheral arterial disease, patients with ABI values between 0.9 and 1.09 are nonetheless burdened with mild to moderate atherosclerosis which indicates a risk of lesions being present, e.g., in the coronary arteries. In the light of the aforementioned results from large studies, it appears that the stage of atherosclerosis within the lower limb arteries, and therefore in the coronary arteries, of the study group patients was much higher than that suggested by the ABI value used to define peripheral arterial disease (PAD).

An attempt to demonstrate the relationship between ABI and the extent of lesions in the coronary arteries was made by Papamichael et al. [15]. They performed coronary angiography examinations and calculated ABI values in 165 patients to assess the advancement of lesions in patients with stable

coronary disease. The results of this study provided evidence for the existence of correlation between reduced ABI and the extent of lesions in coronary arteries. Of 44 people with three-vessel disease as diagnosed by angiography, 13 had ABI of <0.9; for the sake of comparison, only 4 out of 37 subjects with confirmed one-vessel disease presented with ABI values providing the grounds for the diagnosis of peripheral arterial disease. The logistic regression analysis carried out by the study authors led to the final conclusion that ABI of <0.9 was a predictor of cardiovascular incidents. This result coincides with our findings obtained in a much smaller group. However, does identification of a correlation between low ABI and three-vessel disease justify coronary angiography being performed on a routine basis in each patient with ABI < 0.9? While the answer remains unclear, it seems that such patients should be subject to special monitoring, active screening for other kinds of TOD, and possible qualification for noninvasive studies, such as angio-CT of the coronary arteries.

Another factor identified in the logistic regression analysis as being correlated with the risk of ACS was the elevated concentration of C-reactive protein. For many years, the CRP levels have been associated with the presence of generalized inflammation. CRP is synthesized mainly in the liver, but also in smooth muscles, including those within the walls of the coronary arteries [16]. Ridker et al. claim that of all the acute phase proteins, elevated CRP levels have the strongest association with elevated cardiovascular risk [17]. Pasceri and other researchers demonstrated that C-reactive protein directly contributes to the initiation of the atherogenesis by inducing adhesion molecules on the endothelial cell, opsonizing LDL molecules for their subsequent absorption by macrophages leading to the formation of foam cells, and stimulating and activating monocytes to produce various growth tissue factors [18]. Goldstein et al. observed that elevated CRP levels as determined during hospitalization due to ACS were related to a larger extent of coronary atherosclerotic lesions, increased risk of death, and higher incidence of recurrent myocardial infarction and need for revascularization [19]. These observations were consistent with those obtained in a multicenter study conducted in 1773 patients with acute coronary syndrome. Patients with CRP > 10 mg/l had significantly higher risk of death within 30 days of the coronary incident, regardless of troponin levels [20]. There are 3 levels of risk for cardiovascular events based on hsCRP concentration, namely, low risk for hsCRP < 1.0 mg/l, medium risk for hsCRP of 1.0 to 3.0 mg/l, and high risk for hsCRP > 3 mg/l. On the basis of data from the FHS study, Wilson et al. concluded that only hsCRP > 3.0 mg/l is associated with increased cardiovascular risk [21]. However, it should be noted that the mean CRP levels in our control group were higher than 3 mg/l. Perhaps higher cut-off values should be adopted for prognostic purposes as observed in the study by Oltrona et al. [20].

When evaluating the results of our study, we also decided to verify whether the combined use of two hypertension-related target organ damage markers, i.e., ABI and carotid IMT, would differentiate the study group from the control group in a statistically significant manner. The analysis using the Mann-Whitney U test revealed an intergroup difference at the significance level of $p = 0.001$ which means that the IMT/ABI ratio was significantly lower in the control group as compared to the study group. At present, it is difficult to conclude whether the predictive value of this "complex" parameter could be higher than the predictive value of either parameter assessed separately. No mention has been found in the available literature on the use of complex risk assessment markers that would simultaneously describe the stage of atherosclerosis at different levels of the arterial bed. Instead, attempts were made to create a scoring system based on ABI and IMT values. On the basis of their research, Hayashi et al. assumed that correct ABI and IMT values would be assigned the score of 0 while abnormal values would be assigned the score of 1. Next, they divided their study population into 3 groups depending on the score. A total score of 0 was assigned when the patient had presented with unremarkable ABI and IMT values, a total score of 2 was assigned when both markers were abnormal, and a total score of 1 was assigned when only one of the marker values was unremarkable. The conclusion stemming from the use of this simple scoring system was that the incidence of cardiovascular diseases was statistically significantly higher in subjects who scored 1 or 2 points [22]. This means that the lesion on the arterial bed should be sought at many levels, as identification of their absence in only one part of the cardiovascular system may lead to incorrect evaluation of a patient's condition and, therefore, to a failure to implement appropriate management.

The results are indicative of the high value of ABI measurements in the assessment of ACS risk in patients with arterial hypertension. Notably, none of the subjects within the study or the control group reported any signs of intermittent claudication or had previously undergone diagnostic screening for peripheral arterial disease. This is due to the fact that the symptomatic course of the disease is observed in patients with very advanced atherosclerotic lesions due to the development of collateral circulation. Postexercise drops in systolic blood pressure values as observed in exercise tests are even better for identification of disturbed supply of blood to the lower limbs [23]. Perhaps postexercise ABI values would be the strongest predictor of ACS in our study group. However, sensitive postexercise assessment of blood pressure within the lower limb arteries is also a method which cannot be used in general clinical practice, particularly in primary care.

With no doubt, very interesting information could be provided by prospective observation of patients with arterial hypertension and by identification of target organ damage parameter that would, either alone or combined, ensure the best prediction of future coronary episodes. It cannot be ruled out that acute coronary episodes would occur in the near future in all control group subjects. However, it is important to demonstrate that despite the same risk factors, arterial beds may differently respond to the same damaging stimuli, obviously as a consequence of individual traits. Thus, "individualization" of cardiovascular risk assessments becomes particularly important today. Further studies are needed to assess the predictive strength of individual TOD parameters, possibly facilitating the development of new

scales which would differentiate the predictive values of individual TOD parameters. Our study shows that from among numerous easily measurable TOD parameters, the ankle-brachial index has a very high ACS predictive value. Therefore, as well as for the ease of its estimation, it should be particularly recommended for practitioners who encounter the problem of identifying high cardiovascular risk patients in their everyday practice.

5. Conclusions

Our study originated from doubts regarding the equivalence of target organ damage parameters as used in the assessment of cardiovascular risk. From among numerous kinds of total organ damage examined in the study, the ankle-brachial index was the only strong predictor of acute coronary syndrome in our study population.

Data Availability

All data are included in the tables within the article.

Conflicts of Interest

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

Acknowledgments

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Review Article

Advances in Exosomes Derived from Different Cell Sources and Cardiovascular Diseases

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Exosomes can reach distant tissues through blood circulation to communicate directly with target cells and rapidly regulate intracellular signals. Exosomes play an important role in cardiovascular pathophysiology. Different exosomes derived from different sources, and their cargos have different mechanisms of action. In addition to being biomarkers, exosomes also have a certain significance in the diagnosis, treatment, and even prevention of cardiovascular diseases. Here, we provide a review of the up-to-date applications of exosomes, derived from various sources, in the prognosis and diagnosis of cardiovascular diseases.

1. Introduction

Exosomes are endosomal-derived vesicles that play a critical role in cell-to-cell communication and are secreted in several biological fluids including serum, saliva, urine, ascites, and cerebrospinal fluid among others [1]. Exosomes are small (30~150 nm diameter) with a distinctive bilipid protein structure. They can carry and exchange various cargos between cells and are used as a noninvasive biomarker for several diseases. Moreover, exosomes are considered the best biomarkers for disease diagnosis, owing to their unique characteristics [2].

Recent studies have shown that the number of exosomes, exosomal encoded and noncoding RNA, and exosomal proteins may act as biomarkers for the diagnosis and prognosis of cardiovascular diseases [3], whose death brings a great threat to global health [4, 5]. Some heart protection strategies have been shown to increase the number of exosomes in the blood [6–8]. The release of exosomes in the circulation of rats with the ischemia-reperfusion model increased significantly [9], in which the main exosomes containing HSP-60 were found [10]. A group of exosomes rich extracellular vesicles purified from the blood and proved to protect rat heart and myocardial cells from acute ischemia and reperfusion injury when administered *in vivo* or *in vitro* [9]. The exosomes containing HSP70 exist in the outer membrane of normal cardiac myocytes, which can activate the MAPK/ERK1/2 signal pathway through interaction with the TLR4 receptor and thus play a role in myocardial protection [9]. Exosomes rich in miR-143 and miR-145 have atherosclerotic protective effects in mouse models [11]. In addition, the exosomes containing miR-208a are related to the level of cardiac troponin I after coronary artery bypass grafting, while miR-208a in the blood circulation does not find this relationship, which indicates that the exosomal miRNAs may be helpful for the diagnosis of cardiovascular diseases [12]. Moreover, miR-192 and miR-194 in exosomes are also considered as prognostic biomarkers of cardiovascular diseases [13]. It is worth noting that miR-34a is highly expressed in myocardial infarction and preferentially integrated into the exosomes derived from cardiomyocytes and fibroblasts [14], making it possible for early diagnosis of myocardial infarction.

