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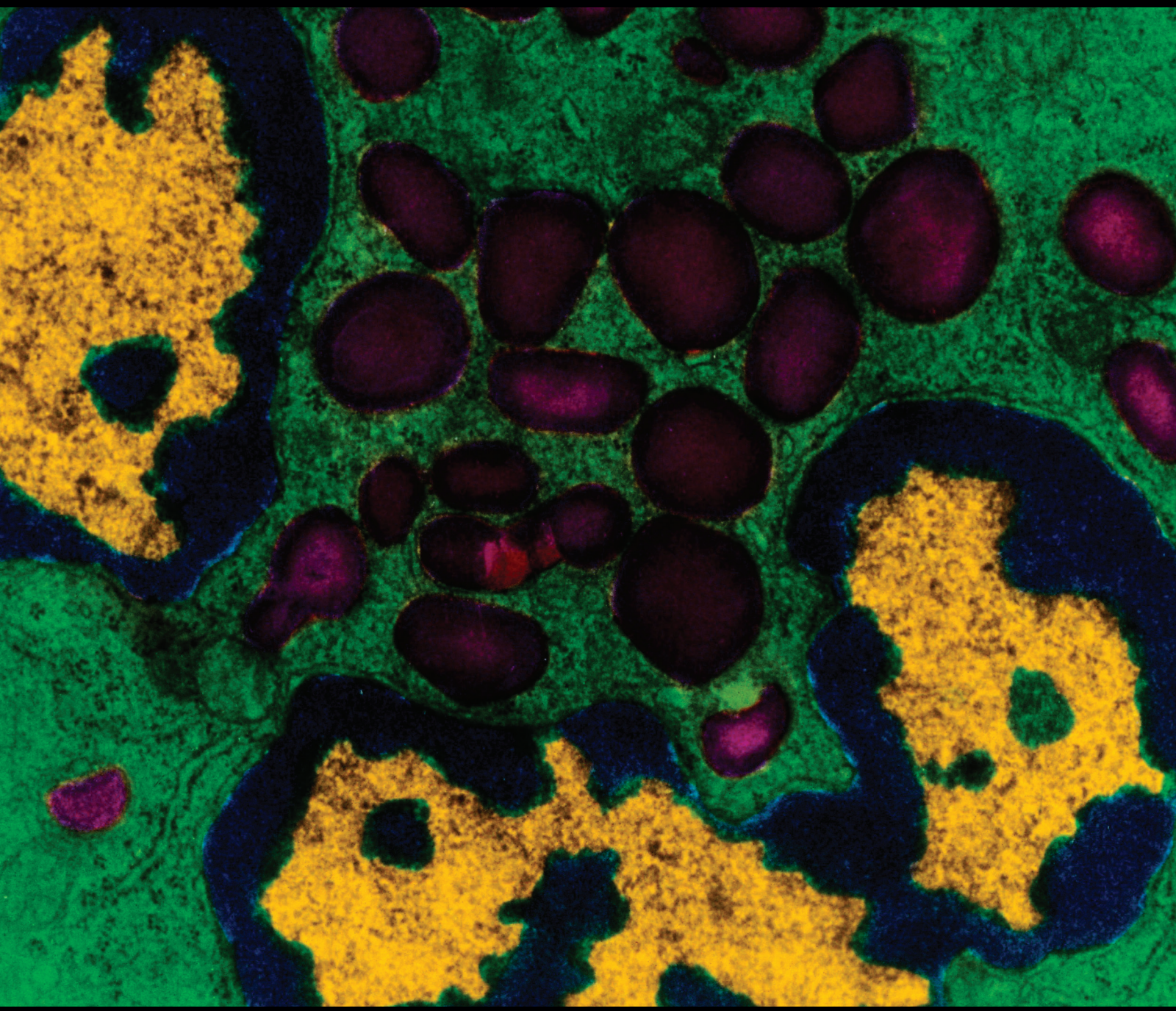
Inflammation and Cardiovascular Cross Talk in Ischemic Vascular Diseases

Guest Editors: Giorgio Zauli, Veronica Tisato, Joseph Raffetto, and Mauro Vaccarezza



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Editorial

Inflammation and Cardiovascular Cross Talk in Ischemic Vascular Diseases

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Ischemic vascular diseases include different pathological events characterized by distinctive features but share the common hallmark of inflammation. In this light, myocardial infarction can be a good paradigm to summarize the different connections linking inflammation and the cardiovascular system during an ischemic event. The immune system and inflammation, through several cellular and soluble inflammatory mediators, play a crucial role in the local tissue structural changes of ischemic heart disease, with a different impact and outcome during acute myocardial infarction compared to the more chronic long-term inflammation [1]. In response to acute damage and hemodynamic stress, there is expansion of resident immune cells and recruitment of extra cells involved in a critical cross talk with parenchymal cells [2, 3]. In other words, postischemic tissue repair is crucial to survival. Recruited inflammatory cells can remove debris and facilitate the repair process; conversely, unrestrained inflammation inhibits optimal healing leading to adverse events. Moreover, other mediators such as some key coagulation factors might influence innate immunity as well as cell-mediated reactions like healing, response to tissue injury, or inflammatory processes [4, 5]. Overall, as recently suggested, the different immune/inflammatory cell subsets act as messengers implicated in novel inflammatory networks that link different organ systems enlarging the continuum beyond the myocardium and blood vessels in a more integrative pathophysiology standpoint [6].

This special issue aims to collect insights about this cross talk with a dual purpose: on the one hand to expand the comprehension on the mechanisms of action and impact of "old" inflammatory mediators and on the other to bring out "new" potential pathways and intermediates. The overall aim is to increase knowledge on the pathophysiological processes of ischemic vascular disease to improve diagnosis and treatment.

The first set of articles draws attention to soluble and cellular circulating mediators with a role in vascular inflammation. M. L. Morieri et al. have considered an "old" inflammatory factor such as interleukin 6 (IL-6) that shows different effects on the acute inflammatory responses compared with that of chronic low-grade systemic inflammation. In particular, since the "transsignalling" mediated by IL-6 is more linked to the harmful actions of this cytokine especially in the cardiovascular setting, the specific targeting of this pathway by using inhibitors such as soluble glycoprotein 130 might be a promising therapeutic strategy. The article of B. Toffoli et al. addresses the role of two other cytokines belonging to the TNF family. Osteoprotegerin (OPG) and TNF-related apoptosis-inducing ligand (TRAIL) are two factors showing controversial effects on several physiopathological contexts [7, 8]. In a preclinical model, the authors show that dyslipidaemia and diabetes, two risk factors for cardiovascular disease, modify the vascular and cardiac expression of OPG and TRAIL leading to an increased OPG/TRAIL ratio. This alteration could contribute to the changes in circulating OPG/TRAIL ratio observed in patients with diabetes and cardiovascular disease, and they could mediate/contribute to atherosclerosis development and cardiac remodelling. As far as cellular mediators, H. Li et al. have addressed the role of neutrophil granulocytes in the pathophysiology of vascular disease in a wide cohort of patients undergoing chronic haemodialysis. In the same line,

R. Helseth et al. deal with neutrophil cell activation in acute myocardial infarction. The profile and role of neutrophil extracellular traps (NET), namely, spindle-like networks made in the extracellular space by neutrophil, have been addressed in patients with ST-elevation myocardial infarction suggesting a role of NET in acute and chronic atherothrombosis in line with previous studies [9, 10]. Finally, W. Zhong et al. have a methodological and "laboratory-oriented" approach and propose an experimental method to evaluate the role of CD137 (41-BB) signalling in the pathogenesis of the atherosclerotic plaque and vascular damage.

Other authors have indeed contributed to the main topic of the special issue by exploring possible mechanisms underlying the pathophysiology of ischemic diseases. S. Wang et al. and M. Neri et al. address ischemia reperfusion injury, one of the Achilles heels of the current therapy for ischemic vascular diseases. In their work, S. Wang et al. highlight autophagy as a fundamental mechanism involved in ischemic pathology in an experimental model of diabetes. In the same line but from a different perspective, M. Neri et al. review the current literature on the ischemia reperfusion injury following myocardial infarction, highlighting the relevance of a calpain system, oxidative and NO-linked pathways, and ventricular remodelling discussing the evidences from a medico-legal standpoint to better understand sudden deaths following myocardial infarction [11, 12]. Finally, M. V. Arcidiacono et al. change perspective and address the cross talk between cardiovascular and kidney systems showing that patients with chronic kidney disease are prone to develop cardiovascular events in a mutual relationship. Indeed, they demonstrate that the higher incidence of microangiopathy of the common carotid artery wall drives the higher incidence of atheromatous disease in these patients.

In conclusion, we are pleased to introduce the reader to this translational research topic on inflammation and vascular cross talk in ischemic diseases. We hope that the research articles/reviews selected and presented might be an opportunity for further discussions and future deeper investigations to move forward by improving knowledge in the field, with the overall aim of increasing the available diagnostic and therapeutic options in the context of ischemic diseases.

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Giorgio Zauli
Veronica Tisato
Joseph D. Raffetto
Mauro Vaccarezza

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Research Article

High Neutrophil-to-Lymphocyte Ratio Predicts Cardiovascular Mortality in Chronic Hemodialysis Patients

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The neutrophil-to-lymphocyte ratio (NLR) is a novel simple biomarker of inflammation. It has emerged as a predictor of poor prognosis in cancer and cardiovascular disease in general population. But little was known of its prognostic value in chronic hemodialysis (HD) patients. Here we investigated the association between NLR and cardiovascular risk markers, including increased pulse pressure (PP), left ventricular mass index (LVMI) and intima-media thickness (IMT), and mortality in HD patients. Two hundred and sixty-eight HD patients were enrolled in this study and were followed for 36 months. The primary end point was all-cause mortality and cardiovascular mortality. Multivariable Cox regression was used to calculate the adjusted hazard ratios for NLR on all-cause and cardiovascular survival. We pinpointed that higher NLR in HD patients was a predictor of increased PP, LVMI, and IMT; HD patients with higher NLR had a lower survival at the end of the study; furthermore, high NLR was an independent predictor of all-cause and cardiovascular mortality when adjusted for other risk factors. In conclusion, higher NLR in HD patients was associated with cardiovascular risk factors and mortality.

1. Introduction

Cardiovascular disease is the major cause of death in patients with chronic kidney disease, especially in end-stage renal disease (ESRD) patients with chronic hemodialysis (HD). The cardiovascular disease (CVD) mortality in HD patients is much higher than that in general population, and it is not yet entirely explained by traditional risk factors for CVD [1]. Microinflammation is an important factor in the pathogenesis of CVD in HD patients, and it can further accelerate the progression of atherosclerosis [2].

Neutrophil-to-lymphocyte ratio (NLR) is obtained by dividing absolute neutrophil to absolute lymphocyte count. NLR is a novel simple and inexpensive index for assessing inflammation [3]. Emerging evidence suggested that increased NLR was a potential marker of poor prognosis in multiple tumors [4–6] and cardiovascular diseases [7–9] in general population. Cho et al. [10] demonstrated the potential utility of NLR in risk stratification of patients with severe calcific aortic stenosis. Isaac et al. [11] reported that increased NLR was associated with mortality among medical inpatients with multiple chronic conditions. Erturk et al. [12] also demonstrated that an increased NLR was related to higher cardiovascular mortality in patients with peripheral arterial occlusive disease, who were admitted with critical limb ischemia or intermittent claudication. Recently, Ahbap et al. [13] found a significant positive correlation of NLR with hsCRP levels in ESRD patients. In 2012, An et al. [14] reported that NLR was a strong predictor for overall and cardiovascular mortality in peritoneal dialysis patients. Recently, Ouellet et al. [15] reported about NLR as a predictor marker of all-cause survival in incident hemodialysis patients. But to date, little was known of its prognostic value in HD patients. In this present study, we investigated the association between NLR and cardiovascular risk factors, including pulse pressure (PP), left ventricular mass index (LVMI), intima-media thickness (IMT), carotid-femoral pulse wave velocity (cfPWV), and mortality in HD patients.

2. Methods

2.1. Data Sources. A total of 268 ESRD patients on chronic hemodialysis (146 men, 122 women) who were admitted to

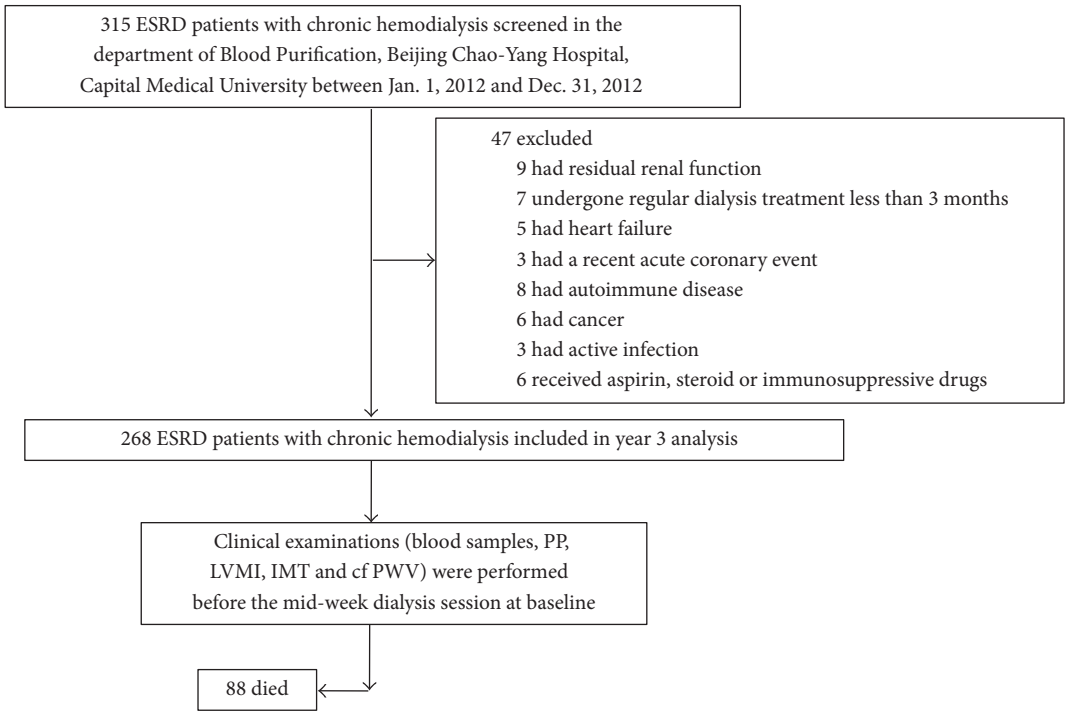

Figure 1: Study flow chart.

the department of Blood Purification, Beijing Chao-Yang Hospital, Capital Medical University were recruited from January 1, 2012, to December 31, 2012. The inclusion criteria included ESRD patients having no residual renal function and having undergone regular dialysis treatment for at least 3 months, but without clinical evidence of heart failure, a recent acute coronary event, autoimmune disease, cancer, and active infection and taking aspirin, steroid, or immunosuppressive drugs. A standard questionnaire was adopted from every patient to obtain systematic information regarding conventional cardiovascular risk factors, including hypertension, hyperlipidaemia, diabetes, and family history of cardiovascular disease. All the patients were followed for 36 months. The primary end point was all-cause mortality and cardiovascular mortality. The flow chart of the study was shown as in Figure 1.

The ESRD patients underwent hemodialysis three times a week with standard bicarbonate dialysate (Na^+ 138 mmol/L, HCO_3^- 35 mmol/L, K^+ 2.0 mmol/L, Ca^{2+} 1.5 mmol/L, and Mg^{2+} 0.5 mmol/L) and 1.6 m^2 polysulphone membrane dialysers. Patients were separated into two groups according to common carotid artery plaque, HD patients with and without plaque. The study was performed conform with the declaration of Helsinki and approved by the ethics committee of Beijing Chao-Yang Hospital, Capital Medical University. The written informed consent was obtained from each participant.

2.2. Cardiovascular Measurement. Cardiovascular risk markers measurements, including pulse pressure (PP), left ventricular mass index (LVMI), intima-media thickness (IMT), and carotid-femoral pulse wave velocity (cfPWV), were performed before the mid-week dialysis session at baseline.

Blood pressure was measured with a mercury sphygmomanometer after 15 minutes of recumbency. PP was calculated as the systolic blood pressure (SBP) minus the diastolic blood pressure (DBP).

LVMI was evaluated by echocardiography. Left ventricular end diastolic dimension (LVDD), interventricular septum thickness (IVST) and left ventricular posterior wall thickness (LVPWT) were measured. LVMI was calculated and normalized by height$^{2.7}$ (LVMI = LVM/height$^{2.7}$) as previously [16].

IMT was evaluated by common carotid artery ultrasonography as described previously [17]. The mean IMT was calculated as the average of the three readings of bilateral carotid arteries. HD patients with plaque were defined as localized thickening of IMT $\geq$ 1.2 mm that did not uniformly involve the whole wall of carotid artery.

The common carotid artery stiffness was evaluated by cfPWV. The cfPWV value was measured with the participants in a supine position by using the Complior SP System (Alam Medical, Vincennes, France) [18].

2.3. Laboratory Investigations. The fasting blood samples of HD patients were taken from the arterial end of the vascular access immediately before initiation of the mid-week HD session at baseline. The levels of albumin (Alb), alanine transaminase (ALT), aspartate aminotransferase (AST), triglycerides (TG), total cholesterol (Tch), low density lipoprotein-cholesterol (LDL-C), high sensitivity C reactive protein (hsCRP), creatinine (Cr), blood urea nitrogen (BUN), calcium (Ca), and phosphorus (P) were measured by standard laboratory methods using an autoanalyzer. Serum intact parathyroid hormone (iPTH) was determined by immunoradiometric assay.

The blood samples were drawn in plastic vacutainers using EDTA (1 mg/mL of blood) for differential white blood cells count. NLR was calculated as the ratio of neutrophils to lymphocytes from the differential white blood cells count.

2.4. Statistical Analysis. All the data were analyzed using statistical software package (SPSS for Windows, Version 20.0, SPSS, USA). Continuous variables data were presented as mean ± standard deviation (±SD). Comparison between groups was performed using independent-samples t-test. In addition, spearman correlation was used for univariate analysis and logistic regression was used for multivariate analysis (confidence interval of 95%). Variables entered in multivariate analysis were age, gender, diabetes mellitus, HD duration, LDL-C, hsCRP, PP, LVMI, and IMT (≥1.2 mm, plaque). NLR cut-off value used in survival curves was determined by a receiver operating characteristic (ROC) curve. Survival curves were estimated by Kaplan-Meier analysis and compared by the log rank test. A Cox regression model was used to identify predictors of mortality. A P value < 0.05 was considered statistically significant.

3. Results

3.1. Demographic, Clinical, Laboratory, and Vascular Parameters of the Studied Population. A total of 268 HD patients with a mean age of 48.7 ± 10.9 years (range 21–78 years) and a mean dialysis period of 45.9 ± 32.5 months (range 4–146 months) were enrolled in this study. The baseline demographic, clinical, biochemical, and vascular characteristics of patients were described as shown in Table 1.

3.2. Characteristics of HD Patients with and without Common Carotid Artery Plaque. According to the localized thickness of IMT, we found that about 44.4% HD patients had plaque in common carotid artery. Mean level of NLR in all HD patients was 3.36, but the HD patients with plaque had higher level of NLR ($n = 119$). There were no significant differences with respect to the following variables between both groups: age, sex distribution, dialysis duration, diabetes, smoking, KT/V, Hb, serum creatinine, BUN, TG, Tch, and LDL-C in HD patients with plaque or without plaque. But interestingly, HD patients with plaque also had higher serum hsCRP level (Table 2).

3.3. Correlation of NLR with Cardiovascular Risk Factors in HD Patients. As shown in Figures 2(a), 2(b), and 2(c), HD patients with higher PP (≥65 mmHg), LVMI (≥50 g/height$^{2.7}$), and IMT (≥1.2 mm) had significantly higher NLR levels (all $P < 0.01$).

By testing using univariate analysis, NLR levels were positively correlated with LVMI ($r = 0.566$; $P < 0.01$), PP ($r = 0.579$; $P < 0.01$), cfPWV ($r = 0.935$; $P < 0.01$), IMT ($r = 0.578$; $P < 0.01$), plaque ($r = 0.776$; $P < 0.01$), and hsCRP ($r = 0.552$; $P < 0.01$). There was no correlation with age, gender, dialysis duration, smoking, diabetes, and serum LDL, as shown in Table 3.

Furthermore in multivariate analysis, NLR was an independent predictor of cardiovascular risk markers, high PP (PP ≥ 65 mmHg, OR = 3.056, 95% CI: 2.051–4.553, $P < 0.01$), high LVMI (LVMI ≥ 50 g/height$^{2.7}$, OR = 3.457, 95% CI: 2.271–5.264, $P < 0.01$), and plaque (IMT ≥ 1.2 mm, OR = 5.248, 95% CI: 3.178–8.667, $P < 0.01$).

Table 1: Demographic and biochemical parameters of the studied population.

Items	Patients ($n = 268$)
Age, years	48.7 ± 10.9
Gender, male, n (%)	149 (55.6%)
Primary disease	
Chronic glomerulonephritis, n (%)	106 (37.1%)
Hypertensive nephropathy, n (%)	31 (11.6%)
Diabetic nephropathy, n (%)	45 (16.8%)
Chronic interstitial nephritis, n (%)	22 (8.2%)
Polycystic kidney disease, n (%)	15 (5.6%)
Unknown, n (%)	49 (18.3%)
Dialysis duration, months	45.9 ± 32.5
Smoking, n (%)	71 (26.5%)
Diabetes, n (%)	49 (18.3%)
NLR	3.36 ± 1.65
LVMI, g/height$^{2.7}$	53.3 ± 10.5
LVMI > 50 g/height$^{2.7}$, n (%)	140 (50.2%)
PP, mmHg	67.6 ± 18.1
PP > 65 mmHg, n (%)	105 (39.2%)
cfPWV (mm/s)	14.7 ± 5.9
IMT (mm)	1.15 ± 0.16
Plaque, n (%)	119 (44.4%)
KT/V	1.36 ± 0.03
Hb, g/L	114.9 ± 12.7
Alb, g/L	36.3 ± 3.5
ALT, U/L	14.9 ± 5.8
AST, U/L	14.3 ± 6.2
TG, mmol/L	1.82 ± 1.17
Tch, mmol/L	4.24 ± 0.86
LDL-C, mmol/L	2.24 ± 0.63
hsCRP, mmol/L	4.85 ± 3.40
Cr, mol/L	913.6 ± 167.9
BUN, mmol/L	24.8 ± 6.0
Ca, mmol/L	2.29 ± 0.33
P, mmol/L	1.93 ± 0.60
iPTH, pg/ml	270.6 ± 125.8
RASI, n (%)	203 (75.7%)
CCB, n (%)	209 (78.0%)
β-Blocker, n (%)	79 (29.1%)

Values are means ± SD, unless specified otherwise.
NLR: neutrophil to lymphocyte ratio; LVMI: left ventricular mass index; PP: pulse pressure; cfPWV: carotid–femoral pulse wave velocity; IMT: intima-media thickness; Hb: hemoglobin; Alb: albumin; ALT: alanine transaminase; AST: aspartate aminotransferase; TG: triglyceride; Tch: total cholesterol; LDL-C: low density lipoprotein-cholesterol; hsCRP: high sensitivity C reactive protein; Cr: creatinine; BUN: blood urea nitrogen; Ca: calcium; P: phosphorus; iPTH: intact parathyroid hormone; RASI: renin angiotensin system inhibitor; CCB: calcium channel blocker; β-blocker: β-receptor blocker.

Table 2: Characteristics of HD patients with and without plaque.

Items	HD/nonplaque group (n = 149)	HD/plaque group (n = 119)	t/χ^2 value	P value
Age, years	48.3 ± 10.9	49.3 ± 10.9	0.773	0.440
Gender, male/female	32/28	30/23	0.082	0.774
Dialysis duration, months	44.5 ± 30.8	47.7 ± 34.6	0.787	0.432
Smoking, no. (%)	16 (26.7)	14 (26.4)	0.181	0.671
Diabetes, no. (%)	11 (18.3)	10 (18.9)	1.064	0.302
NLR	2.38 ± 0.63	4.59 ± 1.72[a]	14.484	0.000
KT/V	1.36 ± 0.28	1.36 ± 0.29	0.293	0.770
Hb, g/L	115.5 ± 12.9	114.2 ± 12.5	0.840	0.402
Alb, g/L	36.4 ± 3.6	36.2 ± 3.4	0.438	0.662
ALT, U/L	15.2 ± 6.0	14.6 ± 5.5	0.779	0.437
AST, U/L	14.6 ± 6.8	13.9 ± 5.5	0.940	0.348
TG, mmol/L	1.89 ± 1.26	1.72 ± 1.05	1.141	0.255
Tch, mmol/L	4.31 ± 0.95	4.15 ± 0.74	1.561	0.120
LDL-C, mmol/L	2.26 ± 0.65	2.22 ± 0.61	0.466	0.642
hsCRP, mmol/L	3.06 ± 1.88	7.09 ± 3.53[a]	11.958	0.000
Cr, mol/L	917.0 ± 167.6	909.3 ± 168.9	0.372	0.710
BUN, mmol/L	25.0 ± 5.8	24.6 ± 6.1	0.588	0.557
Ca, mmol/L	2.28 ± 0.31	2.30 ± 0.35	0.513	0.608
P, mmol/L	1.96 ± 0.59	1.89 ± 0.62	1.023	0.307
iPTH, pg/ml	274.6 ± 126.4	265.4 ± 125.3	0.590	0.556
RASI, no. (%)	43 (71.7)	42 (79.2)	0.285	0.593
CCB, no. (%)	45 (75.0)	43 (81.1)	0.691	0.406
β-Blocker, no. (%)	17 (28.3)	17 (32.1)	2.325	0.127

Values are means ± SD, unless specified otherwise.
[a] $P < 0.01$, compared with HD/nonplaque group.
NLR: neutrophil to lymphocyte ratio; Hb: hemoglobin; Alb: albumin; ALT: alanine transaminase; AST: aspartate aminotransferase; TG: triglyceride; Tch: total cholesterol; LDL-C: low density lipoprotein-cholesterol; hsCRP: high sensitivity C reactive protein; Cr: creatinine; BUN: blood urea nitrogen; Ca: calcium; P: phosphorus; iPTH: intact parathyroid hormone; RASI: renin angiotensin system inhibitor; CCB: calcium channel blocker; β-blocker: β-receptor blocker.

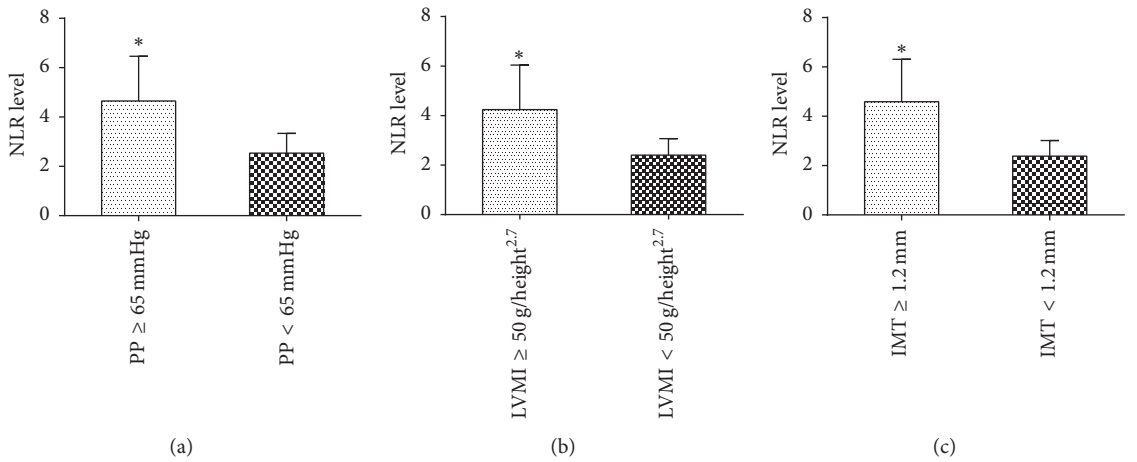

Figure 2: NLR level in HD patients with CVD risk factors. (a) HD patients with higher PP (≥65 mmHg) had higher NLR level. ∗ indicates a significant difference between the PP (≥65 mmHg) group and PP (<65 mmHg) group ($P < 0.01$); (b) HD patients with higher LVMI (≥50 g/height$^{2.7}$) had higher NLR level. ∗ indicates a significant difference between the LVMI (≥50 g/height$^{2.7}$) group and LVMI (<50 g/height$^{2.7}$) group ($P < 0.01$); (c) HD patients with higher IMT (≥1.2 mm) had higher NLR level. ∗ indicates a significant difference between the IMT (≥1.2 mm) group and IMT (<1.2 mm) group ($P < 0.01$).

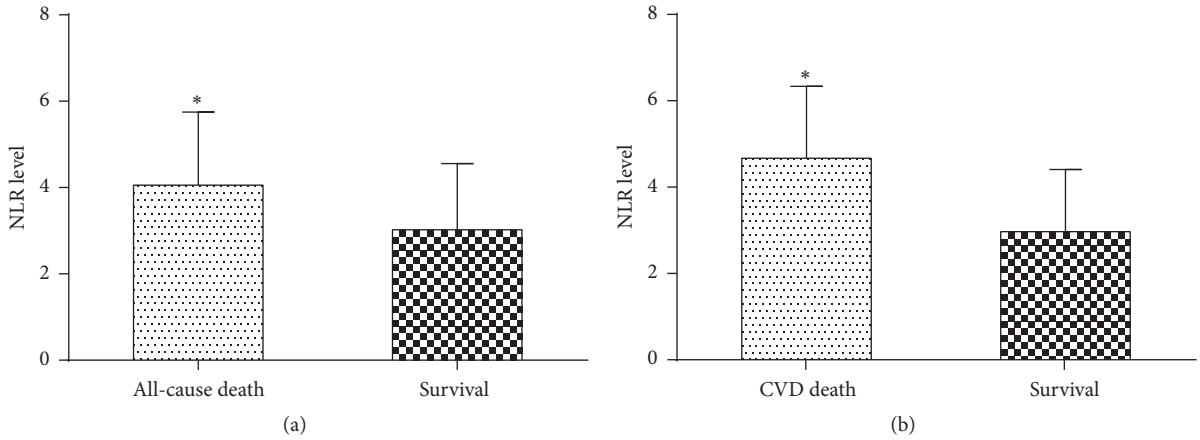

Figure 3: NLR level in HD patients with CVD and overall death. (a) HD Patients who died from overall causes had higher NLR level. ∗ indicates a significant difference between the all-cause death group and survival group ($P < 0.01$); (b) HD Patients who died from cardiovascular causes had significantly higher NLR level. ∗ indicates a significant difference between the CVD death group and survival group ($P < 0.01$).

Table 3: Correlation coefficients for NLR and other variables in HD patients.

Variables	R	P value
Age	0.005	0.931
Gender	0.008	0.899
Smoking	0.006	0.919
Diabetes	0.042	0.489
Dialysis durations	0.055	0.369
LDL-C	0.002	0.978
hsCRP	0.552	0.000
LVMI	0.566	0.000
PP	0.579	0.000
IMT	0.578	0.000
Plaque	0.776	0.000
cfPWV	0.935	0.000

LDL-C: low density lipoprotein-cholesterol; hsCRP: high sensitivity C reactive protein; LVMI: left ventricular mass index; PP: pulse pressure; IMT: intima-media thickness; cfPWV: carotid–femoral pulse wave velocity.

3.4. NLR Level in HD Patients with Cardiovascular Death and All-Cause Death. In this study, 88 of 268 (32.8%) patients died from overall causes during the 36-month period, and 62 of 88 (70.5%) patients died from cardiovascular causes. HD patients with cardiovascular death had higher level of NLR (CVD death versus survival, 4.67 ± 1.66 versus 2.96 ± 1.43; $P < 0.01$). And HD patients who died from overall causes had higher NLR level (4.06 ± 1.69 versus 3.02 ± 1.53; $P < 0.01$) (Figures 3(a) and 3(b)).

3.5. NLR More than or Equal to 3.5 Was Associated with High All-Cause and Cardiovascular Death in HD Patients. The cut-off value of NLR determined by ROC curve analysis was 3.5 (AUC: 0.847; 95% CI: 0.801–0.892; 98.4% sensitivity; 79.1% specificity). Kaplan-Meier analysis showed that overall causes (log rank = 15.28; $P < 0.01$) and cardiovascular diseases (log rank = 43.54; $P < 0.01$) were responsible for a significant lower 36-month survival in HD patients with mean NLR level more than and equal to 3.5 (Figures 4(a) and 4(b)).