Here, we critically review the advances in exosomes derived from different cell sources and cardiovascular diseases (Figure 1), and we mainly highlight the exosomes derived from cardiomyocytes, cardiac progenitor cells, fibroblasts, and mesenchymal stem cells, which communicate intensively to facilitate proper cardiac function through

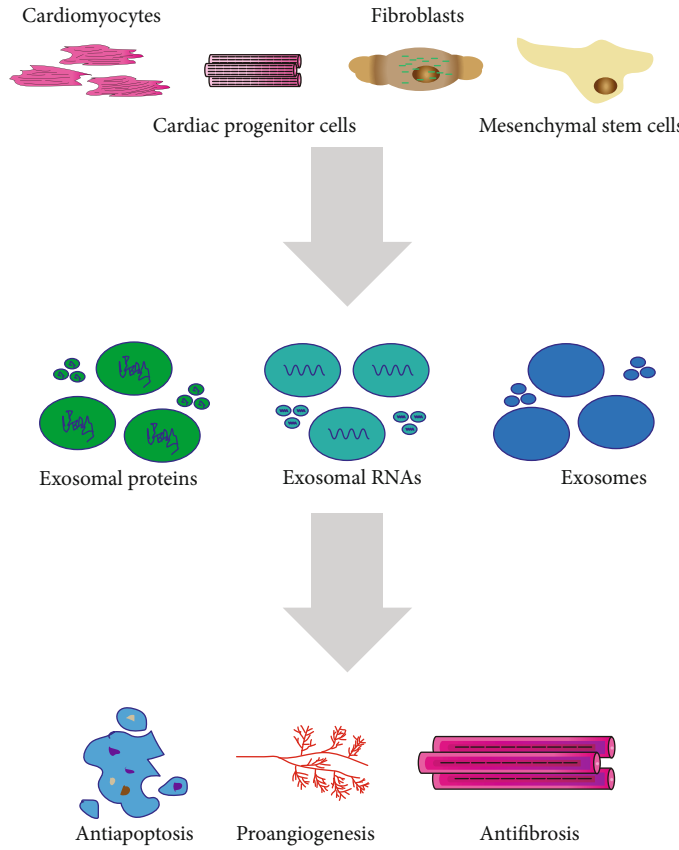

Figure 1: Exosomes derived from different cell sources and cardiovascular diseases.

direct cell-cell contact and paracrine interactions [15, 16]. This knowledge may be helpful to promote and develop diagnostic markers and therapeutic approaches, which may be beneficial to the management of cardiovascular patients.

2. Exosomes Derived from Different Cell Sources

2.1. Exosomes Derived from Cardiomyocytes. Exosomes derived from cardiomyocytes were initially found under hypoxia and reoxygenation conditions and may contain biological molecules [17, 18]. HSPs, which play essential roles in cellular survival and adaptation under numerous stresses [19], are found to be enriched in cardiac exosomes. HSP20 contained in cardiomyocyte-derived circulating exosomes is considered to be a new type of cardiac motility factor, which can promote the formation of myocardial neovascularization by activating VEGFR2 [20] and AKT signaling pathway, repressing TNF-α and IL-1β factors to alleviate myocardial infarction [21]. HSP60, which is thought to be a danger signal to the immune system and is also highly immunogenic [22], is released via exosomes, and that within the exosome, HSP60 is tightly attached to the exosome membrane [23]. The cardiomyocyte-derived exosomes are enriched for HSP70 involved in regulating cardiomyocyte growth and survival under stress [16]. The myocyte-derived HSP90 orchestrates not only p65-mediated IL-6 synthesis but also its release in exosomal vesicles, and such exosomes and myocyte-secreted IL-6 are responsible in unison for the biphasic activation of STAT-3 signaling in cardiac fibroblasts that culminates in excess collagen synthesis, leading to severely compromised cardiac function during cardiac hypertrophy [24]. TNF-α can also be isolated from cardiomyocyte-derived exosomes and interact with HIF-1α to contribute to cardiac remodeling [25]. Furthermore, exosomes derived from cardiomyocytes were found to carry functional GLUT (GLUT4 and GLUT1) and glycolytic enzymes (lactate dehydrogenase) and were shown to have specialized functions in glucose transport and metabolism in endothelial cells [26].

Many studies have demonstrated that exosomes derived from cardiomyocytes can also carry DNA/RNA [19]. Exosomes secreted by cells derived from cardiomyocytes can improve myocardial function, inhibit cell apoptosis, and promote the proliferation of cardiomyocytes when expressed in a mouse myocardial infarction model, which may be related to the enhancement of the expression of vascular gene SIS [27]. These effects can also be achieved by increasing the expression of miR-146a, which is significantly enriched in exosomes [27]. In addition, in an adriamycin-induced dilated cardiomyopathy mouse model, systemic administration of these exosomes can reduce apoptosis and fibrosis [28]. Cardiomyocytes exert an antiangiogenic function in type 2 diabetic rats through an exosomal transfer of miR-320 into endothelial cells [29]. MiR-30a, which is derived from hypoxic cardiomyocytes, is efficiently transferred between cardiomyocytes

via exosomes and regulates autophagy by affecting the expression of Beclin-1, ATG12, and the ratio of LC3II/LC3I, which are important regulators of autophagy [30]. Exosomes from cardiomyocytes were enriched for certain miRNAs (particularly miR-29b, miR-323-5p, miR-455, and miR-466) and mediated the regulation of MMP9, which is involved in matrix degradation and leads to fibrosis and myocyte uncoupling [31]. Additionally, miR-208a was upregulated in cardiomyocyte-derived exosomes and contributed to increased fibroblast proliferation and differentiation into myofibroblasts via targeting Dyrk2 [32]. In a different way, exosomes from the cardiac spheroid are used to activate fibroblasts, which can increase the secretion of angiogenic factor, SDF1 and VEGF by fibroblasts [33, 34]. When injected into the heart of chronic myocardial perfusion model rats, activated fibroblasts were found to significantly promote angiogenesis and cardiac protection [34]. Recent evidence established that exosomes secreted from cardiomyocytes can deliver a wide variety of biomolecules into other types of cells and regulate gene expression in these cells [19].

2.2. Exosomes Derived from Cardiac Precursor Cells. Exosomes of cardiac precursor cells can be obtained from auricular implants of patients undergoing valve surgery [35]. When these exosomes were used in myocardial infarction model rats, it was found that myocardial apoptosis decreased, angiogenesis increased, and left ventricular ejection fraction improved significantly [36]. Saphenous vein-derived pericyte progenitor cells transplantation produces a long-term improvement of cardiac function through a novel paracrine mechanism involving the secretion of miR-132 and inhibition of its target genes [37]. Microarray analysis of exosomes secreted by hypoxic cardiac precursor cells identified 11 miRNAs that were upregulated compared with exosomes secreted by cardiac precursor cells grown under normoxic conditions, and those miRNAs improved cardiac function and reduced fibrosis [38]. Cardiac precursor cell-derived exosomes have a high-level expression of GATA4-responsive-miR-451 and can protect H9C2 from oxidative stress by inhibiting caspase 3/7 activation *in vitro* and inhibit cardiomyocyte apoptosis *in vivo* [39]. Regardless of the type of stem cells used, most cells die or lose shortly after implantation. In order to solve this problem, some researchers found that cotransfection of cardiac progenitor cells and nonviral small ring plasmids carrying HIF1 can improve their resistance to hypoxia [40]; this cotransfection resulted in high expression of HIF1 in endothelial cells, production of exosomes with a high content of miR-126 and miR-210, active internalization by receptor cardiac progenitor cells, activation of kinase, and induction of glycolysis [40]. Cardiac precursor cell-derived exosomal miR-21 had an inhibiting role in the apoptosis pathway through downregulating PDCD4 [41]. Moreover, miR-21 bonded with PTEN and suppressed PTEN expressions to downregulate both infarction size and injury marker expressions *in vivo* and promote endothelial cell proliferation, inhibit apoptosis, and stimulate angiogenesis *in vitro* [42]. Human cardiac precursor cell-derived exosomes were highly enriched in miR-146a-5p and attenuated doxorubicin/trastuzumab-induced oxidative stress in cardiomyocytes through suppressing miR-146a-5p target genes Traf6, Smad4, Irak1, Nox4, and Mpo [43]. Additionally, miR-133a-cardiac progenitor cells clearly improved cardiac function in a rat myocardial infarction model by reducing fibrosis and hypertrophy and increasing vascularization and cardiomyocyte proliferation [44]. The cardiac precursor cell-secreted exosomes promote the infarct healing through the improvement of cardiomyocyte survival and angiogenesis, and the cardioprotective effects can be enhanced by hypoxia conditioning of cardiac precursor cells and are partially contributed by MALAT1 via targeting the miRNA [45]. In contrast, exosomes from cardiac progenitor cells have also been shown to stimulate cell migration [46].

2.3. Exosomes Derived from Fibroblasts. The results showed that the exosomes derived from fibroblasts rich in miR-21-3p could induce cardiomyocyte hypertrophy by targeting SORBS2 and PDLIM5. Inhibition of miR-21-3p reduced cardiac hypertrophy in animals treated with Ang II [47]. Besides, circulatory miR-29 and miR-30 are considered as biomarkers of left ventricular hypertrophy, the correlation of circulatory miR-21 in the diagnosis and prognosis of cardiac hypertrophy deserves further study [48]. In addition, exosomes extracted from endothelial cells expressing KLF2 can attenuate the formation of atherosclerosis [49]. It is also important that exosomes from atherosclerotic plaques and macrophages from peripheral blood participate in the development of atherosclerosis [50]. Patients with atherosclerosis have a higher level of extracellular vesicles derived from leukocyte compared with healthy participants [51].

2.4. Exosomes Derived from Mesenchymal Stem Cells. The exosomal proteins derived from mesenchymal stem cells can reduce the infarct area by half [52], inhibit the proliferation and migration of vascular smooth muscle [53], reduce cardiomyocyte apoptosis, promote angiogenesis, reduce ventricular remodeling, and protect cardiac function [54, 55]. Exosomes isolated from macrophage migration inhibitory factor-pretreated mesenchymal stem cells protected cardiomyocytes from apoptosis [56] through the lncRNA-NEAT1/-miR-142-3p/FOXO1 signaling pathway [57]. At the same time, the exosomes derived from mesenchymal stem cells can also reduce the levels of inflammatory factors, such as IL-6 and MCP-1 [53], through activating the signal pathways involved in IGF-1/PI3K/Akt and GSK-3p [52, 54, 58, 59]. In the hypoxia-induced pulmonary hypertension mouse model, intravenous infusion of the exosomes derived from mesenchymal stem cells can also inhibit vascular remodeling and hypertension, which may be achieved by inhibiting the STAT3 signaling pathway [60]. Another interesting study confirmed the potential of mesenchymal stem cell-derived exosomes in reversing pulmonary hypertension in mice and further showed that exosomes from drug-induced pulmonary hypertension mice can induce pulmonary hypertension when injected into nondiseased animals [61]. These differences may be related to different miRNA expression patterns of exosomes.