Cox regression analysis showed that NLR was a significant predictor of all-cause mortality (HR = 1.695; 95% CI = 1.288–2.231; $P < 0.01$) and cardiovascular mortality (HR = 1.379; 95% CI = 1.162–1.637; $P < 0.01$) in HD patients, using models adjusted for demographic and clinical covariates, which were age, gender, diabetes mellitus, HD duration, LDL-C, hsCRP, PP, LVMI, and plaque (IMT ≥ 1.2 mm).

4. Discussion

In this study, we evaluated the prognostic value of NLR for cardiovascular risk factors and mortality in HD patients. The results indicated that NLR was an independent predictor of higher PP, LVMI, and IMT. Interestingly, we further found that NLR more than or equal to 3.5 was a predictor of all-cause mortality and cardiovascular mortality in HD patients.

Previous studies have illustrated the predictive value of NLR as a novel inflammation marker in patients with cardiovascular diseases in general population. In the hypertension patients, the NLR value increased and positively correlated with hyperhomocysteinemia [19]. In the pathogenesis of aneurysm of the ascending aorta in hypertensive patients, NLR as a marker of inflammation may play an important role [20]. In patients with symptomatic intermediate carotid artery stenosis, NLR was increased and the increased NLR value was an independent variable for carotid artery plaques to become symptomatic [21]. In ischemic stroke patients, dynamic change of NLR has been shown to predict hemorrhagic transformation after thrombolysis [22]. In patients with ST-segment elevation myocardial infarction, NLR was related to electrocardiographic sign of spontaneous reperfusion [23]. In patients undergoing nonurgent percutaneous coronary intervention, a higher NLR increased the risk of

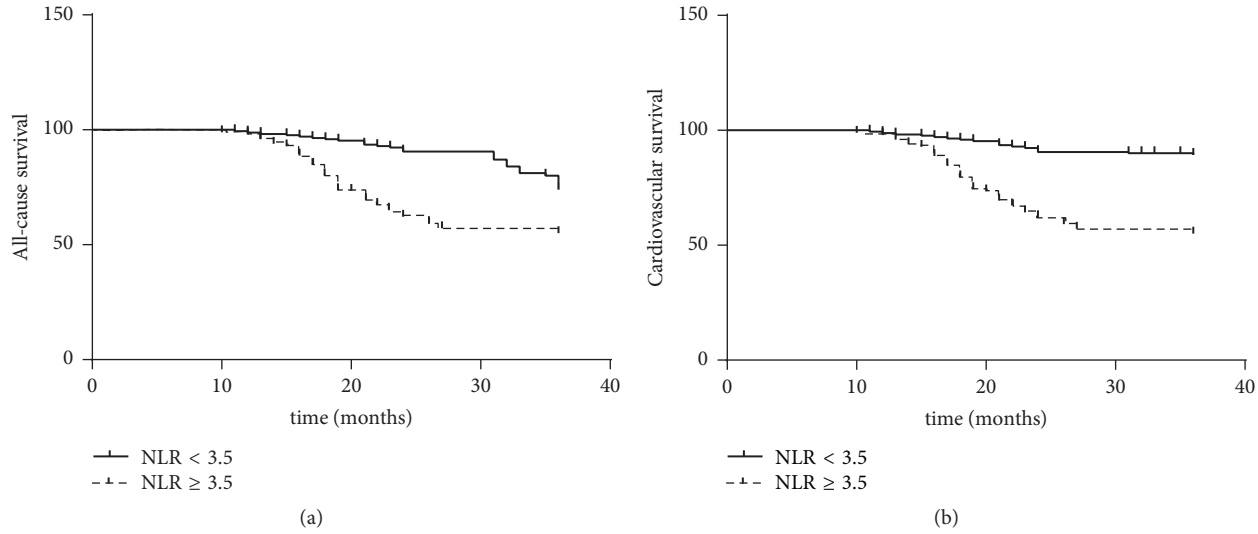

Figure 4: NLR ≥ 3.5 was associated with higher all-cause and cardiovascular death. (a) NLR ≥ 3.5 had a significant higher 36-month all-cause mortality in HD patients (log rank = 15.28; $P < 0.01$); (b) NLR ≥ 3.5 had a significant higher 36-month cardiovascular mortality in HD patients (log rank = 43.54; $P < 0.01$).

periprocedural myocardial infarction [24]. NLR was also significantly associated with microvascular disease in asymptomatic subjects [25]. Furthermore, it has been reported recently that elevated NLR was associated with worse overall survival in noncancer patients [26]. In patients with peripheral arterial occlusive disease, an increased NLR was related to higher mortality [27]. In patients with advanced heart failure, elevated NLR was associated with increased mortality or heart transplantation risk [28]. Meanwhile, Durmus et al. [29] found that NLR was higher in heart failure patients and a cut-off value of 5.1 for NLR can predict death in heart failure patients.

However, the association between NLR and cardiovascular disease has been little investigated in CKD patients. Tatar et al. [30] found the basal NLR was an independent predictor of death in geriatric patients with stage 3–5 chronic kidney disease. Kocyigit et al. [31] demonstrated that patients with a high NLR had worse prognosis and significantly faster progression to the dialysis compared with those with a low NLR. Solak et al. [32] reported that NLR was independently related to endothelial dysfunction and could predict composite cardiovascular endpoints independent of traditional confounding factors in patients with moderate to severe CKD. But thus far, little was known of the prognostic value of NLR in hemodialysis patients. In this present study, we investigated the association between NLR and cardiovascular risk factors and mortality in HD patients, and we believe that the current study will provide us new enlightenment and direction in this area.

Chronic inflammation is prevalent in patients with chronic kidney disease and may contribute to morbidity and mortality among dialysis patients [33]. Increased inflammation in ESRD contributes to cardiovascular morbidity, a leading cause of mortality in these patients. Biomarkers have played a significant role in the prediction, diagnosis, and treatment of cardiovascular disease outcomes including myocardial infarction, congestive heart failure, and stroke [34]. The role of inflammatory markers in cardiovascular diseases has been studied extensively and a consistent relationship between C reactive protein and cardiovascular diseases has been established in the past [35]. NLR, a novel biomarker for assessing inflammation, has been getting widely used to identity patients with various illness. NLR is a biomarker that integrates two WBC subtypes representing two inversely and related immune pathways. It was easily calculated from differential WBC counts, more stable for measurement than the individual WBC counts, and less affected by conditions that could change the individual cell counts [36]. The recent remarkable observation has been that NLR has a greater predictability than total WBC count or neutrophil count as a marker in cardiovascular diseases and was slowly emerging as an independent useful prognostic parameter in cardiovascular diseases [37]. According to our present study, an easy and inexpensive laboratory measure of NLR might provide significant information regarding cardiovascular risk factors and mortality in HD patients.

Neutrophil extracellular traps (NETs), first discovered in 2004 by Brinkmann et al. [38], are formed and released by activated neutrophils during the process of NETosis in which the nuclear material is released into extracellular space, including DNA, citrullinated histones, and enzymes of neutrophil granule [39]. This discovery casts a new light on the role of neutrophils in the nonspecific immune response of the body. Although the beneficial effect of NETs in the fight against pathogens has been confirmed in many clinical findings, further evidence has been provided that NETs may promote inflammatory reactions and cause damage to tissues [40]. Additionally, circulating cell-free DNA, a maker of NETs formation, has been demonstrated to promote inflammation and to predict mortality in HD patients [41, 42]. Meanwhile, Qin et al. reported that NETosis markers, including neutrophil elastase and proteinase 3, were positively

correlated with absolute neutrophil count in type 1 diabetes patients [43]. All of these findings suggest that the formation of NETs may be one of the possible mechanisms by which an increased NLR is related to higher mortality. Unfortunately, there is little research on the relationship between NETs and NLR in HD patients.

Some limitations of this study should be acknowledged. First, in our study, the patients were selected only from the dialysis center in our hospital instead of from a general population; thus this may not be an accurate reflection of the general population. Second, we measured NLR for only one time while serial measurements would have been more informative. Third, we only investigated the effect of the NLR value on the cardiovascular risk factors and mortality in HD patients. But we have not yet compared the predictive role of NLR with other simple inflammatory markers, such as total white blood count and platelet to lymphocyte ratio; thus we did not draw a conclusion which was the best biomarker to predict the cardiovascular risk factors and mortality in HD patients. Meanwhile, although we found that an increased NLR was related to higher mortality in HD patients, its possible molecular mechanism was still not clear.

5. Conclusions

This study demonstrated that a high NLR value was associated with the increased risk of cardiovascular disease. NLR more than or equal to 3.5 predicted all-cause and cardiovascular death in HD patients. Thus, NLR, which is easy to access and inexpensive, may be a novel biomarker for assessing inflammation and identifying high risk for cardiovascular disease and death in HD patients. However, there are still many problems needing further research, such as the mechanism of the effect of high NLR value on the cardiovascular disease and death in HD patients and the effect of high NLR on the specific kind of cardiovascular diseases, so that we will finally find a cheap, reliable, and independent prognostic biomarker of cardiovascular disease and death in HD patients.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Review Article

Ischemia/Reperfusion Injury following Acute Myocardial Infarction: A Critical Issue for Clinicians and Forensic Pathologists

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Acute myocardial infarction (AMI) is a leading cause of morbidity and mortality. Reperfusion strategies are the current standard therapy for AMI. However, they may result in paradoxical cardiomyocyte dysfunction, known as ischemic reperfusion injury (IRI). Different forms of IRI are recognized, of which only the first two are reversible: reperfusion-induced arrhythmias, myocardial stunning, microvascular obstruction, and lethal myocardial reperfusion injury. Sudden death is the most common pattern for ischemia-induced lethal ventricular arrhythmias during AMI. The exact mechanisms of IRI are not fully known. Molecular, cellular, and tissue alterations such as cell death, inflammation, neurohumoral activation, and oxidative stress are considered to be of paramount importance in IRI. However, comprehension of the exact pathophysiological mechanisms remains a challenge for clinicians. Furthermore, myocardial IRI is a critical issue also for forensic pathologists since sudden death may occur despite timely reperfusion following AMI, that is one of the most frequently litigated areas of cardiology practice. In this paper we explore the literature regarding the pathophysiology of myocardial IRI, focusing on the possible role of the calpain system, oxidative-nitrosative stress, and matrix metalloproteinases and aiming to foster knowledge of IRI pathophysiology also in terms of medicolegal understanding of sudden deaths following AMI.

1. Introduction

Acute myocardial infarction (AMI) is a leading cause of morbidity and mortality in the world [1]. Reperfusion strategies are the current standard therapy for AMI [2, 3]. They may, however, result in paradoxical cardiomyocyte dysfunction and worsen tissue damage, in a process known as "reperfusion injury" [4–9]. Ischemic reperfusion injury (IRI) typically arises in patients presenting with an acute ST-segment elevation myocardial infarction (STEMI), in whom the most effective therapeutic intervention is timely and effective myocardial reperfusion [7, 10–14]. Reperfusion itself is known as a "double-edged sword" [4, 15] due to the spectrum of reperfusion-associated pathologies. Outcomes subsequent to IRI accrue in a time-dependent fashion [16], beginning with oxidative stress, inflammation, intracellular Ca^{2+} overload, and rapidly proceeding to irreversible cell death by apoptosis and necrosis [13, 16]. Different forms of myocardial IRI are recognized, of which only the first two are reversible: reperfusion-induced arrhythmias, myocardial stunning, microvascular obstruction, and lethal myocardial reperfusion injury [13].

In particular, sudden death is the most common pattern for ischemia-induced lethal ventricular arrhythmias (VAs) during the acute phase of myocardial infarction [17], and it is well known that reperfusion itself can lead to life-threatening VAs [17] and, ultimately, induce sudden mortality.

The exact mechanisms of IRI are not fully known [18]. Molecular, cellular, and tissue alterations such as cell death,

inflammation, neurohumoral activation, and oxidative stress are considered to be of paramount importance for IRI development [10, 19]. However, comprehension of the exact pathophysiological mechanisms of IRI [20, 21] remains a challenge for clinicians [22, 23], and the existence of reperfusion injury is still a matter of debate in the scientific community, essentially due to a lack of a definitive clinical documentation. Many gaps still exist between experimental animal models and human clinical experience, with subsequent difficulties in translating experimental results on cardioprotection to clinical practice [22–24]. Despite the difficulties that still exist in fully comprehending myocardial IRI, early and aggressive reperfusion strategies remain the most important intervention and are strongly advocated. The development of ischemic conditioning strategies to limit the extent of infarcted tissue caused by ischemia/reperfusion injury markedly enhances the ability of the heart to withstand an ischemic insult [25].

Finally, myocardial IRI is a critical issue also for forensic pathologists since sudden death may occur despite timely reperfusion following AMI, that is one of the most frequently litigated areas of cardiology practice [26, 27].

In this paper we explore the literature regarding the pathophysiology of myocardial IRI, focusing on the possible role of the calpain system, oxidative-nitrosative stress, and matrix metalloproteinases. We discuss these mechanisms within the broad scenario of IRI, also discussing the medicolegal issues related to sudden deaths occurring during the acute phase of myocardial infarct following reperfusion interventions.

2. The Calpain System

The process of IRI is not yet completely understood in its underlying pathophysiological mechanisms. Several pathways have been proposed, including cytosolic and mitochondrial Ca^{2+} overload, release of reactive oxygen species (ROS), acute inflammatory response, and impaired metabolism [20, 21]. These alterations may collaboratively act and produce irreversible damage to ischemic reperfused cardiomyocytes.

The possibility that the calpain system could play a role in generating myocardial IRI has been experimentally investigated in the literature [28–32], and several studies have focused on the effects of calpain inhibitors in improving myocardial dysfunction in different animal models [33–37]. Calpains are a family of Ca^{2+}-dependent nonlysosomal cystein proteinase localized in the cytosol in their inactive form [38]. Calpain activation, which may occur under several conditions, is thought to be a key mechanism in activating a number of substrates such as growth factor receptors, cytoskeletal proteins, microtubules associated proteins, and mitochondria, so playing a crucial role in cell cycle, apoptosis, and differentiation [38–40].

The calpain superfamily is complex, and more than 25 calpains or calpain-like molecules have been discovered. Calpains 1 and 2 are biologically activated when they arrange as dimer with a 30 kDa subunit. Both biologically active calpains are usually called μ-calpain (calpain 1 + 30 kDa subunit) and m-calpain (calpain 2 + 30-kDa subunit). The terms μ-calpain and m-calpain indicate, respectively, the micromolar and millimolar Ca^{2+} concentrations required for their activation [19]. Calpains may appear in the form of both "ubiquitous" isoenzymes that are present in almost all cells (such as μ-calpain, m-calpain, and calpains 5, 7, 10, 13, and 15) and "tissue specific" calpains expressed only in special tissues and cells, such as calpains 3 and 6 and others [31].

In brief, it has been hypothesized that, under physiological conditions, inactive calpains are stored in cellular cytosol and bound in a substrate competitive manner to their endogenous inhibitor calpastatin. The elevation of intracellular calcium levels is the key to the calpain activation process. Calpain conformational changes permit its translocation into cellular membrane, where phospholipids reduce the Ca^{2+} threshold for calpain activation or close the Ca^{2+} channels leading up to protein activation [41]. Several pathological cardiac events are associated with an imbalance of calcium homeostasis related to myocardial ischemia/reperfusion injury [29–31]. Experimental studies on isolated perfused mammalian hearts demonstrated an increase in intracellular Ca^{2+} concentrations in response to ischemia/reperfusion [31, 41]. Myocardial ischemia favours intracellular ion accumulation (sodium, calcium) till dropping in pH and tissue acidosis. Reperfusion evokes rapid alterations in ion flux and interacts with ischemia in altering the physiology of ion exchange [42]. Among others, a final result of the dangerous interplay between ischemia and reperfusion is intracellular calcium overload.

The kinetics of calpain activation are not completely understood, and whether or not translocation to the sarcolemma is needed for calpain activation during IRI remains undetermined [43]. In their elegant experiment, Hernando et al. [37] suggested that calpain translocation to the cardiomyocytes membranes during ischemia is independent of its activation since intracellular acidosis occurring during ischemia is likely to inhibit calpain activation. As intracellular pH normalizes following reperfusion, calpain activation occurs. Despite translocation, calpain seems to remain inactive even after 60 minutes of ischemia and only on reperfusion is it activated [37].