Stem cells can be injected into the heart muscle to prevent myocardial ischemia and reperfusion injury and improve myocardial function through repair and gradual regeneration [62]. Transplantation of human $CD34^{+ve}$ hematopoietic stem cells into ischemic tissue can induce neovascularization in preclinical models, which has been proved to be related to the treatment of angina pectoris and the improvement of exercise time in phase II clinical trials. However, the benefits in these experiments seem to depend more on the effect of paracrine signals than on stem cell transplantation of cardiomyocytes [63]. At present, exosomes have been used to regulate paracrine benefits [16, 64]. In fact, the exosomes of $CD34^{+ve}$ have angiogenic activity *in vitro* and *in vivo*. Interestingly, although the benefits of $CD34^{+ve}$ exosomes on angiogenesis have been observed, $CD34^{+ve}$ hematopoietic stem cells do not have cardioprotective effects in the expression of the SHH gene after acute myocardial infarction [65]. Injection of exosomes from embryonic stem cells into the heart of mice with myocardial infarction increased neovascularization and cardiomyocyte survival and reduced fibrosis, which is related to the transmission of miR-294 and C-kit^{+ve} cardiac progenitor cells in the myocardium, thus increasing their regeneration activity [66]. In the peripheral blood of patients with chronic heart failure, $CD34^{+}$ stem cell-derived exosomes rich in angiogenesis-related miR-126 and miR-130a were significantly reduced [67].

3. Future Perspectives

Exosomes act as messengers of intercellular communication among cardiomyocytes, fibroblasts, smooth muscle cells, and endothelial cells and participate in the regulation of cardiac regeneration, ventricular remodeling, and angiogenesis in cardiovascular diseases [68]. Because of its perfect properties as a carrier of signal molecules, circulating exosomes transmit both protective and harmful information [69, 70]. Since it is difficult to obtain the heart tissue samples of patients, it may be a useful strategy to detect the changes of exosomes in peripheral blood circulation to obtain the pathophysiological process information of cardiovascular diseases [8, 71] and guide the treatment of patients [61, 72]. In this new and exciting field of exosome research, there are still many problems. In contrast, there are relatively few studies on exosomal protein content and its significance in diagnosis, treatment, and prevention, although they have been proved to be accurate prognostic tools for predicting negative cardiovascular events, including $CD31^{+}$/Annexin V^{+} and CD144-positive exosomes [73, 74]. In addition, the preparation and purification of exosomes have some limitations. Biologically, the exact mechanism of exosomes remains to be verified. The ability to study the role of exosomes *in vivo* will be greatly enhanced by the discovery of a specific production or absorption inhibitor. In terms of treatment, a better understanding of the pharmacokinetics and pharmacodynamics of exosomes is essential. All available preclinical and clinical data strongly support the hypothesis that exosomes have therapeutic value or play an important role in the treatment of many diseases [75].

The release of exosomes can also be regulated by different exogenous stimuli. Inflammatory response following myocardial infarction dramatically increased the number of circulating exosomes carrying alarmins such as IL-1α, IL-1β, TNF-α, and Rantes [76, 77]. In fact, recent studies demonstrated that exosome numbers may be increased by some specific chemical compounds. Monensin induces the formation of exosome and stimulates exosome secretion in K562 cells [78], which are human myeloid leukemia cells that secrete exosomes [79], in a Ca^{2+}-dependent manner through an increase in transferrin [78]. Quantitative analysis demonstrates activation of histamine H1 receptor in HeLa cells which increases Ser110 phosphorylation of SNAP23, promoting multivesicular body-plasma membrane fusion and the release of CD63-enriched exosomes [80]. In addition to compounds, some drugs can also affect the secretion of exosomes. Atorvastatin enhances the numbers of exosomes derived from mesenchymal stem cells, which improve cardiac function and promote blood vessel formation, thus enhancing the therapeutic efficacy for myocardial infarction [81]. Ticagrelor, another commonly prescribed for cardiovascular diseases, can be leveraged to modulate the release of antihypoxic exosomes from resident human cardiac-derived mesenchymal progenitor cells through acute phosphorylation of ERK42/44 [82]. Besides, recent studies have shown that exosomes are also a safe alternative to drug delivery systems [83]. Ideally, a method of targeting the heart or target cells is needed to avoid intramyocardial or nonspecific systemic administration. Despite these barriers, the potential of exosomes as a therapeutic drug for cardiovascular diseases is still exciting, and there has been a blueprint for treatment based on extracellular vesicles in clinical trials [84]. However, it is important to do a lot of research on exosomes.

Exosomes can transfer proteins, RNAs, and other bioactive molecules to recipient cells to influence their biological properties [85], suggesting the potential functional diversity of exosomes [86]. The exosomes from different cardiac cells deliver a specific protein or RNA. The cardiac precursor cell-secreted exosomes rich in PAPP-A had a cardioprotection profile through releasing IGF-1 via proteolytic cleavage of IGFBP-4, resulting in IGF-1R activation and intracellular Akt and ERK1/2 phosphorylation [87]. It was found that exosomes secreted by endothelial progenitor cells derived from patients with coronary atherosclerotic heart disease could inhibit the migration and angiogenesis through expressing more miR-146a-5p and miR-146b-5p [88]. Another study indicated that normoxia endothelial progenitor cell-derived exosomes rich in miR-10b-5p could significantly ameliorate cardiac fibroblast activation *in vitro* [89].

Increasing studies also have shown that exosomes play an active role in patients with cardiovascular crisis and patients with complications. Acute myocardial infarction is a common critical disease of cardiovascular diseases. Exosomes released by chronically hypoxic cardiac precursor cells deliver a pool of miRNAs (miR-15b, miR-17, miR-20a, miR-103, miR-199a, miR-210, and miR-292) that enhance angiogenesis, reduce profibrotic gene expression, preserve myocardial contractile function, and improve cardiac function in the early hours after the onset of acute myocardial

Table 1: Summary of exosomes derived from different cell sources in cardiovascular diseases.

Source	Cargos	Biological effects	Evidences	References
Cardiomyocytes	HSP20	Promote angiogenesis by activating VEGFR2, and activate AKT signaling pathway and repress TNF-α and IL-1β factors to alleviate myocardial infarction	Preclinical evidences (*in vivo* and *in vitro*)	[20, 21]
	HSP60	Promote immune responses	Preclinical evidences (*in vitro*)	[23]
	HSP70	Activate monocytes alone, resulting in monocyte adhesion to endothelial cells; improve cardiac function	Preclinical evidences (*in vitro*)	[16, 17]
	HSP90 and IL-6	Active STAT-3 signaling in cardiac fibroblasts that culminates in excess collagen synthesis, leading to severely compromised cardiac function during cardiac hypertrophy	Preclinical evidences (*in vivo* and *in vitro*)	[24]
	TNF-α	Interact with HIF-1α to contribute to cardiac remodeling	Preclinical evidences (*in vitro*)	[25]
	GLUT	Increase glucose transport	Preclinical evidences (*in vitro*)	[26]
	miR-15b, miR-17, miR-20a, miR-103, miR-199a, miR-210, and miR-292	Enhance angiogenesis, reduce profibrotic gene expression, preserve myocardial contractile function, and improve cardiac function	Preclinical evidences (*in vivo* and *in vitro*)	[90]
	miR-29b, miR-323-5p, miR-455, and miR-466	Mediate the regulation of MMP9, which is involved in matrix degradation and leads to fibrosis and myocyte uncoupling	Preclinical evidences (*in vitro*)	[31]
	miR-30a	Regulate autophagy by affecting the expression of Beclin-1, ATG12, and the ratio of LC3II/LC3I	Preclinical evidences (*in vitro*)	[30]
	miR-34a	Biomarkers of myocardial infarction	Preclinical evidences (*in vitro*)	[14]
	miR-146a	Inhibit apoptosis and promote proliferation of cardiomyocytes, while enhancing angiogenesis	Preclinical evidences (*in vivo* and *in vitro*)	[27]
	miR-208a	Increase fibroblast proliferation and differentiation into myofibroblasts via targeting Dyrk2	Preclinical evidences (*in vivo* and *in vitro*)	[32]
	miR-320	Inhibit proliferation, migration, and tube-like formation	Preclinical evidences (*in vivo* and *in vitro*)	[29]
	miR-451	Protect H9C2 from oxidative stress by inhibiting caspase 3/7 activation and inhibit cardiomyocyte apoptosis	Preclinical evidences (*in vivo* and *in vitro*)	[39]
	NA	Reduce apoptosis and fibrosis	Preclinical evidences (*in vivo* and *in vitro*)	[28]
	NA	Activate fibroblasts, which can increase the secretion of angiogenic factor, SDF1 and VEGF by fibroblasts	Preclinical evidences (*in vivo* and *in vitro*)	[33, 34]
	NA	Promote angiogenesis and cardiac protection	Preclinical evidences (*in vivo* and *in vitro*)	[34]
Cardiac progenitor cells	PAPP-A	Cardioprotection profile through releasing IGF-1 via proteolytic cleavage of IGFBP-4, resulting in IGF-1R activation, intracellular Akt and ERK1/2 phosphorylation	Preclinical evidences (*in vivo* and *in vitro*)	[87]
	miR-15b and miR-20a	Stimulate angiogenesis	Preclinical evidences (*in vivo* and *in vitro*)	[38]
	miR-17 and miR-103	Promote angiogenesis, inhibit myocardial fibrosis	Preclinical evidences (*in vivo* and *in vitro*)	[38]

Table 1: Continued.

Source	Cargos	Biological effects	Evidences	References
	miR-21	Inhibit cardiomyocyte apoptosis through downregulating PDCD4; downregulate both infarction size and injury marker expressions *in vivo* and promote endothelial cell proliferation, inhibit the apoptosis, and stimulate angiogenesis *in vitro* by targeting PTEN	Preclinical evidences (*in vivo* and *in vitro*)	[41, 42]
	miR-126 and miR-210	Active kinase and induce glycolysis	Preclinical evidences (*in vivo* and *in vitro*)	[40]
	miR-132, miR-210, and miR-146a-3p	Decrease myocardial apoptosis, increase angiogenesis, and improve left ventricular ejection fraction	Preclinical evidences (*in vivo* and *in vitro*)	[27, 36, 37]
	miR-133a	Improve cardiac function by reducing fibrosis and hypertrophy and increasing vascularization and cardiomyocyte proliferation	Preclinical evidences (*in vivo* and *in vitro*)	[44]
	miR-146a-5p	Attenuate doxorubicin/trastuzumab-induced oxidative stress in cardiomyocytes through suppressing target genes Traf6, Smad4, Irak1, Nox4, and Mpo	Clinical evidences	[43]
	miR-181a and miR-323-5p	Promote angiogenesis	Preclinical evidences (*in vivo* and *in vitro*)	[27, 36]
	miR-210	Promote angiogenesis, inhibit cardiomyocyte apoptosis, improve heart function	Preclinical evidences (*in vivo* and *in vitro*)	[38]
	lnc RNA MALAT1	Promote the infarct healing through improvement of cardiomyocyte survival and angiogenesis by targeting the miRNA	Preclinical evidences (*in vivo* and *in vitro*)	[45]
	NA	Stimulate cell migration	Preclinical evidences (*in vitro*)	[46]
Fibroblasts	miR-21-3p	Induce cardiomyocyte hypertrophy by targeting SORBS2 and PDLIM5	Preclinical evidences (*in vivo* and *in vitro*)	[47]
	miR-21, miR-29, and miR-30	Biomarkers of left ventricular hypertrophy	Preclinical evidences (*in vivo* and *in vitro*)	[48]
	miR-34a	Biomarkers of myocardial infarction	Preclinical evidences (*in vitro*)	[14]
Mesenchymal stem cells	NA	Reduce the infarct area, inhibit the proliferation and migration of vascular smooth muscle, reduce cardiomyocyte apoptosis, promote angiogenesis, reduce ventricular remodeling, and protect cardiac function	Preclinical evidences (*in vivo* and *in vitro*)	[52–55]
	NA	Protect cardiomyocytes from apoptosis through lncRNA-NEAT1/miR-142-3p/FOXO1 signaling pathway	Preclinical evidences (*in vitro*)	[56, 57]
	NA	Reduce the levels of inflammatory factors, such as IL-6 and MCP-1, through activating the signal pathways involved in IGF-1/PI3K/Akt and GSK-3p	Preclinical evidences (*in vivo* and *in vitro*)	[52–54, 58, 59]
	NA	Inhibit vascular remodeling and hypertension by inhibiting STAT3 signaling pathway	Preclinical evidences (*in vivo* and *in vitro*)	[60]
	NA	Reverse pulmonary hypertension	Preclinical evidences (*in vivo*)	[61]
	miR-126 and miR-130a	Biomarkers of chronic heart failure	Clinical evidences	[67]
	miR-294	Increase neovascularization, cardiomyocyte survival, and reduce fibrosis	Preclinical evidences (*in vivo* and *in vitro*)	[66]

Table 1: Continued.

Source	Cargos	Biological effects	Evidences	References
Endothelial cells	KLF2	Attenuate the formation of atherosclerosis	Preclinical evidences (*in vivo* and *in vitro*)	[49]
	miR-10b-5p	Ameliorate cardiac fibroblast activation	Preclinical evidences (*in vitro*)	[89]
	miR-146a-5p and miR-146b-5p	Inhibit the migration and angiogenesis	Preclinical evidences (*in vitro*)	[88]
Macrophages and leukocyte	NA	Promote vascular smooth muscle cells migration and adhesion, which may be mediated by the integration of extracellular vesicles into vascular smooth muscle cells and the subsequent downstream activation of ERK and Akt	Preclinical evidences (*in vitro*)	[51]
		Biomarkers of atherosclerosis	Clinical evidences	[50]
Cardiac stromal cells	miR-21-5p	Contribute to heart repair by enhancing angiogenesis and cardiomyocyte survival through the phosphatase and tensin homolog/Akt pathway	Pre-clinical evidences (*in vivo* and *in vitro*)	[93]

infarction [90]. Heart failure is the end-stage state of various cardiovascular diseases [91, 92], and heart failure pathological condition altered the miRNA cargos of cardiac-derived exosomes and impaired their regenerative activities. It may be related to miR-21-5p, the exosome released from explant-derived cardiac stromal cells, contributing to heart repair by enhancing angiogenesis and cardiomyocyte survival through the phosphatase and tensin homolog/Akt pathway [93]. Both heart failure and diabetes independently increase the morbidity of another disease and associated with considerable mortality [94, 95]. Diabetic cardiomyocytes exhibited increased secretion of detrimental exosomes containing decreased HSP20 levels, which contributed to diabetes-induced organ damage [96]. In a transgenic mouse model with cardiac-specific overexpression of HSP20, overexpression of HSP20 significantly attenuated cardiac dysfunction, hypertrophy, apoptosis, fibrosis, and microvascular rarefaction, in other words, protected against *in vivo* cardiac adverse remodeling by increasing generation/secretion of exosomes, indicating that elevation of HSP20 in cardiomyocytes can offer protection in diabetic hearts through the release of instrumental exosomes [96]. Poststroke cardiac complications are common, and diabetes exacerbates poststroke cardiac injury. In type 2 diabetes mellitus-stroke mice, exosomes harvested from human umbilical cord blood-derived $CD133^+$ cell treatment significantly increased miR-126 expression in the heart and decreased its target gene expression and decreased myocardial cross-sectional area, interstitial fibrosis, TGFβ, numbers of M1 macrophages, and oxidative stress markers 4-HNE and NOX2 in heart tissue, meaning that exosome treatment significantly improves cardiac function [97]. Additionally, uremic cardiomyopathy contributes to chronic kidney disease-induced morbidity and mortality. Overexpression of exosome-encapsulated miR-26a in muscle attenuated cardiomyopathy via exosome-mediated miR-26a transfer, which may be related to decreased the upregulation of FBXO32/atrogin-1 and TRIM63/MuRF1 and depressed cardiac fibrosis lesions [98]. Although exosomes show exciting and gratifying cardioprotection effects, it should be noted that the evidences of this cardioprotection are mostly from *in vivo* or *in vitro*, which still needs to be verified in the clinic.

4. Conclusion

In this review, ample preclinical and biomedical data were summarized (Table 1), which can provide a reference for the study of exosomes and their application in the diagnosis and prognosis of cardiovascular diseases. Although the current evidences show that exosomes derived from different cell sources are helpful for the diagnosis and prognosis of cardiovascular diseases, most of the evidences come from preclinical studies; solid clinical data are urgently needed. Moreover, there are some limitations in the preparation and purification of exosomes, and their exact mechanisms of action still need to be validated. These challenges need to be addressed before exosomes can proceed to clinical application.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Roles and Clinical Applications of Exosomes in Cardiovascular Disease

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Despite substantial improvements in therapeutic strategies, cardiovascular disease (CVD) is still among the leading causes of mortality and morbidity worldwide. Exosomes, extracellular vesicles with a lipid bilayer membrane of endosomal origin, have been the focus of a large body of research in CVD. Exosomes not only serve as carriers for signal molecules responsible for intercellular and interorgan communication underlying CVD pathophysiology but also are bioactive agents which are partly responsible for the therapeutic effect of stem cell therapy of CVD. We here review recent insights gained into the role of exosomes in apoptosis, hypertrophy, angiogenesis, fibrosis, and inflammation in CVD pathophysiology and progression and the application and mechanisms of exosomes as therapeutic agents for CVD.

1. Introduction

Cardiovascular disease (CVD), with a global prevalence of 10.6%, imposes a large health and economic burden [1]. Numerous intra- and extracellular factors, biochemical complexes, and vesicles have been found to participate in regulating the pathophysiological progression of CVD, and studies have suggested that exosomes play important roles in it as well. Exosomes are a subgroup of extracellular vesicles surrounded by a lipid bilayer membrane of endosomal origin and ranging in size from 40 to 160 nm (average~100 nm) [2, 3]. The contents of exosomes include lipids, proteins, mitochondrial DNA, mRNAs, and noncoding RNAs, which are constantly changing both in quality and in quantity according to the microenvironment where the parent cells are [4, 5]. Exosomes are important in intercellular and interorgan communication by delivering signal molecules to recipient cells and regulating downstream signal pathways which have been associated with CVD progression [6, 7]. Moreover, exosomes released by progenitor cells are bioactive and are the key mediators of stem cell therapy of CVD, which simultaneously overcome some limitations of stem cell therapy [8–10].

In this review, we outline the role and function of exosomes in CVD pathophysiological progression and focus on their use to repair the cardiac injury.

2. Exosomes in Cardiovascular Pathophysiology

CVD progression entails a series of basic pathological changes, including cardiomyocyte apoptosis and hypertrophic growth, angiogenesis, cardiac fibrosis, and inflammation. Exosomes play a critical role in regulating CVD progression via transport and exchange of signal molecules [11, 12] (see Figure 1).

2.1. Apoptosis. The apoptosis of cardiomyocytes is a critical event in CVD progression, including myocardial infarction (MI) and heart failure. By transporting signal molecules between cardiomyocytes and other cells and organs, exosomes

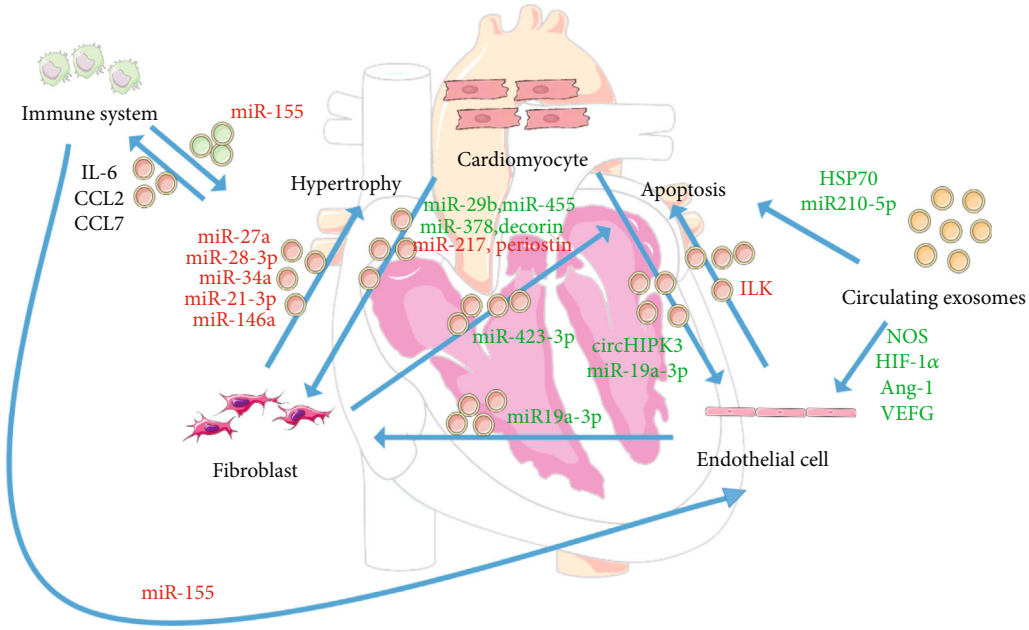

Figure 1: The roles of exosomes in the pathophysiology of cardiovascular diseases. Exosomes serve as the messengers exchanging bioinformation among cardiomyocytes, fibroblasts, endothelial cell, and the immune system. The molecules transported by exosomes regulate the hypertrophy, apoptosis, fibrosis, angiogenesis, and immune response in the recipient cells; those exerting adverse effects are depicted in red, while those depicted in green and black exert beneficial and neutral effects in the corresponding pathological process.