Activated calpain has a number of substrates such as growth factor receptors, cytoskeletal proteins, microtubules associated proteins, and mitochondria, thus playing a crucial role in the processes of cell cycle, apoptosis, and differentiation, negatively affecting cardiomyocyte function.

Firstly, the calpain system is part of the integrated proteolytic system which is crucial to the maintenance of the structure and function of the cardiac sarcomere. An imbalance of this system is the key to the sarcomeric dysfunction linked to several cardiovascular diseases, including hypoxia, IRI, myocardial infarction, and end-stage heart failure. Protein degradation (proteolysis) within cardiac sarcomere is regulated mainly by three systems: the ubiquitin proteasome system (UPS); autophagy/lysosomal degradation; and the calpain system [44]. Degradation of myofibrillar proteins involved in the contractile process is an effect of calpain activation. The degradation process following IRI involves either structural or regulatory proteins of contractile apparatus. In vitro study [45] showed that many of these proteins are

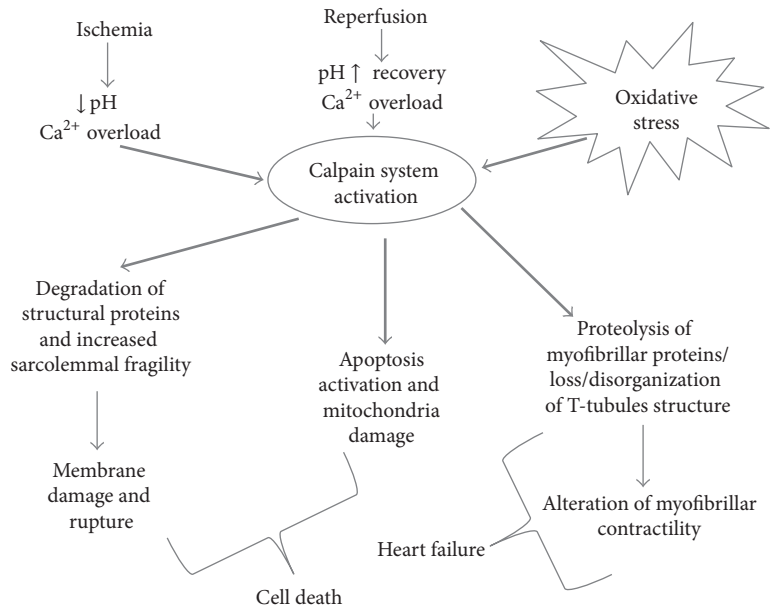

Figure 1: Schematic representation of calpain activation during myocardial IRI. Ca^{2+} overload and pH recovery in reperfusion phase are crucial in the activation of the calpain system. Increased sarcolemmal fragility may lead to membrane rupture and cell death. In addition, both the death-receptor and mitochondrial mediated apoptotic pathways seem to be affected by calpain activation. The degradation of myofibrillar proteins and the loss/disorganization of T-tubules structure are key factors in post-MI heart failure development.

potential targets of activated calpains, thus contributing to the development of postischemic injury in the human myocardium. Several experimental studies demonstrated that the loss/disorganization of T-tubules structure is a key factor in heart failure development [46–48]. Calpain-mediated disruption of T-tubules integrity through the proteolysis of junctophilin is demonstrated to be one of the major factors involved in an experimental model of cardiac muscle failure [49, 50].

Calpain deregulation is known to be an effective mechanism of apoptosis induction in cardiac sarcomeres through different pathways [51–53], and apoptosis of myocardial cells is considered an important mechanism of IRI [54–56].

Conclusively, an uncontrolled activation of calpain has been found to be implicated in the pathophysiology of several cardiovascular disorders [57] including myocardial IRI [58], and the inhibition of calpains has been shown to attenuate myocardial stunning and reduce infarct size after ischemia reperfusion [59] (Figure 1). However, the exact role of calpain in acute myocardial IRI remains controversial [60].

3. Oxidative Stress and Mitochondria

An oxidant and antioxidant imbalance (oxidative stress) favours the accumulation of oxidants, from both increased ROS production and decreased ROS scavenging ability, thus leading to cellular damage in the cardiomyocytes [61]. Oxidative stress is often associated with elevated levels of ROS or reactive nitrogen species (RNS) in the cellular and subcellular levels [61], leading to proteins, lipids, and DNA damage [62]. Furthermore, in cardiomyocytes, increased ROS/RNS levels can induce alterations of proteins involved in excitation-contraction coupling with increased susceptibility to proteolysis [62–65].

In the first few minutes IRI, and especially myocardial reperfusion, induces a high production of ROS by a variety of sources [66–69]. Since Arroyo et al. provided direct evidence of ROS formation during myocardial ischemia and postischemic reperfusion by trapping these free radicals using nitrone DMPO [70], several preclinical and clinical studies [71–74] have demonstrated the potential cardioprotective value of antioxidants. While small amounts of ROS could result in cardioprotection via preconditioning [75], the excessive production of ROS during reperfusion seems especially important in inducing injury.

Mechanisms leading up to the dysfunction and the initial sources of ROS during IRI are not completely clear [76]. Nitric oxide (NO) production is considered a key factor in IRI. NO is an important bioactive substance which plays an important role in the regulation of normal body function and disease occurrence, and it is recognized as an ubiquitous signalling molecule with a multitude of biological actions and targets. Signalling may involve direct reactions between NO and a molecular target or can occur through indirect reactions of secondary ROS [77]. In fact, actions of NO are multifaceted, and its interactions with oxygen or oxygen-related reactive intermediates (e.g., superoxide) yield numerous RNS and ROS. These account for most of the so-called indirect effects attributed to NO through oxidation, nitrosation, and nitrate reactions referred to as oxidative, nitrosative, and nitrative stress, respectively. The physiological production of NO in the heart maintains coronary vasodilator tone and inhibits platelet aggregation and neutrophil and platelet adhesion, so performing an active

role in cardioprotection [78–80]. Beyond its beneficial effects, it has been speculated that NO excess can induce cellular injury either due to direct toxicity [81, 82] and to the reaction with superoxide ($O2^{-}$) to form the potent oxidant peroxynitrite (ONOO2) [83] which in turn exerts cytotoxicity via its reaction with a variety of molecular targets [84, 85]. The formation of highly reactive species, such as peroxynitrite, is a possible mechanism by which NO elicits its dangerous effects [83].

Much about NO biological actions remains contradictory, especially with regard to pathophysiologic disturbances in NO signalling. There is an ongoing debate about the levels of NO involved and whether there is a clearly defined threshold at which NO shifts from being beneficial to being destructive. Some authors hypothesize that the biological function of NO depends mostly on concentration and time course of exposure to NO, supposing that cytotoxic events, such as arrest of the cell cycle, cell senescence, or apoptosis, can occur at high NO concentrations [86]. However, other authors suggest that the chemical and biological reactivity of NO that has been studied using very high NO concentrations is of doubtful physiological relevance [87].

Zhang and Cai have shown that exogenously applied netrin-1 exerts robust cardioprotective effects against IRI, via an increase in NO formation [88]; the same group have further demonstrated that endogenously increased NO production could mediate cardioprotection by modulating oxidative stress and mitochondrial function [76]. Under physiological oxidative stress, NO mediates S-nitrosylation of critical protein thiols and thus averts them from further oxidative modifications by ROS, thereby rendering cardioprotection [89]. It is argued that NO protects the heart against IRI [79, 90]; however, excessive NO formation is thought to contribute to contractile dysfunction [91, 92].

During reperfusion NO release may be stimulated through a number of mechanisms including the change in shear stress in the coronary vasculature during reperfusion, increased intracellular Ca^{2+} levels as a result of ischemia, and the thermodynamically favoured production of NO from L-arginine and molecular oxygen due to reperfusion [93, 94]. NO is produced endogenously within the myocardium by three distinct isoforms of NO synthase (NOS) [95]. Neuronal NOS (NOS1) and endothelial NOS (NOS3) are constitutively expressed within cardiomyocytes while inducible NOS (NOS2) is only expressed within cardiomyocytes during inflammatory responses which occur during many pathophysiological conditions of the myocardium [96].

Mitochondria play a critical role in the pathogenesis of myocardial IRI. They occupy 30–50% of the cardiomyocyte cytoplasmic volume and are critical in cardiac energy balance since energy supply for cardiomyocytes is mostly derived from mitochondrial oxidative phosphorylation (OXPHOS). On the other hand, they are a favoured target of intracellular damage [97–99]. These cell organelles are the major contributors of ROS as well as the major target for ROS-caused damage [100–106]. Mitochondrial dysfunction, reflected in the structure, function, and number of mitochondria within the cardiomyocyte, leads to diminished energy production, loss of myocyte contractility, altered electrical properties, and eventual cardiomyocyte cell death [100]. In this context, the mitochondrial permeability transition pore (MPTP) is thought to play a critical role in myocardial IRI (Figures 2 and 3)

MPTP refers to a mitochondrial channel which mediates the abrupt change, or transition, in inner mitochondrial membrane permeability which occurs under certain conditions [107]. The opening of the MPTP renders the inner mitochondrial membrane nonselectively permeable to molecules less than 1.5 kDa and elicits mitochondrial membrane depolarization and uncoupling of oxidative phosphorylation. It also favours collapsing the mitochondrial membrane potential, and uncoupling oxidative phosphorylation, thus leading to impairment of energy and ATP metabolism and cell necrosis [108–111]. MPTP opening also causes mitochondrial swelling, and outer mitochondrial membrane rupture, thus favouring the deposition of proapoptotic factors such as cytochrome *c* and SMAC/Diablo from the intermembranous space into the cytosol, thereby initiating apoptotic cell death [107].

During ischemia/reperfusion, intertwined biochemical events occur leading to MPTP opening. In the ischemic period, following factors such as Ca^{2+}, long-chain fatty acids, and ROS accumulation, the likelihood that MPTP will occur upon reperfusion gradually increases [112, 113]. During ischemia, due to increased glycolysis, an accumulation of lactic acid and reduction of pH occur. To restore the pH, the Na^{+}/H^{+} antiporter is activated, but it acts inefficiently because Na^{+} cannot be pumped out of the cell, as the Na^{+}/K^{+} ATPase is inhibited by the absence of intracellular ATP. Consequently, the cytosolic Ca^{2+} concentration increases. Moreover, the existing decrease in the adenine nucleotide concentration, which is associated with an increased phosphate concentration, is likely to sensitize MPTP opening in response to Ca^{2+}; however, low pH inhibits the opening. When reperfusion occurs, the mitochondria recover their ability to respire and rescue the sustained mitochondrial membrane potential, which is required for ATP synthesis. In addition, strong production of ROS occurs when the inhibited respiratory chain is reexposed to oxygen. Thus, the following resulting conditions are nearly optimal for MPTP opening: high Ca^{2+} levels within the mitochondrial matrix, increased levels of phosphate and oxidative stress, depletion of adenine nucleotide concentration, and rapid restoration of physiological value of pH [113–115].

In their milestone paper, Griffiths and Halestrap [116] demonstrated that MPTP are closed during ischemia and open the first few (2-3) minutes of reperfusion. Subsequent data has confirmed that pore opening occurs during reperfusion of the heart after ischemia, but not in the ischemic period [107]. Thus MPTP is an important new target for cardioprotection during reperfusion [114].

4. The Matrix Metalloproteinases

One group of enzymes that is important in mediating IRI injury is the family of matrix metalloproteinases (MMPs). The MMPs are a large family of calcium-dependent,

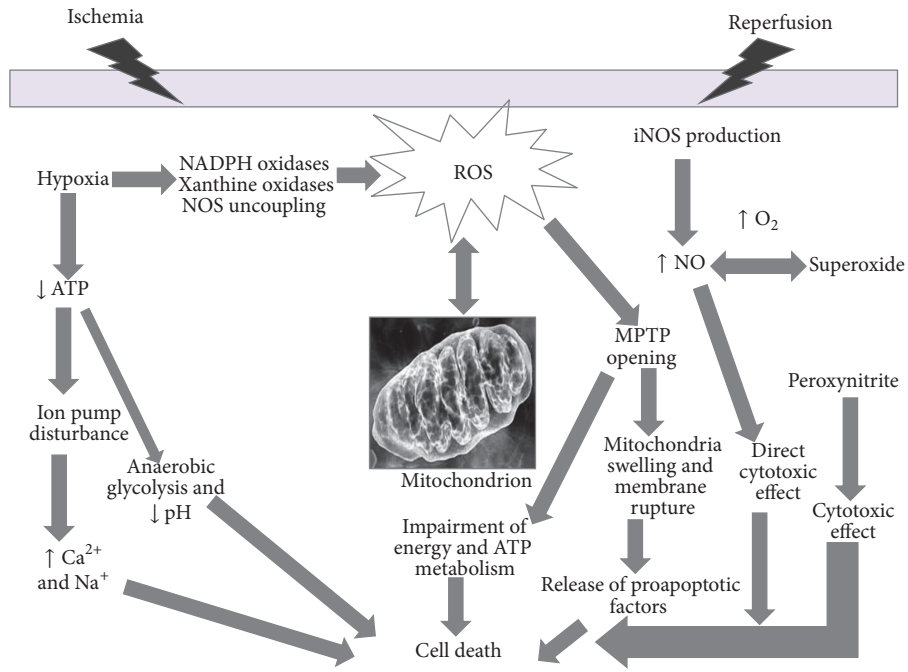

Figure 2: Schematic representation of oxidative stress contributing to tissue injury and cell death in IRI. Following ischemia, hypoxia results in reduction of ATP production, ion pump function unbalance, leading to overload of Na^{+} and Ca^{2+}, activation of anaerobic glycolysis, and, finally, reduction of pH. During the initial ischemic phase, the activation and upregulation of enzymes (such as NADPH oxidase, a superoxide-generating enzyme comprising a membrane-bound catalytic subunit) occurs, that are capable of producing ROS, when molecular oxygen is reintroduced in the reperfusion phase. ROS induces cell dysfunction and death via other mechanisms: activation of metalloproteinases and calpains, mitochondrial permeability transition pore (MPTP) opening which contributes to swelling and lysis of cells. This may elicit the release of proapoptotic factors in the cytosol, thus contributing to cell death. ROS indirectly interact with nitric oxide (NO) production, partly mediated by the inducible NOS (iNOS), the high-capacity NO-producing enzyme. Unlike the other two NOS isoforms, iNOS is not constitutively expressed in cells, and its production is elicited by several stimuli like IRI. NO cytotoxic effects are both direct and indirect mediated by NO reaction with superoxide to form the potent oxidant peroxynitrite which in turn exerts cytotoxicity.

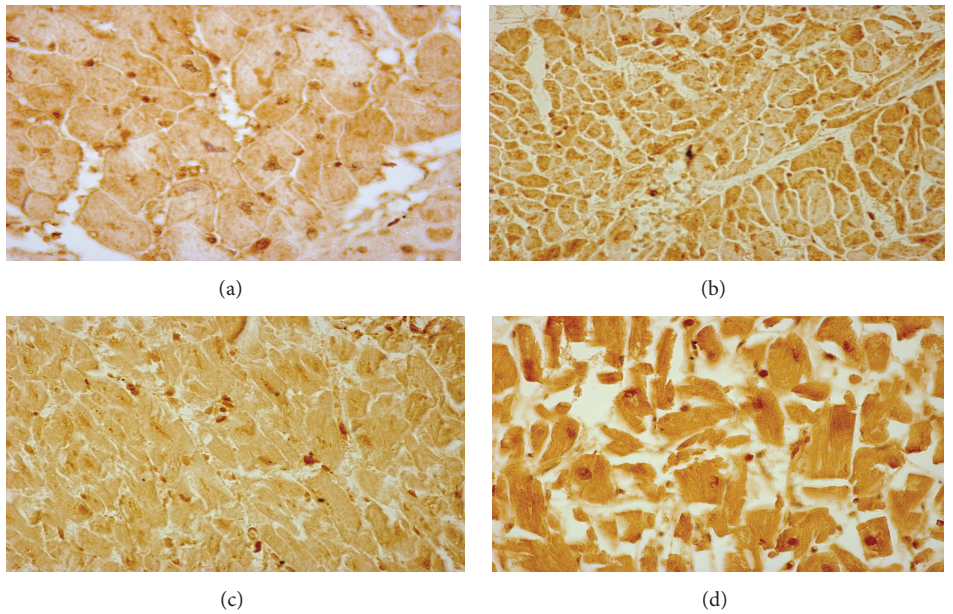

Figure 3: Histomorphological pictures showing the phenotypic results of altered pathways in IRI. (a) Mild calpain 1 expression in the left ventricle cardiac tissue of a patient who died following early reperfused AMI (calpain 1, antibody anti-calpain 1, Santa Cruz, USA). (b) NOX2 expression in the left ventricle cardiac tissue of a patient who died following prompt fibrinolysis in acute STEMI. (c) Strong immunopositivity to anti-nitrotyrosine antibody (Abcam, Cambridge, UK). (d) Mild immunopositivity to anti-iNOS (inducible nitric oxide synthase) antibody (Santa Cruz, CA, USA) in the left ventricle sample of a patient who died following reperfusion therapy in STEMI.

zinc-containing endopeptidases that have the ability to remodel the extracellular matrix in both physiological and pathological processes. MMPs are regulated at different levels including transcriptional, posttranscriptional, and posttranslational levels. Moreover, they are controlled via their endogenous inhibitors, the tissue inhibitor of metalloproteinases (TIMPs), and by their intra- and extracellular localization [117]. Of all MMPs, MMP-2 (also known as gelatinase A or type IV collagenase) plays a critical role in cardiovascular diseases [117]. MMP-2 activity is also regulated via nonproteolytic posttranslational modifications of the full-length zymogen form, by *S*-glutathiolation, *S*-nitrosylation, and phosphorylation [118–120]. The NO product, $ONOO^-$, directly activates MMP-s 2 [118] via a nonproteolytic mechanism involving the *S*-glutathiolation of the propeptide cysteine sulfhydryl group in a reaction requiring only micromolar concentrations of $ONOO^-$ in conjunction with normal intracellular levels of glutathione [119]. In turn, it was demonstrated that $ONOO^-$ inactivate TIMP-4 and TIMP-1, leading to a net increase in MMP activity [118].