regulate the apoptosis of cardiomyocyte apoptosis. For example, exosomes released by cardiac fibroblasts rescue cardiomyocytes from ischemia-reperfusion injury. Mechanistically, miR-423-3p, which is remarkably enhanced in cardiac fibroblasts and exosomes, fulfills its cardioprotective effect by downregulating RAP2C in H9C2 cells and consequently reducing apoptosis [13]. The bioactive factors in the exosomes can also be proteins. For instance, circulating exosomes isolated from healthy volunteers and rats protect cardiomyocytes from apoptosis by the interaction between exosomal HSP70 and TLR4 on the cardiomyocytes and subsequent activation of the TLR4-ERK-p38MAPK-HSP27 pathway [14]. Even the exosomes secreted by cardiomyocyte itself, which is pretreated with lentivirus to overexpress HSP20, can reduce apoptosis via amplifying the AKT signaling pathway [15]. Exosomes also can be negative regulators of apoptosis. In systemic inflammation as mimicked by *il-10* knockout, cardiac endothelial cell-derived exosomes aggravate cell apoptosis post infarction by increasing ILK in exosomes and activating the NF-κB pathway in recipient cells [16]. Moreover, the biological function of exosomes is highly dependent on the status quo of the cell type of origin. While exosomes derived from cardiomyocytes from healthy volunteers significantly promote proliferation and reduce apoptosis of neonatal rat cardiomyocytes in vitro, exosomes from patients with heart failure yield the opposite outcome, which may be secondary to decreased exosomal miR-21-5p which activates PTEN and downregulates Akt phosphorylation in cardiomyocytes. PDCD4, a pro-apoptotic protein, is also a target of miR-21-5p [17].

2.2. Hypertrophy. In response to TNF-α stimulation, cardiac fibroblasts secrete miR-27a-, miR-28-3p-, and miR-34a-enriched exosomes, which are taken up by cardiomyocytes and suppress the expression of Nfn2 and promote expression of cardiac hypertrophy-related genes such as ANP and β-MHC in cardiomyocytes [18]. miR-21-3p-enriched exosomes are also released by cardiac fibroblasts in the mouse model of cardiac hypertrophy, and exosomal miR-21-3p silences SORBS2 and PDLIM5 in cardiomyocytes resulting in cardiac hypertrophy [19]. Exosomes also intensify cardiac hypertrophy by promoting Ang II production and its receptor content in cardiomyocytes [20]. In a heart failure model, miR-146a is overexpressed in active myofibroblasts and secreted through exosomes. Exosomal miR-146a is taken up by cardiomyocyte leading to the dysfunction of SUMOylation [21].

2.3. Angiogenesis. The density of myocardial capillary is a critical pathological index of cardiac function and angiogenesis is of great importance in cardiac repair and regeneration post injury [5, 22]. In the MI microenvironment, proinflammatory M1-type macrophages have increased the expression of proinflammatory miRNAs, such as miR-155, and transport them to endothelial cells through exosomes. These proinflammatory exosomes inhibit Sirt1/AMPKα2/eNOS and RAC1/PAK2 pathways in endothelial cells which reduces angiogenic potential and aggravate myocardial injury [23]. Besides macrophage, cardiomyocytes in hypoxic environment release exosomes with significantly upregulated circHIPK3, which acts as an endogenous miR-29a sponge, abrogates the inhibition of IGF-1, and relieves oxidative

stress-induced dysfunction in cardiac microvascular endothelial cells [24]. Exosomes derived from cardiac telocytes, which is a subgroup of interstitial Cajal-like cells, increase the proliferation, migration, and tube-formation of endothelial cells post MI [25]. Interestingly, a previous study suggests that pretreating the bilateral hindlimbs of rats with tourniquets could enhance the angiogenesis and alleviate the apoptosis of endothelial cells post MI, likely secondary to the proangiogenic contents of circulating exosomes, such as NOS, HIF-1α, Ang-1, and VEGF [26]. A novel therapy, low-energy shock wave therapy, also has been proven to enhance cardiac regeneration post MI by improving vascularization. The shock wave therapy can stimulate endothelial cells in the ischemic myocardium to release angiogenic exosomes, and miR-19a-3p is the effective cargo to promote endothelial tube formation and proliferation [27].

2.4. Cardiac Fibrosis. Cardiac fibroblasts are indispensable for normal myocardial physiology. However, pathologically activated cardiac fibroblasts deposit excess extracellular matrix, which negatively affects myocardial compliance and stiffness and cardiac function, leading to, for instance, heart failure with preserved ejection fraction [28–30]. Cardiac fibrosis can be modulated by different types of exosomes. Under exercise conditions, diabetic cardiomyocytes release exosomes with higher content of miR-29b and miR-455, which can bind to the $3'$ region of MMP9, suppressing its expression and reducing cardiac fibrosis [31]. In the chronic heart failure model, cardiomyocyte-derived miR-217-containing exosomes target PTEN and aggravate cardiac fibrosis both *in vivo* and *in vitro* partly by promoting the proliferation of fibroblasts [32]. Mechanical stress increases the secretion of exosomal miR-378 from cardiomyocytes, and exosomal miR-378 inhibits excessive cardiac fibrosis by suppressing the p38 MAPK-Smad2/3 pathway [33]. Exosomes derived from macrophages are also involved in the pathological activation of cardiac fibroblasts. Exosomes derived from macrophages in a diabetic microenvironment exacerbate cardiac fibrosis, which is abrogated by human antigen R (HuR) knockdown (either in macrophages or in exosomes) [34]. Outsider intervention can also regulate the crosstalk inside a cardiac microenvironment and affect the process of fibrosis. Injection into the ischemic myocardium of exosomes derived from endothelial cells stimulated by shock wave therapy decreases myocardial fibrosis [27]. Simvastatin significantly attenuates collagen deposition and cardiac fibrosis in rats treated with Ang II, which appears secondary to simvastatin-mediated induction of decorin and reduction of periostin in Ang II-treated cardiomyocyte-derived exosomes [35].

2.5. Inflammation. The interaction between the immune and cardiovascular systems plays an important and complex role in CVD progression, with exosomes mediating the exchange of signals among cells and organs [36]. Inflammation promotes the secretion of miR-155-enriched exosomes by activated macrophages. Compared with the control group, the myocardium treated with miR-155-enriched exosomes exhibits markedly increased expression of proinflammatory cytokines, including IL-1β, IL-6, TNF-α, and CCL-2, likely secondary to the suppression of Socs1 [37]. Systematic inflammation is also reported to impair the beneficial effect of endothelial exosomes on cardiac repair post cardiac injury [16]. The "cardioimmune" regulation appears to be reciprocal, as myocardium-derived exosomes can also influence the localization and function of immune cells post cardiac injury. Post MI, cardiac exosomes are rapidly released to the interstitial space but also rapidly disappear, which is reported to be related to immune cell infiltration into the ischemic region. Migrating immune cells, mainly monocytes, engulf cardiac exosomes and increase the expression of IL-6 and chemokines CCL2 and CCL7, which shapes the inflammation post MI [38].

The roles and functions of exosomes in the cardiovascular system have also been characterized in the normal conditions. For example, a study revealed that both in the basal level and in the mild stress state, exosomal HSP60 is continuously released by cardiomyocytes [39]. However, the scarcity of such studies hinders us from figuring out the physiological role of exosomes.

3. Application of Exosomes in Cardiovascular Disease Treatment

Exosomes are deemed as the bioactive ingredient responsible for the beneficial effects of stem cell therapy in repairing cardiac injury. Exosomes as therapeutic agents can overcome some drawbacks of stem cell therapy, such as the low stem cell retention rate. We here summarize the therapeutic effects of exosomes and their underlying mechanisms (see Figure 2).

3.1. Mesenchymal Stromal Cell-Derived Exosomes. Mesenchymal stromal cell- (MSC-) derived exosomes have become attractive candidates for cardiac injury repair. MSC-derived exosomes enhance the polarization of M1 macrophages to M2 macrophages which significantly alleviates the inflammation in the heart and reduces the infarct size. miR-182 knockout in exosomes or TLR4 knockout in macrophages attenuates the immunomodulation function of MSC-derived exosomes [40]. In terms of apoptosis, the content of miR-185 is dramatically increased in exosomes released from MSCs, which suppresses SOCS2 and rescues cardiomyocytes from apoptosis post infarction [41]. MSC-derived exosomal miR-19a also exhibit antiapoptosis effects, by targeting SOX6, activating AKT, and inhibiting JNK3/caspase-3 pathway in cardiomyocytes [42].

Despite the aforementioned salutary effects, the efficacy of exosomes isolated from original and nonartificial MSCs remains limited, which has triggered efforts to optimize and engineer MSCs. Exosomes obtained from MSCs overexpressing MIF show a better cardioprotective effect on reducing mitochondrial fragmentation and cardiomyocyte apoptosis compared to normal MSC-derived exosomes [43]; this is also the case for exosomes secreted by MSCs cultured in a medium containing extra MIF. Mechanically, the exosomal transfer of lncRNA-NEAT1 modulates miR-142-3p and FOXO1 in cardiomyocytes and ameliorates oxidative stress [44]. Interestingly, circulating exosomes isolated from serum

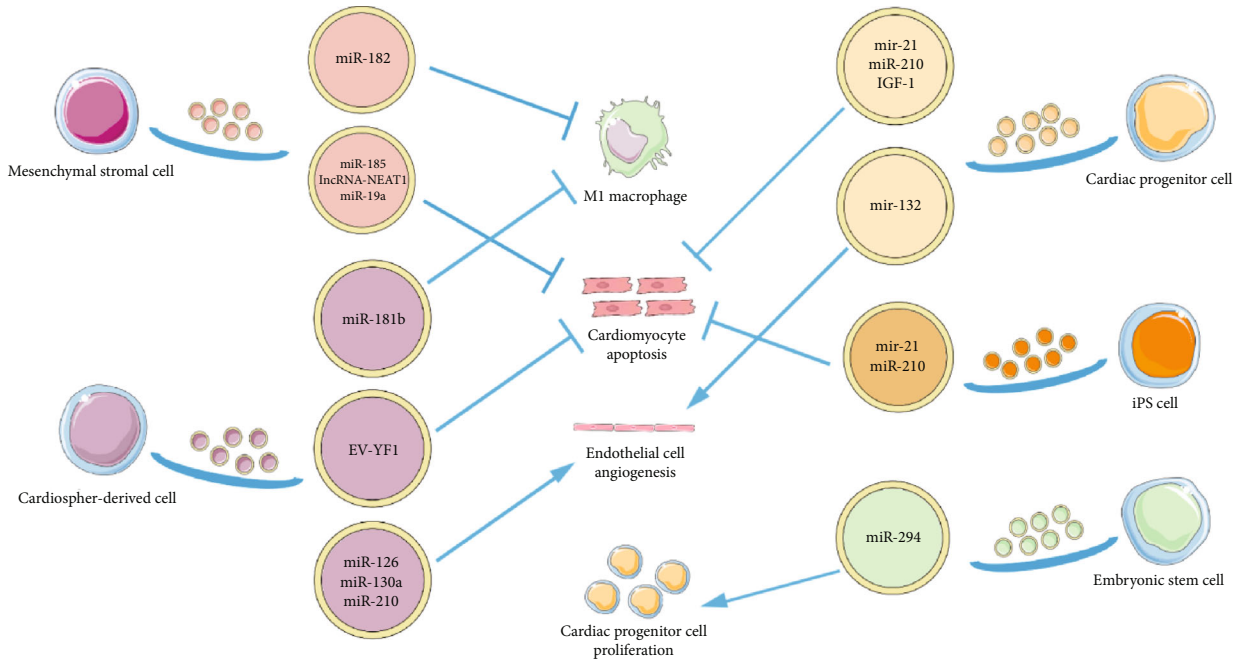

Figure 2: Application of exosomes in the treatment of cardiovascular diseases. Multiple stem cells, namely, mesenchymal stromal cells, cardiosphere-derived cells, cardiac progenitor cells, induced pluripotent stem cells, and embryonic stem cells, secrete exosomes containing therapeutic molecules to modulate the macrophage polarization, cardiomyocyte apoptosis, angiogenesis, and other responses in cardiac injury.

post MI can also be a stimulant of MSCs. The serum exosomes, which are proved to mainly come from the kidney and ischemic myocardium, transfer miR-1956 to MSCs, downregulate Notch-1 pathway, and significantly enhance the pancreatic function of MSCs [45]. The efficacy of MSC-derived exosomes is also greatly influenced by the origin of MSCs. For instance, exosomes secreted by human fetal amniotic fluid stem cells (hAFSCs) possess higher cardioprotective potential than exosomes derived from traditional adult MSCs [46].

3.2. Cardiosphere-Derived Cell-Derived Exosomes. Exosomes released by cardiosphere-derived cells (CDCs) alleviate cardiac hypertrophy induced by Ang II and reduce myocardial infarct size post infarction. The Y RNA fragment EV-YF1, which is the maximum small RNA inside exosomes, can mimic such cardioprotective effect by altering the IL-10 expression [47, 48]. CDC-derived exosomes alleviate cardiac fibrosis, enhance tube formation of endothelial cells, and attenuate the cardiomyocyte apoptosis post MI [49–51]. Macrophage polarization is also regulated by CDC-derived exosomes after MI. Exosomal miR-181b hinders PKCδ expression and enhances M1 to M2 macrophage shift, which underlies the beneficial effect of CDC-derived exosomes [52]. Such a protective effect also has been documented in large animals such as pigs, further underscoring the therapeutic effect of CDC-derived exosomes following intravenous delivery in a myocardial infarction model [53]. Moreover, exosomes obtained from CDCs of neonatal rats or pediatric donors induce cardiac rejuvenation in old ones, as evidenced by the decreased cardiac hypertrophy and myocardial fibrosis, improved cardiac systolic and diastolic function, and enhanced exercise capability [54].

Like MSCs, exosomes obtained from engineered CDCs exhibit an improved protective effect. Hypoxic treatment, as the most widely used preconditioning method, increases the efficacy of CDC-derived exosomes by enriching proangiogenic miRs, such as miR-126, miR-130a, and miR-210, in exosomes [50]. The antiapoptotic effect of CDC-derived exosomes is also reinforced after hypoxic treatment [51]. Preclinical research has revealed that the decreased potency of CDC and its exosomes are associated with the inactivation of Wnt/β-catenin pathway, and β-catenin can efficiently reverse the therapeutic efficacy of the low potency CDCs [55]. To promote the cardiac tropism of CDC-derived exosomes, CDCs are engineered to connect a cardiomyocyte-specific binding peptide to the N-terminus of Lamp2b, which is a transmembrane protein on the exosomes. This engineered exosomes show better cardiomyocyte-specific uptake and enhanced protective effect [56].

3.3. Cardiac Progenitor Cell-Derived Exosomes. Cardiac progenitor cells (CPCs) are also emerging as a promising candidate in stem cell-based therapy for cardiac repair. CPC-derived exosomes effectively attenuate cardiomyocyte apoptosis induced by oxidative stress *in vitro* in an exosomal miR-21-dependent manner, which targets the gene PDCD4 in cardiomyocytes [57]. *In vivo*, reduced apoptosis and improved ejection fraction are observed in the infarcted hearts injected with exosomes released by CPCs, which has been associated with increased miR-210 in exosomes and decreased ephrin A3 and PTP1b in cardiomyocytes [58].

Moreover, tube formation in endothelial cells is promoted by CPC-derived exosomes because the miR-132 in exosomes inhibits the RasGAP-p120 [58]. Exosomes from CPCs perform better in alleviating ischemic cardiac injury than those from bone marrow-derived MSCs. The beneficial effects are associated with the activation of PAPP-A, the release of IGF-1 in exosomes, and the enhancement of intracellular Akt and ERK1/2 [59].

As for expanding the efficacy of CPC-derived exosomes, ticagrelor appears to be an effective stimulant, which can improve both the quantity and quality of CPC-derived exosomes. Ticagrelor enhances the mitotic activity of CPCs and thus increases the number of the exosome level. Meanwhile, exosomes from CPC precondition with ticagrelor show improved antiapoptotic activity through the activation of the ERK1/2 pathway [60].

3.4. Other Stem Cell-Derived Exosomes. Exosomes secreted by induced pluripotent stem (iPS) cells protect the heart against multiple stress [61]. iPS cell-derived exosomes enhance the autophagic flux by inhibiting mTOR pathway and promoting cardiomyocyte survival both *in vitro* and *in vivo* [62]. Cardioprotective miR-21 and miR-210 are transported to cardiomyocytes by exosomes obtained from iPS cells, and they suppress caspase 3/7 and improve the function of an ischemic myocardium [63].

Embryonic stem cell-derived exosomes are also effective in relieving cardiac injury [64]. For example, cardiomyocyte pyroptosis induced by doxorubicin can be greatly attenuated by the treatment of embryonic stem cell-derived exosomes, which can decrease the inflammation in the injured myocardium by blocking caspase-1-dependent cell death [65]. Cardiac injury induced by doxorubicin is also ameliorated by the increased number of M2 macrophages and levels of anti-inflammatory cytokines, which is mediated by embryonic stem cell-derived exosomes [66]. Besides promoting neovascularization and cardiomyocyte survival in infarcted hearts, exosomes from embryonic stem cells augment the survival and proliferation of CPCs and promote endogenous repair, which is mediated by the exosomal miR-294 [67].

3.5. Nonstem Cell-Derived Exosomes. Cardiac injury also can be alleviated by the exosomes derived from nonstem cells. For example, upon hypoxia stress, cardiac fibroblasts would release miR-423-3p-enriched exosomes and reduce apoptosis of cardiomyocytes [13]. Cardiomyocyte-derived exosomes can reduce the cardiac fibrosis caused by mechanical stress through expanding the content of miR-378 [33]. The response partially belongs to the body's self-repair system, and the targeted activation of the process can be the new treatment strategy of cardiovascular diseases.

3.6. Exosome Delivery Method. Exosomes are most commonly delivered via the intravenous route and have been shown to be effective in many animal models and in patients with graft versus host disease [68, 69]. Exosomes also alleviate cardiac injury when administered via an intracoronary route, intramyocardial injection, and cardiac patch [53, 70]. The huge benefits of exosome-based therapy have fueled exploration of more clinically feasible and effective exosome delivery methods, which will greatly promote the clinical applications of exosomes [71, 72].

4. Conclusion

Exosomes play critical and important roles in the regulation of physiological and pathophysiological processes, recognition and diagnosis, and treatment of CVD [11]. In this review, we focus on the regulation of pathophysiological process by exosomes and the therapeutic potential, which is of great importance in understanding the mechanism of CVDs and exploring therapeutic approaches. However, there remain many hurdles, including tropism and pharmacokinetics of exosomes, to surmount for their clinical application. Research to further assess the contents of exosomes and the signals exchanged by exosomes will continue, as will efforts to optimize efficacy and delivery methods for potential clinical applications.

Abbreviations

CVD: Cardiovascular disease
MI: Myocardial infarction
RAP2C: Ras-related protein Rap-2c
ILK: Integrin-linked kinase
NF-κB: Nuclear factor κB
Nfn2: Nuclear factor erythroid 2-related factor 2
SORBS2: Sorbin and SH3 domain-containing protein 2
PDLIM5: PDZ and LIM domain 5
Ang II: Angiotensin II
Sirt1: Sirtuin 1
AMPK: Protein kinase AMP-activated catalytic subunit
eNOS: Endothelial nitric oxide synthase
RAC1: Rac family small GTPase 1
PAK2: p21 (RAC1)-activated kinase 2
MMP9: Matrix Metalloprotease 9
MSCs: Mesenchymal stromal cells
TLR4: Toll-like receptor 4
SOCS2: Suppressor of cytokine signaling 2
MIF: Macrophage migration inhibitory factor
FOXO1: Forkhead class O1
CDCs: Cardiosphere-derived cells
PKCδ: Protein kinase Cδ
CPCs: Cardiac progenitor cells
PDCD4: Programmed cell death 4
PAPP-A: Pregnancy-associated plasma protein-A
IGF-1: Insulin-like growth factor-1
iPS cells: Induced pluripotent stem cells
mTOR: Mammalian target of rapamycin
HSP60: Heat shock protein 60.