In IRI, the sudden availability of molecular oxygen during reperfusion reenergizes mitochondria and reactivates the electron transport chain, causing a significant increase in the biosynthesis of ROS (including $ONOO^-$) [94, 121] which stimulates MMP-2 activity [118].

It has been demonstrated that MMP-2 exerts rapid effects in modulating different cellular functions independent of its action on the extracellular matrix (ECM). These include effects on platelet aggregation [122], vascular tone [123, 124], and acute mechanical dysfunction of the heart immediately after ischemia and reperfusion [125, 126]. In ischemia/reperfusion, injury may result in the partial proteolysis of the thin-filament regulatory protein troponin I (TnI) [60, 124, 125, 127–129], and studies on animal models have validated this observation, showing that MMP-2 degrade Tn I myofilaments [130].

MMP-2 has a proapoptotic role as demonstrated in adult rat cardiomyocyte by Menon et al. who show that inhibition of MMP-2 inhibits β-AR-stimulated apoptosis [131, 132]. Furthermore, MMP-2 is present in mitochondria [130], and cardiac-specific transgenic expression of active MMP-2 causes abnormalities in mitochondria ultrastructure, impaired respiration, increased lipid peroxidation, cell necrosis, and reduced recovery of contractile performance during post-IRI [133].

Finally, a complex interplay exists between the calpain and MMP systems since there appears to be overlap in the substrates and/or biological actions of MMP-2 and calpains in various cellular pathways [117]. Kandasamy et al. have hypothesized that either MMP-2 targets a subset of proteins similar to calpain, or calpain has been incorrectly identified as the protease responsible for some intracellular proteolytic activities. Indeed, much of the evidence for calpain degradation of substrates in cardiac cells rests on the use of calpain inhibitors such as calpastatin, which has been found to inhibit MMP-2 activity in vitro [117].

Other MMPs are thought to be involved in myocardial injury following AMI, such as MMP-9, first known as 92-kDa type IV collagenase or gelatinase B, a structurally complex metalloproteinase that intervenes in the degradation of ECM in a large spectrum of physiology and pathophysiology processes involving tissue remodelling, including cardiac remodelling after AMI.

MMP-9 is expressed in the heart by endogenous cardiac cell types (e.g., cardiomyocytes, endothelial cells, and fibroblasts) and is also produced by nonresident cells that infiltrate the infarct in response to ischemic injury (e.g., leukocytes) [134–136].

Different and opposite functions have been hypothesized for MMP-9. Potential detrimental consequences of MMP-9 release and activation may include stimulating inappropriate extracellular matrix degradation, activating inflammatory mediators, and/or increasing capillary permeability [137, 138]. On the other hand, potential beneficial effects of early MMP-9 activation include removing matrix and necrotic myocytes, releasing growth factors and cell surface receptors, remodelling the extracellular matrix for scar formation, processing inflammatory mediators such as interleukin-1β, and influencing angiogenesis [137]. MMP-9 has been correlated with an increase in infarct size and left ventricle fibrosis after experimental AMI [138–142].

Furthermore, increased myocardial MMP-9 expression or activity has been found in experimental myocardial injuries such as permanent coronary artery occlusion [143, 144] or reperfusion injury model in animals [145, 146], and the possible role of MMP-9 activation in myocardial IRI has been explored [147].

Following myocardial acute ischemia and reperfusion, neutrophil-derived MMP-9 is released in the myocardium and its levels increase as early as several minutes after AMI, remaining high for the first week in many animal models [137, 144, 148]. In the early phase of reperfusion, MMP-9 activation is likely to be localized in the perineutrophil area and might be initiated by neutrophils adhering to the ECM [137]; its temporal trend mirrors leukocyte infiltration [149]. The action of MMP-9 appears to be complex; it directly degrades ECM proteins and activates cytokines and chemokines to regulate tissue remodelling. MMP-9 deletion or inhibition has proven to be beneficial in a variety of animal models of cardiovascular disease. On the other hand, MMP-9 cell-specific overexpression has also proven beneficial [137, 144, 146, 150] (Figure 4).

5. Ischemia/Reperfusion Injury and Medicolegal Issues

Early reperfusion reduced mortality in AMI so that, in many countries, the hospital mortality has declined to about 5% [151]. There is no doubt that early reperfusion, both pharmacological and mechanical, is the only way to prevent progression to myocardial necrosis and thus to limit the size of the infarct. However, myocardial IRI has been described following reperfusion therapies including percutaneous coronary intervention (PCI), thrombolysis, and coronary bypass grafting [7, 13, 16].

VAs upon reperfusion have been recognized since the advent of recanalization techniques [152]; however their pathophysiological and prognostic significance is still controversial [153]. Several arrhythmogenic mechanisms have been

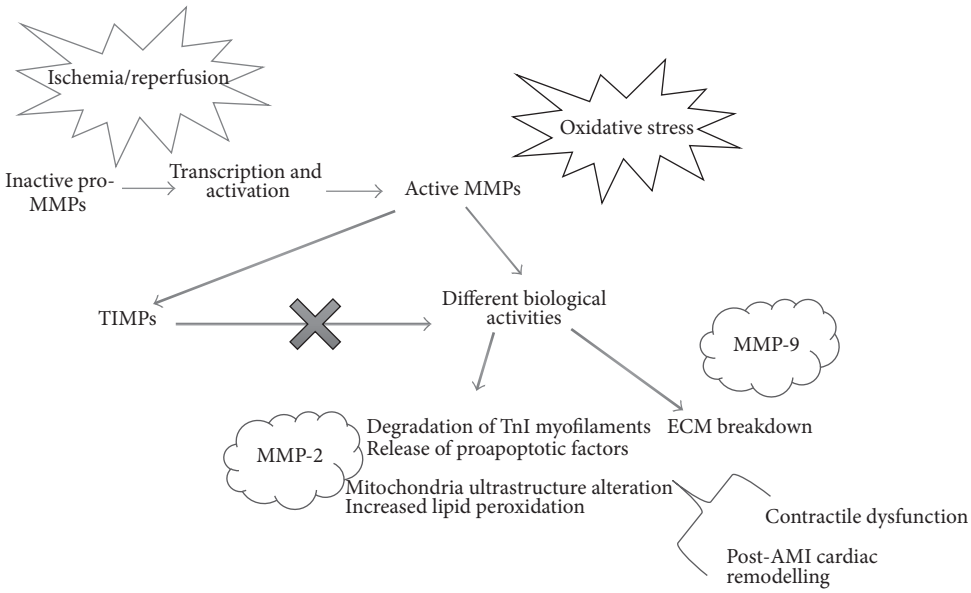

Figure 4: The matrix metalloproteinases system. MMPs activity results from different levels of regulation: transcription, activation, and inhibition by tissue inhibitors of metalloproteinases (TIMPs). During ischemia/reperfusion, oxidative stress stimulates the activity of MMPs, like MMP-2. Several biological activities of MMPs may contribute to myocardial contractile dysfunction and cell death. MMPs can both degrade extracellular matrix (ECM) and modulate different cellular mechanisms, thus leading to contractile dysfunction and modulation of cardiac remodelling and healing.

proposed to be involved in IRI-induced arrhythmias [154, 155]. Some of VAs that occur almost directly at the moment of reperfusion (namely, ventricular premature beats and accelerated idioventricular rhythms) are usually harmless and well tolerated [152]; however it has been reported that ventricular tachycardia and ventricular fibrillation occurring immediately after reperfusion remain the most important causes of sudden death following restoration of blood flow [156, 157].

Severe arrhythmias may not be common but the fact that they are life threatening makes them a relevant issue also for pathologists. In fact, the occurrence of such fatal events may represent a potential source of malpractice claims for cardiologists and it is noteworthy that AMI, in some manner, remains one of the most challenging areas with an associated high risk of alleged medical malpractice [158] and one of the clinical settings in which claims are most likely to arise [159, 160].

There is no doubt that prompt mechanical or/and pharmacological myocardial reperfusion represents the only realistic strategy in STEMI and that it has greatly improved AMI outcome. However, patients may have increasing optimistic expectations about the benefits of the procedures, as well as in many other cardiological clinical settings [161–163], and especially in cases with fatal outcome litigations and malpractice claims may arise thus leading to medicolegal autopsies which are critical in proving or excluding medical malpractice. It is now recognized that there are a spectrum of responses of the myocardium to ischemia/reperfusion [16, 157], and knowledge on the biochemical and molecular substrates of myocardial IRI has considerably improved. Reperfusion induces typical patterns of myocardial injury; contraction bands, calcium loading in the irreversibly injured myocytes, and hemorrhage in the region due to leakage of blood out of damaged blood vessels have been associated with IRI [16]. There is a growing appreciation that the pathobiologic response to ischemia/reperfusion injury is characterized by changes involving, among others, oxidative stress, mitochondria, and Ca^{+} homeostasis disturbance, with each leading to unique histomorphological footprint. As the cellular and molecular processes of myocardial IRI are more and more unravelled, the histopathology of reperfused AMI has been revisited and deserves further studies [3, 164]. We believe that, in postmortem examination in cases of fatal outcome of reperfused AMI, forensic experts should be very careful as this type of postmortem examination requires a deep knowledge and investigation of the complex ionic and biochemical alterations which could result in an unstable electrical substrate capable of initiating and sustaining arrhythmias. A sound knowledge of the pathophysiological changes underlying myocardial IRI and, namely, reperfusion arrhythmias is critical for forensic pathologists to make correct opinions concerning the real mechanism of death. Forensic pathologists, like clinicians, must think correlatively and move towards the explanation of the death on the basis of the underlying complex mechanisms. As a general concept, but mostly when deep pathophysiological derangements occur potentially leading to death, structural and anatomical knowledge obtained from autoptic observation is not so useful and cannot provide satisfactory explanations, independently of functional knowledge.

6. Therapeutic Challenges

Although there is no doubt that in AMI the reopening, as soon as possible, of occluded coronary arteries using either thrombolytic therapy or primary percutaneous coronary intervention is of vital importance for limiting the infarct size, thus representing an effective tool in AMI [162, 163], currently no similarly valid options exist in the treatment of myocardial IRI. Since the 1980s research has been focused on therapeutic agents that would render myocardial cells more resistant to the deleterious effects of ischemia and reperfusion [3, 164], the concept of "cardioprotection" encompasses the manipulation of the cellular events by different therapeutic tools during ischemia and reperfusion to reduce the amount of myocardial cells death [3, 164]. Ischemic conditioning strategies (ischemic preconditioning, IPC; remote ischemic preconditioning, RIPC; and ischemic postconditioning, iPOST) have been widely investigated in laboratory settings. Nevertheless, there still exists some difficulty in translating experimental results and controlled animal models into a heterogeneous population of human patients [12, 13, 24, 165]. An incomplete understanding of how cardioprotective signalling may be initiated at the level of the cardiomyocytes may, in part, explain the lack of success [166]. A deeper knowledge of the cellular and molecular mechanisms underlying IRI has led to the development of cardioprotective strategies, focusing on epigenetic regulation, limitation of cell death (both necrosis and apoptosis), stem cell regenerative therapies, gene therapy, and the use of growth factors [167]. Among the mechanisms through which postischemic myocardial damage has been shown to occur, mitochondrial dysfunction and the opening of MPTP are key steps. This crucial role renders them very attractive targets for therapeutic intervention [168–170]. In this context, a potential cardioprotective effect of intracoronary administration of 4-chlorodiazepam (4-CLD, a benzodiazepine derivative of diazepam) in animal models of IRI, has been recently demonstrated [171], thus suggesting a therapeutic role of intracoronary infusion of 4-CLD in AMI [171].

Furthermore, several lines of evidence support a potential role of platelet-rich plasma (PRP), an autologous product rich in growth factors obtained from a blood sample, in the healing of MI injury [172–175]. Platelets contain a wide amount of growth factors that are crucial in the reparative process following ischemic myocardial injury. In addition, they are rich in Factor XIII, a plasma transglutaminase, that has been shown to be critical in post-MI healing [176–178]. Factor XIII influences several steps of the reparative process, the formation of the three-dimensional fibrin meshwork, and the ECM components. Furthermore it is essential in adult stem cells recruitment, neoangiogenesis, and collagen deposit, thus playing a pivotal role at the intersection of several pathways involved in myocardial healing [179, 180].

7. Conclusions

AMI is a major cause of mortality worldwide. Early and successful myocardial reperfusion with either thrombolytic agents or primary percutaneous coronary intervention is the most effective strategy to reduce infarct size and improve clinical outcome. However, the process of restoring blood flow to the ischemic myocardium can induce myocardial reperfusion injury, which can paradoxically reduce the beneficial effects of myocardial reperfusion. Thus reperfusion itself may lead to accelerated and additional myocardial injury beyond that generated by ischemia alone [181]. Different clinical manifestations of this injury exist [13]; however, RAs remain the most important causes of sudden death following reperfusion therapy [182] even when the latter is technically successful. Thus myocardial IRI is both a critical clinical and medicolegal problem. For clinicians a better understanding of the pathophysiology of myocardial IRI may open the way to new therapeutic strategies [25, 182, 183]. For forensic pathologists, the value of fostering a knowledge of IRI pathophysiology should be highlighted as this can lead to an increased awareness of this potentially fatal event related to myocardial IRI, even in the case of optimal and early treatment. The clear investigation and comprehension of IRI may be an additional value which may diminish the risk of exposure of physicians to malpractice claims.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Amelioration of Inflammatory Cytokines Mix Stimulation: A Pretreatment of CD137 Signaling Study on VSMC

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Previous studies showed little CD137 expressed in normal vascular smooth muscle cells (VSMCs) and it is important to find a valid way to elevate it before studying its function. The level of CD137 was detected by RT-PCR, western blot, and flow cytometry, respectively. CD137 signaling activation was activated by agonist antibody and measured through phenotype transformation indicators and cell functions. Proteins in supernatants were detected by ELISA. The total CD137 elevates under different concentrations of CM treatment. Among these, 25 ng/ml CM treatment increases the CD137 expression mostly. However, flow cytometry demonstrates that 10 ng/ml CM elevates surface CD137 more significantly than other concentrations and reaches the peak at 36 h. At 10 ng/ml, but not 25 ng/ml CM pretreatment, the levels of phenotype related proteins such as SM-MHC, α-SMA, and calponin decrease while vimentin and NFATc1 increase, suggesting that VSMCs undergo phenotype transformation. Transwell, CCK-8 assay, and ELISA showed that the ability of VSMCs viability, migration, and IL-2 and IL-6 secretion induced by CD137 signaling was significantly enhanced by the pretreatment of 10 ng/ml CM. This research suggested that 10 ng/ml CM pretreatment is more reasonable than other concentrations when exploring CD137 function in VSMCs.

1. Introduction

CD137, also known as 4-1BB or TNFRSF9, belongs to the tumor necrosis factor (TNF) superfamily (TNFRSF) and is increasingly recognized as a key factor in immune and inflammation responses [1]. CD137 expression is mainly found in T cells, DCs, NKs, and monocytes. Activation of CD137 signal contributes to cytokine production, expansion, and functional maturation which can enhance immune response [2, 3]. Recent study showed that the combined activation of CD137 and programmed death-1 (PD-1) holds the composition for next-generation immunotherapy against tumor [4, 5].

Although CD137 signaling sheds new light on cancer therapy, the potential risk of activating CD137 should not be neglected. For example, it may be an undesirable factor in atherosclerosis (AS). Olofsson et al. found that CD137 is elevated in human plaques and may play an important role in regulating the function of atherosclerosis related cells such as ECs and VSMCs [6]. In animal studies, $CD137^{-/-}$ mice exhibit an obvious reduction of atherosclerosis lesion than WT mice, suggesting that CD137 signal activation may promote plaques formation and destabilization [7–9].

Inflammation is a hallmark of atherosclerosis involving multiple factors and cell types [10]. VSMCs participate in the middle and final phase of plaque formation. VSMCs are highly differentiated cells that are primarily responsible for contraction and the regulation of blood vessel [11]. In middle phase of AS progression, the VSMCs in the media can be transformed from a differentiated phenotype into a dedifferentiated state, which is characterized by accelerated proliferation, migration, and the production of cytokines, to initiate plaque formation once migrated to intima [12]. In final phase, the proliferated VSMCs undergo apoptosis and calcification, which contributes to plaque instability [13].

Recent study suggests that CD137 signaling play an important role in VSMCs function regulation [14]. Normal VSMCs express low CD137 compared with other cells which

makes the signaling investigation difficult. To increase the CD137 level, Olofsson and Jung use inflammatory cytokines mix (CM: IL-1β, IFN-γ, and TNF-α) to treat VSMCs. However, we found that the effect of this method depends on the cytokine concentration strictly, and improper concentration may even interfere with the CD137 function. Thus, in this article, we pretreated VSMCs with different concentrations of CM in order to find the reasonable experiment condition for further CD137 study. The better understanding of CD137 signaling on VSMC may help to explore the atherogenic mechanism in AS progression and guide the clinic therapy of CD137 antibody in tumors to avoid the side effect in future.

2. Materials and Methods

2.1. Mouse Primary Cell Culture. Thirty C57BL/6J mice aged 8 weeks were purchased from Jiangsu University. All the animals were housed under a 12-hour light-dark cycle, and $23 \pm 2^{\circ}$C under $55 \pm 10\%$ humidity, in normal cages with free access to water and food. The Animal Care and Use Committee of Jiangsu University approved animal experiments. Mouse primary aorta smooth muscle cell was extracted as described previously [14]. Generally speaking, mice were euthanized by CO_2 and chest was removed. The thoracic aorta was exposed; separate the lipid and fiber membrane from vessel under a surgical microscope. The artery was removed and washed with PBS several times before being treated with type II collagenase. Then, the vessels were cut into pieces and cultivated in cell culture flasks until the tissue block was removed and the 5th–8th generation was used in experiments. Mouse primary cell was cultured in DMED/F12 (Hyclone) medium containing 15% FBS at 37°C and 5% CO_2.

2.2. Cell Treatment. Inflammatory cytokines IL-1β, IFN-γ, and TNF-α are bought from Peprotech. Cells were divided into groups with different concentration gradients (0, 10, 25, 50, 75, and 100 ng/ml) or time points (0, 6, 12, 24, 36, and 48 h), and cell supernatants are collected for sCD137 detection. In CD137 signal activation assay, cells are pretreated with relative CM for 36 h and further treated with or without 10 ng/ml agonist CD137 antibody (R&D) to activate CD137 axis.

2.3. Quantitative Real-Time PCR. Total RNA was extracted from VSMCs with TRIzol (Invitrogen). Reverse transcription was carried out with 1 ug RNA using Thermo Fisher RT reagents. CD137 primers are forward: CCTCCAAGTACCTTCTCCAGCA and reverse: CCTCCAAGTACCTTCTCCAGCA. GAPDH primers are forward: GGCATTGCTCTCAATGACAA and reverse: TGTGAGGGAGATGCTCAGTG and were synthesized by Sangon (Shanghai, China).

2.4. Western Blot Analysis. The membrane protein and cytoplasm protein were isolated from VSMCs through membrane and cytosol protein extraction kit (Beyotime, China). The proteins were quantified using BCA kit from Vazyme (China), mixed with 5x SDS loading buffer and electrophoresed on a 10% SDS-PAGE gel. The anti-CD137 polyclone antibody (Abcam, USA) was used to detect the monomer or polymer of CD137. Antibodies such as SM-MHC (Abcam, USA), NFATc1 (CST, USA), vimentin (Immunoway, USA), calponin (Abcam, USA), and α-SMA (Sigma, USA) were used to observe the phenotype transformation of CD137 axis.

2.5. Enzyme-Linked Immunosorbent Assay. Cell culture supernatants were collected from VSMCs treated with gradient cytokines mix or CM + agonist-CD137 antibody. Anti-CD137 ELISA kit (Raybio, USA) was used to detect the sCD137 level secreted from VSMCs under the stimulation CM. IL-2 and IL-6 were purchased from Multi-Science (China) to measure the inflammation response of VSMCs induced by CD137 signal activation. The sensitivity of ELISA kit is 6 pg/ml–1500 pg/ml.

2.6. Flow Cytometry. The flow cytometry was performed to observe the CD137 level of membrane, which further validates the results of western blot. Cells were digested and washed with PBS. Anti-CD137-PE (eBioscience, USA) was diluted according to the protocol and was added to 100 μl cell suspension for 30 min at 4°C. After the incubation, the cells were washed one time and then analyzed by flow cytometry (BD Canto).

2.7. Transwell and CCK-8 Assay. The cells were pretreated with different concentrations of CM (0, 10, 25, and 50 ng/ml) for 36 h; 5×10^4 cells/ml were plated in 200 μl of DMEM without FBS in the upper chamber, and the lower chamber was filled with 500 μl DMEM containing 2% FBS with/without agonist CD137. The cells were allowed to migrate for 24 h at 37°C in a 5% CO_2 atmosphere. The cells that remained on the bottom of the lower chamber were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, and enumerated using Image-Pro Plus 6.0 software.

VSMCs were treated as described previously, 2000 cells were plated in 96-well plate and proliferation was determined using a CCK-8 kit (Vazyme Biotech).

2.8. Statistical Analysis. Data are expressed as the mean ± SD of at least three independent experiments (in vitro) and were compared by t-test or ANOVA using SPSS version 12.0 (SPSS, Chicago, IL, USA). A two-tailed $P < 0.05$ was considered significant.

3. Results

3.1. Comparison of CD137 Expression on VSMCs Induced by CM Gradient Treatment. VSMCs were treated with different concentrations of CM (0, 10, 25, 50, 75, and 100 ng/ml). The mRNA and protein levels of CD137 on membrane or cytoplasm were measured separately (Figure 1). Our data shows that CD137 exists in different forms according to the location; it is more likely to exist as tetramer on membrane but monomer or dimer in cytoplasm. The expression of CD137 elevates under all concentrations of CM stimulation except 50 ng/ml and reaches the peak at 25 ng/ml treatment, but only 10 ng/ml CM can induce the expression of CD137 on membrane. Flow cytometry analysis shows the same results (Figure 1(d)).

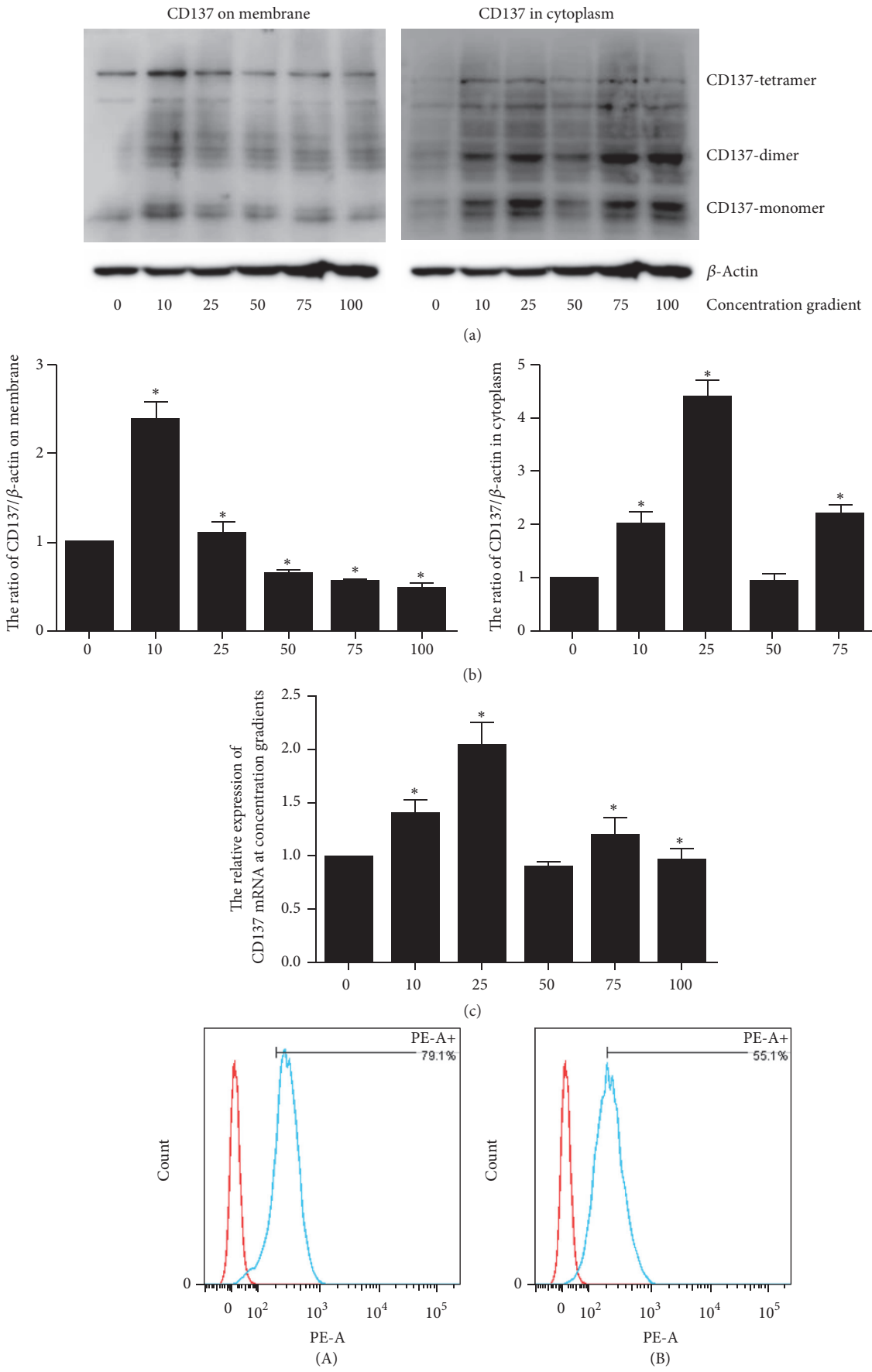


Figure 1: Continued.

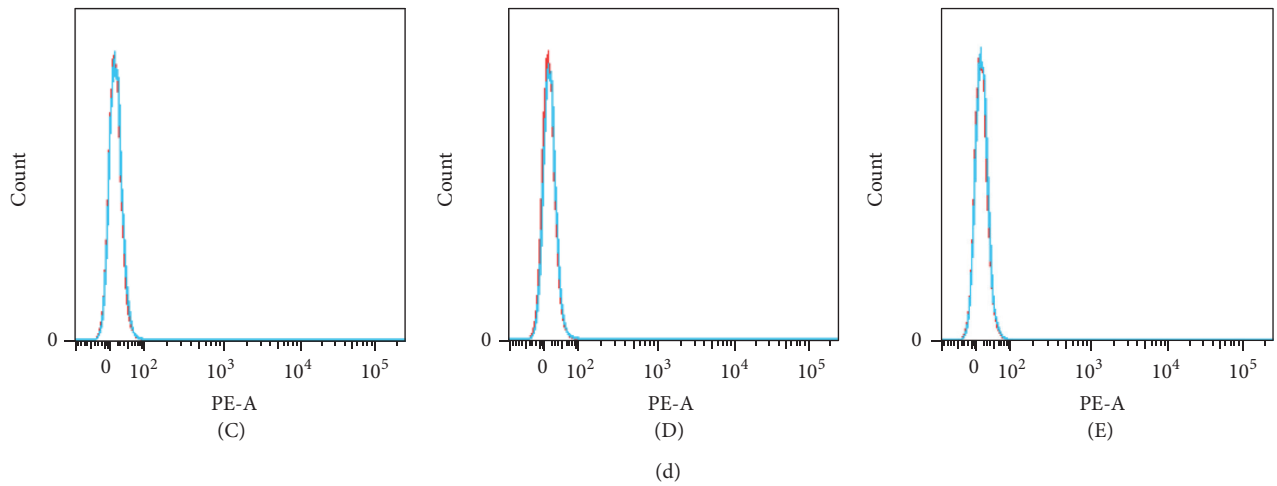


Figure 1: The expression of CD137 treated by CM gradient. (a) The protein level of CD137 on cell surface or in cytoplasm. The different polymer of CD137 exists according to the location. (b) The Gray scale analysis of western blot classified with CD137 location, the mean ± SEM. $^*P < 0.05$. (c) The mRNA level of each group. (d) The flow cytometry of CD137 on cell surface compared to 0 ng/ml CM group ((A–E) represents 10, 25, 50, 75, and 100 ng/ml CM groups, resp.).

3.2. Comparison of CD137 Expression on VSMCs at Different Time Points. We choose the 10 ng/ml concentration of CM and detect the expression of CD137 at 0, 6, 12, 24, 36, and 48 h through western blot, Q-PCR, and flow cytometry. The total level of CD137 elevates with time and reaches the peak at 36 h (Figure 2). The flow cytometry and western blot suggest that the membrane CD137 may exist at a high level from 12 h to 36 h but decrease at 48 h. Interestingly, the level of CD137 in cytoplasm is higher in 0 h and 48 h, showing an opposite tendency with membrane CD137.

3.3. Detection of sCD137 in Cell Supernatants. The CD137 in cytoplasm may be associated with the level of soluble CD137 (sCD137) which is reported to be the potent competitive inhibitor of membrane CD137 [15, 16]. Thus, we use western and ELISA to observe the forms and content of sCD137 secreted from VSMCs induced by CM. Western blot shows that sCD137 in supernatant is dimer, similar to cytoplasm CD137 (Figure 3(a)). ELISA results suggest that the level of sCD137 increases with the CM concentration gradient but shows no difference at different time points (Figures 3(b), 3(c), and 3(d)). The sCD137 levels secreted by VSMC are obviously increased at 25, 50, and 75 ng/ml CM compared to other groups.

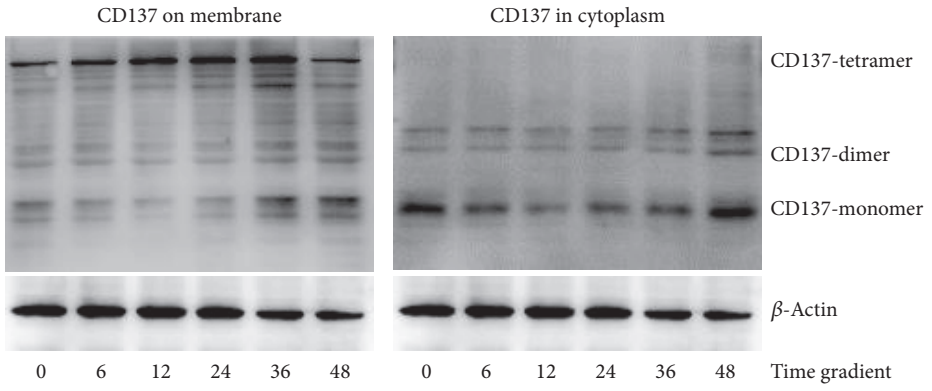
3.4. VSMCs Phenotype Transformation Induced by CD137 Signaling with Different CM Concentrations. In previous study, our groups found that CD137 signaling can induce phenotype transformation in VSMCs which contributed to atherosclerosis plaque formation. In the present study, we investigated the phenotype in VSMCs to identify the CD137 signaling activation.