Conflicts of Interest

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

Authors' Contributions

Dong Guo and Yuerong Xu contributed equally to this work.

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Review Article

Changes of Laboratory Cardiac Markers and Mechanisms of Cardiac Injury in Coronavirus Disease 2019

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Some patients with coronavirus disease 2019 (COVID-19) show abnormal changes in laboratory myocardial injury markers, suggesting that patients with myocardial injury have a higher mortality rate than those without myocardial injury. This article reviews the possible mechanism of myocardial injury in patients with COVID-19. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) affects the patients with COVID-19 in aspects of direct infection of myocardial injury, specific binding to functional receptors on cardiomyocytes, and immune-mediated myocardial injury. During hospitalization, the monitoring of laboratory myocardial injury markers in patients of COVID-19 should be strengthened.

1. Introduction

In December 2019, an outbreak of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) occurred in Wuhan, China. This has attracted global attention because of its high infectivity. COVID-19 has rapidly spread to more than hundred countries worldwide, of which Western Pacific Region and European Region are severely affected, and there are still new cases appearing. As of May 12, 2020, there were 4,088,848 confirmed cases; of them, 283,153 died [1]. A retrospective review of lung injury caused by COVID-19 found that there were varying degrees of changes in laboratory cardiac markers [2]. Therefore, there is a great deal of attention as to whether it causes myocardial injury. In this paper, we searched for laboratory cardiac markers in patients with COVID-19 to investigate the mechanisms through which SARS-CoV-2 caused myocardial injury.

2. Laboratory Cardiac Markers in Patients with COVID-19

The levels of laboratory cardiac markers, lactose dehydrogenase (LDH), creatine kinase (CK), creatinine kinase-muscle/-brain activity (CK-MB), myoglobin (Mb), cardiac troponin I (cTnI), alpha-hydroxybutyrate dehydrogenase (α-HBDH), aspartate aminotransferase (AST), and N-terminal of the prohormone brain natriuretic peptide (NT-proBNP) increase in different proportions in patients with COVID-19 (see Table 1). Although LDH, CK, α-HBDH, and AST are cardiac enzymes, their increases cannot specifically represent myocardial injury. It may be due to damage to the lungs, liver, kidneys, or other organs. However, specific myocardial indicators including CK-MB, cTnI, Mb, and NT-proBNP are increased to varying degrees in patients with COVID-19, especially in ICU and severe patients [3]. A meta-analysis which included 4189 confirmed patients of COVID-19 from 28 studies pointed out that cardiac injury biomarkers rose above normal by the midpoint of hospitalization and spiked immediately before death, which seemed to be the most seen in severe cases [4]. Subsequently, some literatures indicated that increased in troponin I [5], CK-MB, and NT-proBNP [6] were indicators of possible cardiac damaged during SARS-CoV-2 infection. It must be noted that in the clinical scenario of COVID-19 patients, cardiac magnetic resonance or endomyocardial biopsy is rarely feasible; thus, the diagnosis is mainly based on troponin elevation in association with echocardiographic data compatible with

Table 1: Cardiac laboratory markers of COVID-19 patients.

Patients	LDH	CK	CK-MB	cTnI	Mb	α-HBDH	AST	NT-proBNP
$n = 41$ [29]	29/40 (73) ICU 12/13 (92) No-ICU 17/27 (63)	13/40 (33) ICU 6/13 (46) No-ICU 7/27 (26)	—	5/41 (12) ICU 4/13 (31) No-ICU 1/28 (4)	—	—	15/41 (37) ICU 8/13 (62) No-ICU 7/28 (25)	—
$n = 99$ [45]	75/99 (76)	13/99 (13)	—	—	15/99 (15)	—	35/99 (35)	—
$n = 188$ [2]	129/188 (68.6)	21/188 (11.2)	19/188 (10.1)	21/188 (11.2)	—	143/188 (76.1)	—	—
$n = 267$ [31]	57/267 (21.3) 39/217 (18.0) Nonsevere 39/50 (18.0) Severe	50/267 (18.7) 32/217 (14.7) Nonsevere 18/50 (36.0) Severe	—	3/76 (3.9) 0/55 (0) Nonsevere 3/21 (14.3) Severe	8/76 (10.5) 1/55 (1.8) Nonsevere 7/21 (33.3) Severe	—	—	—
$n = 140$ [46]	—	4/60 (6.7) 1/35 (2.8) Nonsevere 3/25 (12.0) Severe	—	—	—	—	—	—
$n = 1099$ [47]	277/675 (41.0)	90/657 (13.7)	—	—	—	—	168/757 (22.2)	—
$n = 12$ [16]	11/12 (91.7)	1/6 (16.7)	1/12 (8.3)	1/12 (8.3)	2/12 (16.7)	—	2/12 (16.7)	—
$n = 62$ [48]	17/62 (27.4)	5/62 (8.1)	—	—	—	—	10/62 (16.1)	—
$n = 101$ [49]	—	—	32/101 (31.68) Admission to ICU 56/101 (55.45) 48 h to death	51/101 (50.50) Admission to ICU 73/101 (72.28) 48 h to death	—	—	—	37/101 (36.63) Admission to ICU 41/101 (40.59) 48 h to death
$n = 19$ [50]	6/19 (31.58)	1/18 (5.56)	—	—	—	6/8 (75.0)	5/18 (27.78)	—
$n = 148$ [51]	52/148 (35.1)	—	—	—	—	—	32/148 (21.6)	—
$n = 29$ [52]	20/29 (69)	—	—	—	—	—	—	—
$n = 2$ [53]	1/2 (50)	—	—	—	—	—	—	—
$n = 82$ [30]	68/73 (93.2)	25/72 (34.7)	21/70 (30.0)	52/60 (86.7)	42/70 (60.0)	—	44/72 (61.1)	—
$n = 46$ [54]	9/46 (19.6)	2/46 (4.4)	—	—	—	—	3/46 (6.5)	—
$n = 102$ [55]	37/102 (36.3)	19/102 (18.6)	11/102 (10.8)	—	4/59 (6.8)	37/102 (36.3)	26/102 (25.5)	—
$n = 200$ [56]	—	—	—	137/200 (68.5) 112/166 (67.5) Alive cases 25/34 (73.5) Dead cases	—	—	—	—
$n = 34$ [57]	10/34 (29.4)	—	—	—	—	—	—	—
$n = 9$ [58]	—	—	—	—	—	—	3/9 (33.3)	—

Laboratory data is reported as percent of patients with abnormalities defined according to the local reference ranges. No./total No. (%); ICU: intensive care unit; LDH: lactose dehydrogenase; CK: creatine kinase; CK-MB: creatinine kinase-muscle/brain activity; cTnI: cardiac troponin I; Mb: myoglobin; α-HBDH: alpha-hydroxybutyrate dehydrogenase; AST: aspartate aminotransferase; NT-proBNP: N-terminal of the prohormone brain natriuretic peptide.

acute myocarditis (i.e., segmental wall motion abnormalities, left ventricular ejection fraction (LVEF) < 50%, or the presence of left ventricular wall thickening > 10 mm and/or pericardial effusion) and ECG changes (ST elevation or ST/T segment changes) [7].

Some patients are improved, and laboratory cardiac markers return to normal, whereas some severe patients show further worsening in laboratory cardiac markers, resulting in an irreversible loss. Persistent elevation of laboratory cardiac markers is a prognostic factor of disease worsening [8], and most of these patients are transferred into an intensive care unit (ICU) ward for treatment. There is no evidence that patients with hypertension and cardiovascular and cerebrovascular diseases are more susceptible to infection with SARS-CoV-2, but it is certain that patients with hypertension and cardiovascular and cerebrovascular diseases are more likely to develop severe/ICU cases [9]. On the other hand, patients with SARS-CoV-2 are prone to cardiovascular complications [10]. Li et al. reported that at least 8.0% patients with COVID-19 suffered the acute cardiac injury [9]. The incidence of acute cardiac injury was about 13 folds higher in ICU/severe patients compared with the non-ICU/severe patients [9].

3. Mechanism Exploration

3.1. Direct Virus Infection. Virus infects cardiomyocytes and replicates intracellularly, resulting in cardiomyocyte degeneration and necrosis, which causes loss of cardiac function and arrhythmia. During this process, the specific binding between viruses and surface receptors in cells is an important event for virus infection of target cells. We investigate the mechanism through which SARS-CoV-2 infects cardiomyocytes to cause direct injury and to analyze receptors that are related to cardiac injury. Hu et al. reported markers of myocardial injury such as troponin T, creatine kinase isoenzyme, and N-terminal of the prohormone brain natriuretic peptide (NT-proBNP) were significantly elevated in patients with nucleic acid test-confirmed COVID-19, who were finally diagnosed coronavirus fulminant myocarditis with cardiogenic shock and pulmonary infection [11]. Subsequently, an otherwise healthy middle-aged white woman with similar symptoms, characteristics, and auxiliary examination results was diagnosed with COVID-19 acute myopericarditis [12]. The two cases suggest that SARS-CoV-2 may directly infect myocardial cells, causing viral myocarditis and impairing myocardial function. However, the above two cases have not undergone pathological examination and cannot confirm the speculation. Recently, endomyocardial biopsy in a 69-year-old patient with acute cardiac injury directly demonstrated low-grade myocardial inflammation and viral particles in the myocardium suggesting either a viraemic phase or, alternatively, infected macrophage migration from the lung, which linked to myocardial localization of SARS-CoV-2 [13]. Subsequent studies had confirmed myocarditis had also been identified with high viral loads and mononuclear infiltration on autopsy of some patients with COVID-19 [14–16]. In fact, one study suggested that up to 7% of COVID-19-related deaths were due to myocarditis [17]. When laboratory myocardial markers elevate and severe arrhythmias appear early in COVID-19, we must be alert to the occurrence of viral cardiomyopathy.