Four concentrations (0, 10, 25, and 50 ng/ml) of CM were chosen as pretreatment for 36 h before adding agonist-CD137 antibody to activate CD137 signaling. Western blot shows that VSMCs pretreated with 10 ng/ml CM undergo significantly phenotype transformation induced by agonist-CD137 antibody. Both 10 and 25 ng/ml CM group exhibited VSMCs phenotype transformation; the expression of phenotype proteins such as SM-MHC, α-SMA, and calponin decreases while expression of vimentin increases in VSMCs (Figure 4). In 0 ng/ml CM concentration groups, the according proteins show no significant difference. Interestingly, in 50 ng/ml CM group, we observed the opposite effect on VSMCs phenotype. SM-MHC increases while vimentin and the phenotype regulation protein NFATc1 decrease in 50 ng/ml CM group compared to 10 ng/ml group.

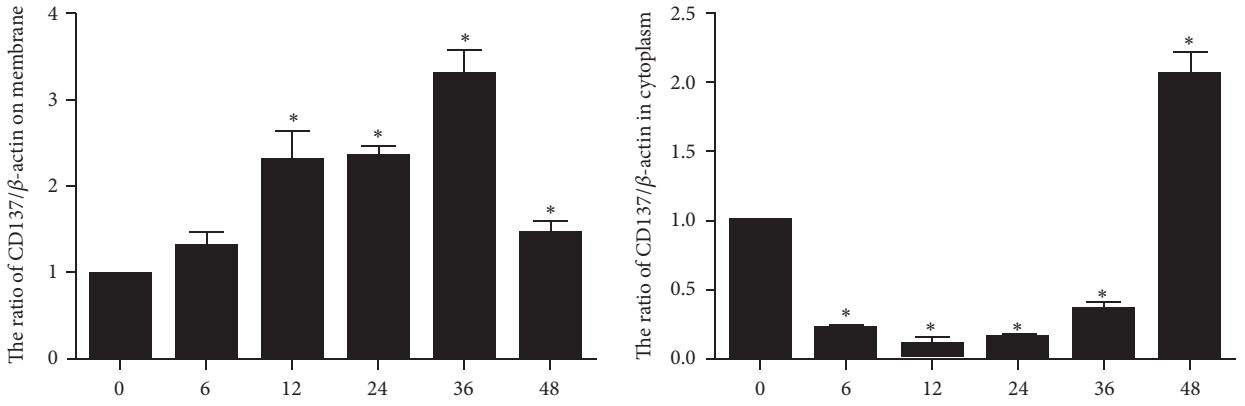
3.5. VSMCs Migration and Proliferation Induced by CD137 Signaling with Different CM Concentrations. The effect of CD137 signaling under different concentrations of CM before treatment was measured by transwell assay to determine the migration ability of cells (Figures 5(a) and 5(b)). In Figure 5, the migration cells number shows no significant difference in 0 ng and 25 ng/ml CM groups when treated with or without agonist-CD137. In 10 ng/ml CM group, CD137 signaling activation induced cell migration while inhibiting it in 50 ng/ml CM group. It should be noted that high concentration (25 ng/ml or 50 ng/ml) CM stimulation can increase basic cell migration.

In cell viability assay, VSMCs in 10 ng/ml CM and 25 ng/ml CM group showed increased proliferation when treated with agonist-CD137 (Figure 5(c)).

3.6. VSMCs Secrete IL-2 and IL-6 When CD137 Signaling Is Activated by Different Concentrations of CM. The concentration of IL-2 and IL-6 in supernatant secreted by VSMCs is measured by ELISA (Figure 5(d)). IL-2 and IL-6 levels were significantly elevated when CD137 signaling was activated by 10 ng/ml CM treatment. We also observed a high basic level of IL-6 when treated by 50 ng/ml CM.



(a)



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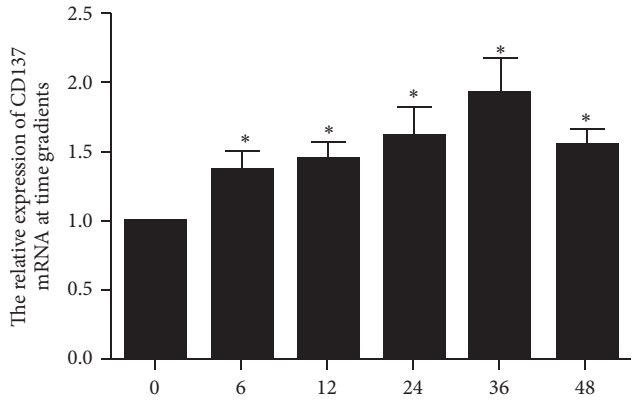

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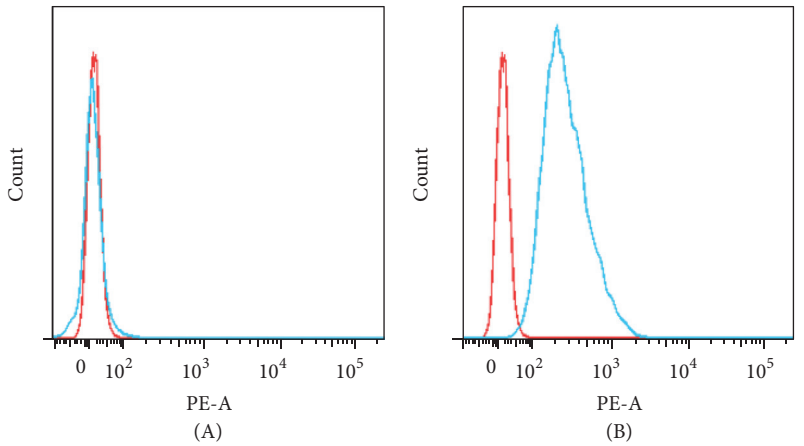


Figure 2: Continued.

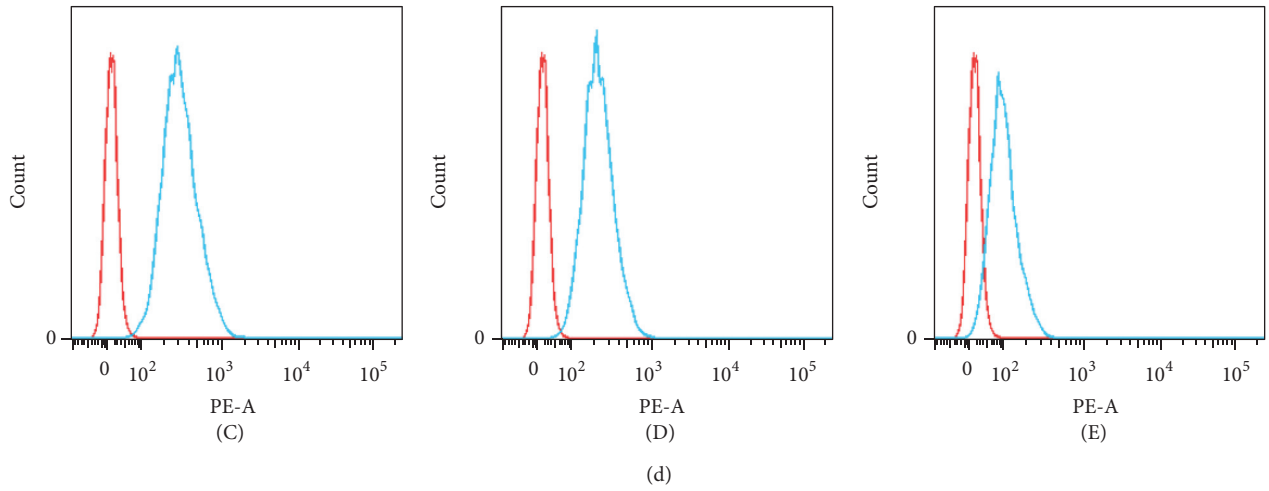


Figure 2: The expression of CD137 in time gradient. (a) The protein level of CD137 on cell surface or in cytoplasm at the time points 0, 6, 12, 24, 36, and 48 h. (b) The Gray scale analysis of according results, the mean ± SEM. $^*P < 0.05$. (c) The mRNA level of each group. (d) The flow cytometry of CD137 on cell surface compared to 0 h group ((A–E) represents 6, 12, 24, 36, and 48 h groups).

4. Discussion

CD137 is one of the costimulators of T cells and through binding with CD137 ligand it activates CD137 signaling and promotes proliferation and cytokine production [17]. In recent years, CD137 signaling is regarded as an important target in tumor therapy because it stimulates immune system. For example, combination of agonist-CD137 antibody and PD-1 blockade eradicated tumors through inducing strong immunity in T cells [4]. Moreover, constructed chimeric antigen receptor modified T cells (CART) with anti-CD20 scFv, and human CD137 and CD3ζ signaling was proved to be an effective treatment modality in patients with relapsed or refractory aggressive diffuse large B cell lymphomas (DLBCL) [18].

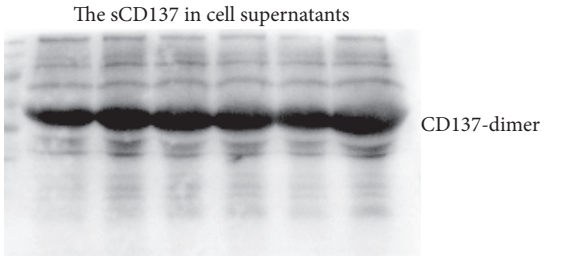
CD137 signaling can enhance immune and inflammation response. But the blessing in tumor therapy may be detrimental in atherosclerosis, a disease which is caused by inflammation and immune overactivation. Many researches, including ours, suggest that activation of CD137 signaling may be a risk in plaque formation. Meanwhile, the study of CD137 in atherosclerosis is still limited due to the low basic level of CD137 expression on several atherosclerosis related cells especially VSMCs [6, 8]. Researchers always pretreated VSMCs with cytokine mix to elevate CD137 expression before studying CD137 signaling, but this method is undefined and without verification. Thus, in this article, we performed a systematic study to validate the effect of the method.

In our study, we found that the concentration of CM is important, because the elevation of CD137 is not linear with CM concentration. In Figure 1, we demonstrate that although 25 ng/ml CM induces the most expression of total CD137, the membrane CD137 that may be functional is elevated upon 10 ng/ml CM stimulation. It should be noticed that CD137 might exist as different forms according to the location and function. For example, the CD137 on membrane is tetramer, the CD137 in cytoplasm is dimer or monomer, and sCD137 always exists as dimer in VSMCs (Figures 1 and 3). Whether CD137 in other tissues is tetramer or dimer and whether different forms of CD137 have different function are not known. It is implied that the different structure of CD137 can be used as a target, which may have the cell specificity to avoid the side effect of agonist-CD137 antibody in clinic trial.

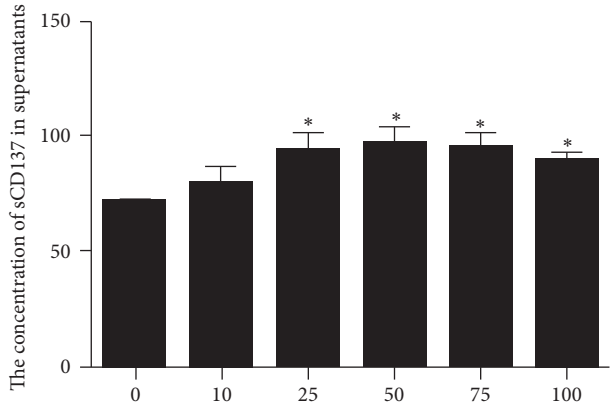
We also detected the time gradient to find the time point that is better for CD137 signaling activation. Interestingly, the expression of membrane CD137 increased from 12 h and reached the top at 36 h while the cytoplasm CD137 had an opposite tendency. The reason for this phenomenon may be that CD137 in cytoplasm undergoes a dynamic translocation to membrane or is secreted to extracellular as sCD137.

As stated above, CM stimulation induced the total CD137 elevation. More than membrane CD137, sCD137 which is regarded as a potent competitive inhibitor of CD137 may also be elevated [15, 16]. Thus, we measure the sCD137 in supernatant by ELISA. Consistent with our hypothesis, sCD137 also increased with the CM gradient. Compared to other concentrations, 10 ng/ml CM shows less sCD137 secretion, which means less interference by CD137 signaling activation.

We finally detect the CD137 signaling activation using agonist-CD137 antibody at different concentrations of CM. Because there has not been an acknowledged standard to measure the CD137 signaling activation in VSMCs [19, 20], we took the VSMCs phenotype transformation and cell function as the indicators which are associated with inflammatory cytokines (IL-2, IL-6) secretion as proved in our previous study. Consistent with our hypothesis, the three indicators exhibit obvious changes induced by agonist-CD137 only at the 10 ng/ml CM pretreatment. Jung et al. used 25 ng/ml CM to pretreated VSMCs for the further CD137 activation and showed a good performance [8]. However, in our study, we found that although 25 ng/ml CM has some effects, further CD137 activation is not stable compared to 10 ng/ml CM pretreatment. The reason for these discrepancies may be due



(a)



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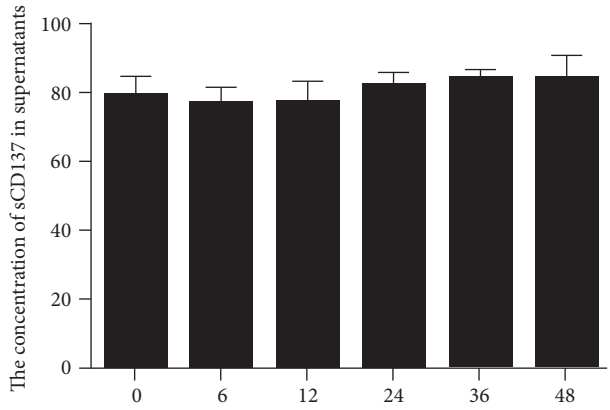

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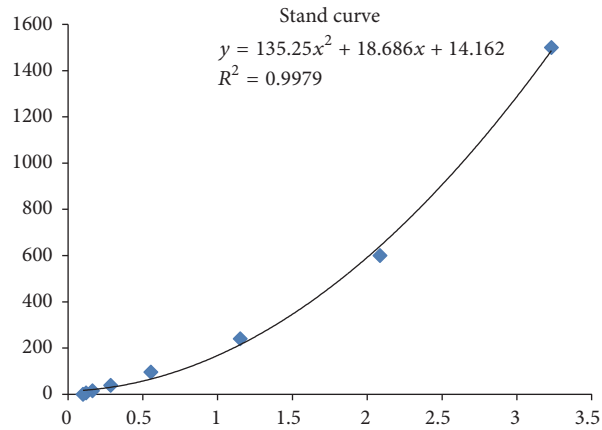

Sample	OD value 1	OD value 2	Content (pg/ml)
std1	3.371	3.092	1500
std2	2.094	2.077	600
std3	1.148	1.154	240
std4	0.526	0.58	96
std5	0.28	0.29	38.4
std6	0.161	0.164	15.36
std7	0.115	0.125	6.14
std8	0.092	0.108	0

Sample	OD value 1	OD value 2	Content (pg/ml)	
0	0.11	0.11	17.85399	71.41594
10	0.158	0.137	19.86072	79.44287
25	0.201	0.204	23.49201	93.96804
50	0.229	0.199	24.35471	97.41885
75	0.23	0.189	24.01287	96.05149
100	0.202	0.175	22.49005	89.96019

Sample	OD value 1	OD value 2	Content (pg/ml)	
0	0.196	0.198	23.09206	92.36824
6	0.184	0.189	22.35124	89.40495
12	0.184	0.191	22.42051	89.68203
24	0.201	0.212	23.78802	95.15209
36	0.214	0.217	24.46988	97.87951
48	0.22	0.212	24.5084	98.0336

(d)

Figure 3: The expression and format of sCD137 in cell supernatant treated with CM. (a) sCD137 exists as dimer which is similar with CD137 in cytoplasm. (b, c) The quantity of sCD137 induced by CM treatment at different concentrations and time points using ELISA, $^*P < 0.05$. (d) The stand curve and OD values above.