3.2. Specific Binding to Functional Receptors on Cardiomyocytes. Angiotensin-converting enzyme 2 (ACE2) is a monocarboxylate that degrades angiotensin II to angiotensin 1–7 [18] and is highly expressed in the lungs and heart [19, 20]. Hofmann et al. proved that ACE2 receptor expression was intimately associated with SARS virus invasion [21], and SARS-CoV infection can lead to infection of ACE2-dependent cardiomyocytes. SARS-CoV-mediated myocarditis was intimately associated with ACE2 [22]. This may be one of the reasons for cardiac insufficiency and poor cardiac outcomes in patients with SARS [22]. The older the person, the poorer the cardiac reserved, which ultimately lead to significant age dependence regarding the SARS mortality rate [23]. Three-dimensional reconstruction and computer simulation experiments that the structure of the receptor binding domain and external region of SARS-CoV-2 is very similar to SARS-CoV, suggesting that ACE2 may be a potential receptor for SARS-CoV-2 and form a tight bond [24]. Letko and Munster successfully proved that SARS-CoV-2 could enter cells expressing human ACE2 [25]. Therefore, it has been fully determined that SARS-CoV-2 enter cells through ACE2 on the surface of human cells [25]. Furthermore, some studies have confirmed that due to the expression of ACE2 cardiomyocytes, SARS-CoV-2 easily invaded cardiomyocytes and caused cardiomyocyte loss [26]. In fact, there are no available data which support that ACE inhibitors (ACEI) increase COVID-19 infection via its binding to ACE2 [27]. However, studies have suggested that although COVID-19 combined with cardiovascular diseases (CVDs) has a higher mortality rate, the use of ACEI/ARB drug intervention has no significant effect on the morbidity and mortality [28]. It is necessary to take ACE2 as the entry point to further study its therapeutic value.

3.3. Immune Damage. A paper published in Nature mentioned that the plasma of newly diagnosed patients with COVID-19 contains IL1B, IL1RA, IL7, IL8, IL9, IL10, basic fibroblast growth factor (basic FGF), granulocyte colony-stimulating factor (GCSF), granulocyte-macrophage colony-stimulating factor (GMCSF), IFN-γ, IP10, monocyte chemoattractant protein 1 (MCP1), macrophage inflammatory protein 1A (MIP1A), MIP1B, platelet-derived growth factor (PDGF), tumor necrosis factor-α (TNF-α), and vascular endothelial growth factor (VEGF) [29]. Compared with non-ICU patients, plasma IL-2, IL-7, IL-10, GCSF, IP10, MCP1, MIP1A, and TNF-α levels in ICU patients were significantly higher [29]. Subsequent researches have suggested that IL-6, IL-17A, and TNF-α are highly expressed in critically ill patients or deaths [30, 31]. Therefore, the blood in the patients with COVID-19, particularly severe cases, contains large number of inflammatory cytokines. The main pathogenesis is characterized by high cell division or "cytokine storm," resulting in an overexuberant immunologic host response [32, 33]. Virus stimulation will result in the innate immune phase, which is manifested by monocyte and

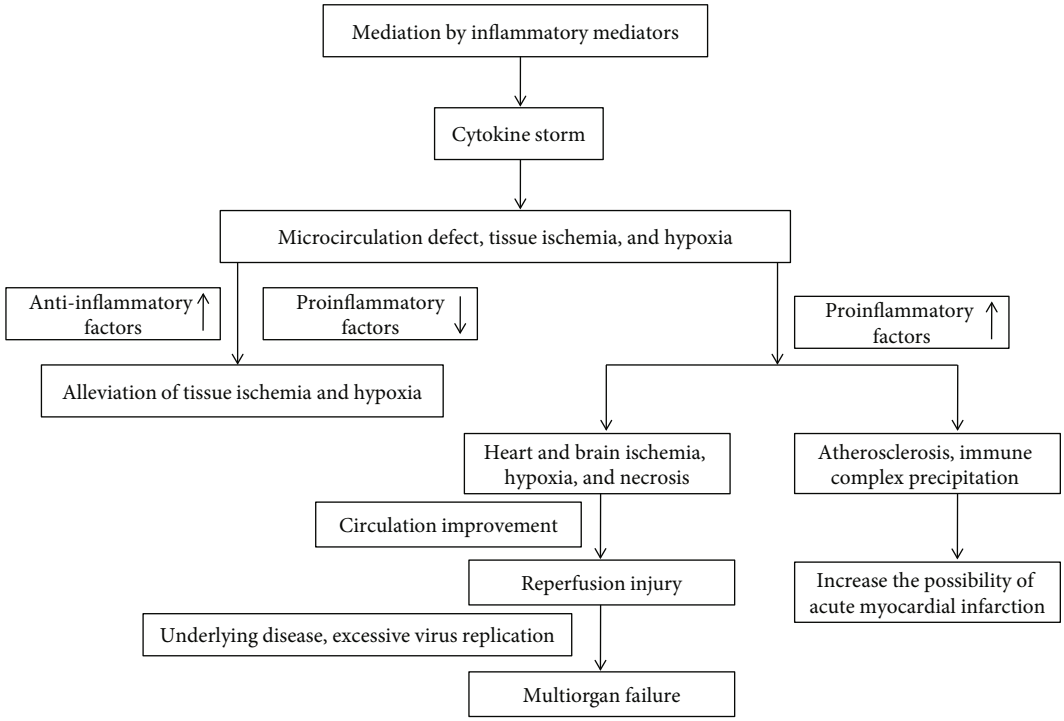

Figure 1: Immune-mediated mechanism of myocardial injury.

macrophage intervention. As inflammatory mediators, monocytes and macrophages play an important role by activating inflammatory cells to release proinflammatory (stress-activated) cytokines, resulting in "cytokine storm." A study showed that proinflammatory (stress-activated) cytokines such as TNF-α, IL-1, and IL-6 played a role in the pathogenesis of congestive heart failure [34], showing that cytokines played an important role in cardiac injury.

These cytokines act on leukocytes, lymphocytes, platelets, and vascular endothelial cells to secrete inflammatory mediators, which can increase blood C-reactive protein (acute phase protein), α2-macroglobulin, and fibrinogen levels while decreasing albumin and transferrin levels [35]. This pathophysiological process causes circulation to be at a high-output and low-resistance state. On the other hand, cytokines such as IL-1, IL-6, and TNF-α act on capillaries, resulting in ischemia and hypoxia in peripheral tissues and increased compensatory pulsation to improve peripheral circulation.

When levels of inflammatory cytokines decrease, high output, low resistance, and peripheral circulation are alleviated, and blood circulation returns to normal, thereby alleviating hypotension and tachycardia. A study found that the clinical presentation of SARS-CoV pneumonia was mostly hypotension, tachycardia, impaired systolic, and diastolic functions in the heart [36], which was usually self-limiting [37] and proved the aforementioned speculation. As the disease progresses, the viral load may have returned to normal, but excessive immune responses further damage various major organs, such as the heart. This causes ischemia and hypoxia in cardiac tissues while the heart is overloaded to maintain a high-output and low-resistance state. The further results in ischemic injury and changes in laboratory cardiac markers such as elevated troponin I [5], CK-MB, and NT-proBNP [6].

Li et al. pointed out that due to severe SARS-CoV-2 infection, the pneumonia caused significant gas exchange obstruction, leading to hypoxaemia, which significantly reduced the energy supply by cell metabolism, and increased anaerobic fermentation, causing intracellular acidosis and oxygen-free radicals to destroy the phospholipid layer of cell membrane [9]. Meanwhile, hypoxia-induced influx of calcium ions also led to injury and apoptosis of cardiomyocytes [9]. Generali et al. suggested that a not well-managed inflammatory status led to accelerated atherosclerosis that precipitates ischemic disease; the precipitation of immune complexes on endocardium was finally responsible of inflammatory infiltration which led to subsequent worsening of the previous damage [38]. The blood in the patients with COVID-19, particularly severe cases, contains large number of inflammatory cytokines, suggesting that patients with COVID-19 also experience an overexuberant immunologic host response. It is possible to exacerbate the atherosclerosis that cytokine responses to infection as activators of vascular cells and as inducers of the acute phase response with consequent heightened production of fibrinogen, the precursor of clots, and of endogenous inhibitors of fibrinolysis [39].

Patients with COVID-19 can produce cytokines to enter the systemic circulation, which can stimulate macrophages within the plaque to augment local cytokine production and provoke an increase in tissue factor expression that renders lesions more thrombogenic [40, 41]. If patients with COVID-19 suffer severe underlying atherosclerotic diseases, extreme cases of acute myocardial infarction are likely to

occur during the course of the disease. Patients with COVID-19 are likely to suffer atherosclerosis, leading to insufficient coronary blood supply and causing myocardial damage. Interpretation of rises in cardiac troponin requires consideration of the context of the clinical situation. We should be alert to the possibility of other types of cardiogenic diseases [39].

The disease is a dynamic process. When ischemia and hypoxia occur in various organs and circulation is not improved, the body will progress to shock. When circulation improves through treatment and ischemic and hypoxic tissues and cells recover, cytokines such as IL-1 [42], IL-6 [43], and TNF-α carry out their effects again by participating in ischemia-reperfusion injury and production of large amounts of free radicals, causing tissue damage. This causes secondary damage to organs. Persistent damage to cardiomyocytes causes persistent LDH elevation. A study showed that LDH elevation can reflect the severity of tissue injury and inflammation [44]. Persistent disease progression may lead to irreversible multiorgan failure and can ultimately lead to death (see Figure 1).

4. Conclusions

SARS-CoV-2 damages myocardial cells and induces changes of laboratory cardiac markers to varying degrees. The mechanisms include direct infection of myocardial injury, specific binding to functional receptors on cardiomyocytes, and immune-mediated myocardial injury. These mechanisms are not independent and exist strictly in a temporal sequence, as there is a large possibility that these three injury modes simultaneously exist and act together to result in permanent cardiomyocyte loss. Therefore, for patients with COVID-19, it is necessary to actively prevent myocardial injury and reduce the possibility of irreversible remodeling of the myocardium with finally preventing the occurrence of congestive heart failure.

Conflicts of Interest

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

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