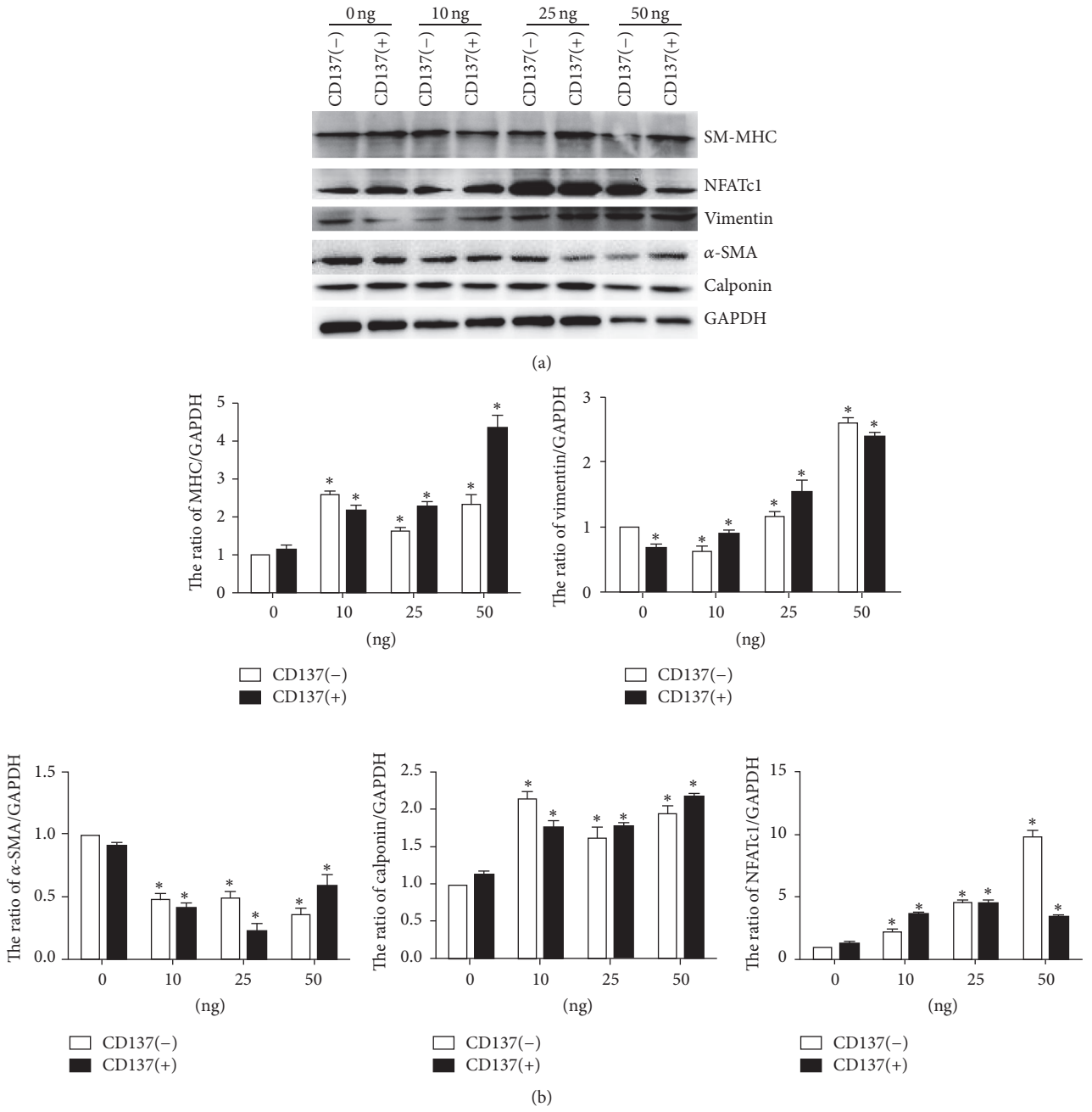

Figure 4: The comparison of phenotype transformation in VSMCs induced by CD137 signaling on different concentration treatment. (a) The western blot of phenotype markers in VSMCs. (b) The Gray scale analysis of western blot, the mean ± SEM, $^{*}P < 0.05$.

to the actual concentration of CM or cell status. In addition, we found that the response of VSMCs induced by CD137 signaling might be dependent on the CM concentration. For example, VSMCs treated by high concentration of CM (25 ng/ml, 50 ng/ml) may execute even the opposite effect when CD137 signaling is activated. In Figure 4, we showed an opposite tendency in phenotype transformation markers when cells were pretreated by 50 ng/ml CM. The similar phenomenon also exists in cell migration and IL-2 secretion.

Thus, our data suggest that the effect of CM pretreated method before CD137 activation assay depends strictly on the cytokine concentration, and improper concentration may even interfere with the CD137 function. It is worth mentioning that Olofsson and Jung found that CD137 signaling activation induced VSMC apoptosis in VSMCs, but we observed a mild increase in cell viability through CCK-8 assay. The difference may also be attributed to the instability of CM pretreatment.

In this article, we explored the function of different polymers of CD137 and performed an investigation on further validation and improvement of the CM treatment method for CD137 signaling study on VSMCs. We found

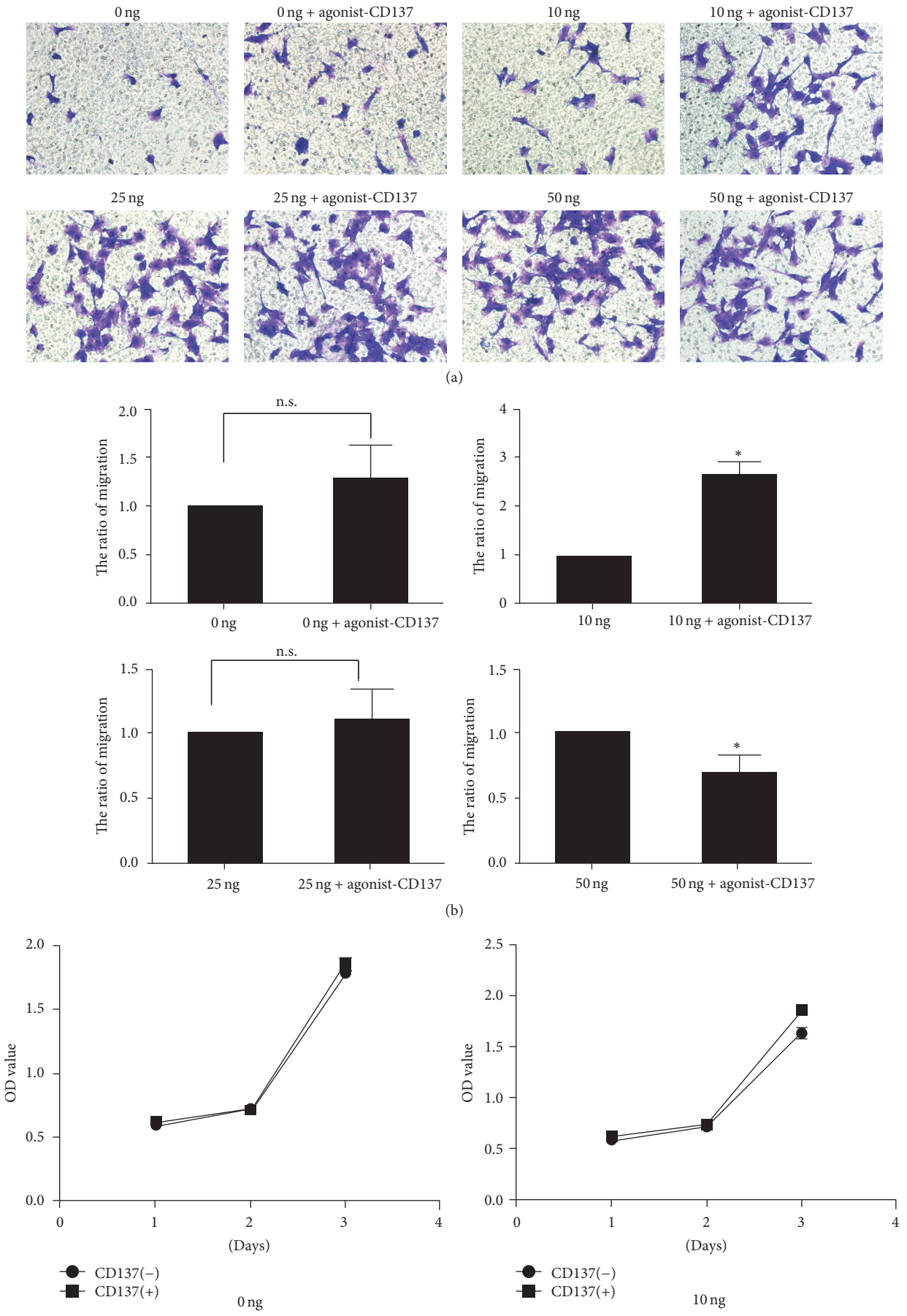

Figure 5: Continued.

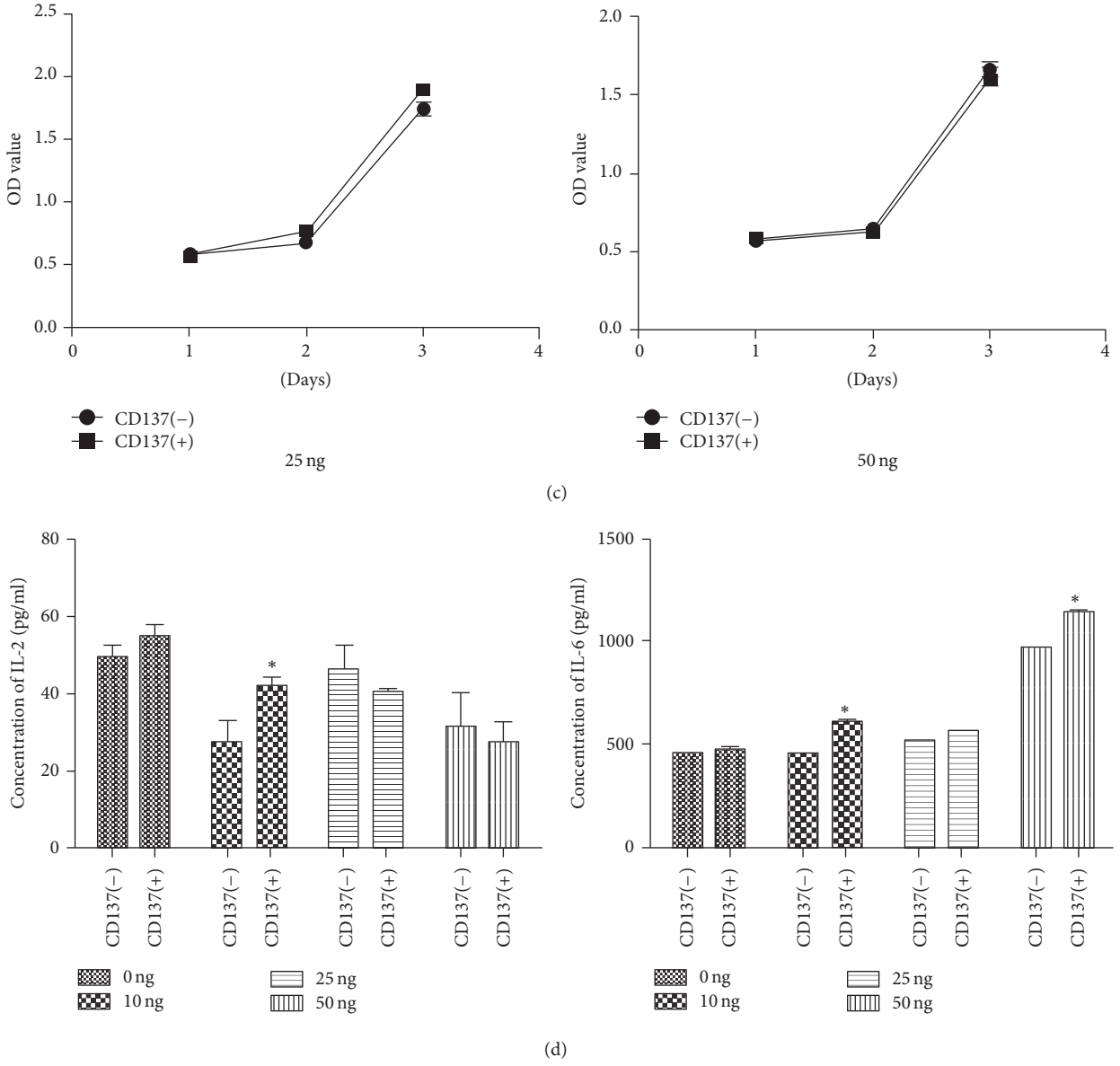

Figure 5: The comparison of cell function changes in VSMCs induced by CD137 signaling on different concentration treatment. (a) The transwell assay of VSMCs with CD137 signaling activation at different CM concentration. (b) The analysis of relative migration ratio in transwell assay, the mean ± SEM. $^{*}P < 0.05$. (c) The cell viability of VSMCs measured by CCK-8 assay during 3 days. (d) The secretion ability of inflammatory cytokines IL-2 and IL-6 induced by CD137 signaling.

that 10 ng/ml CM pretreatment is more reasonable than other concentrations when exploring CD137 function in VSMCs. The results may help us to choose a better condition when studying the mechanism of CD137 signaling in AS. More than this, the relationship between inflammatory cytokines and CD137 activation could be a consideration in future clinic CD137 antibody in tumor therapy to avoid side effects such as AS or other inflammation related diseases.

Competing Interests

The authors have no conflict of interests to declare.

Acknowledgments

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Research Article

N-Acetylcysteine Attenuates Diabetic Myocardial Ischemia Reperfusion Injury through Inhibiting Excessive Autophagy

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Background. Excessive autophagy is a major mechanism of myocardial ischemia reperfusion injury (I/RI) in diabetes with enhanced oxidative stress. Antioxidant N-acetylcysteine (NAC) reduces myocardial I/RI. It is unknown if inhibition of autophagy may represent a mechanism whereby NAC confers cardioprotection in diabetes. *Methods and Results*. Diabetes was induced in Sprague-Dawley rats with streptozotocin and they were treated without or with NAC (1.5 g/kg/day) for four weeks before being subjected to 30-minute coronary occlusion and 2-hour reperfusion. The results showed that cardiac levels of 15-F2t-Isoprostane were increased and that autophagy was evidenced as increases in ratio of LC3 II/I and protein P62 and AMPK and mTOR expressions were significantly increased in diabetic compared to nondiabetic rats, concomitant with increased postischemic myocardial infarct size and CK-MB release but decreased Akt and eNOS activation. Diabetes was also associated with increased postischemic apoptotic cell death manifested as increases in TUNEL positive cells, cleaved-caspase-3, and ratio of Bax/Bcl-2 protein expression. NAC significantly attenuated I/RI-induced increases in oxidative stress and cardiac apoptosis, prevented postischemic autophagy formation in diabetes, and reduced postischemic myocardial infarction (all $p < 0.05$). *Conclusions*. NAC confers cardioprotection against diabetic heart I/RI primarily through inhibiting excessive autophagy which might be a major mechanism why diabetic hearts are less tolerant to I/RI.

1. Introduction

Ischemic heart disease is the most serious complication of diabetes, which increases morbidity and mortality [1]. Restoring of blood flow to the ischemic heart, namely, reperfusion, is the only resolution to salvage the ischemic myocardium. However, reperfusion itself also causes additional damage to ischemic heart, which was termed ischemic reperfusion injury (I/RI). Numerous studies have demonstrated that ischemia and reperfusion induced burst production of reactive oxygen species (ROS) and the consequent oxidative damage and inflammation [2] are major mechanisms of I/RI. Increased ROS also contribute to reduced bioavailability of the cardiovascular protective nitric oxide and the impairments of the prosurvival reperfusion injury salvage kinase (RISK) pathway which involves PI3K/Akt and the survival activating factor enhancement (SAFE) pathway which involves Jak/signal transducer and activator of transcription 3 (STAT3) [3]. The impairment of these prosurvival pathways may lead to the impairment of the molecular identity of the mitochondrial permeability transition pore (mPTP) that is decisive for cardiomyocyte survival [4], which involves the regulation of cell apoptosis and autophagy via the AMPK/mTOR signaling [5]. Hyperglycemia is the major

character of diabetes, which causes ROS overproduction and interferes with oxidant and antioxidant hemostasis and exacerbates myocardial I/RI in diabetes. The antioxidant N-acetylcysteine (NAC) has been shown to reduce cardiac ROS overproduction in diabetes [6] and reduce myocardial I/RI and improve postischemic heart function in diabetic rats [7, 8] through attenuating I/RI-mediated apoptotic cell death. Recent studies show that autophagy is one of the major forms of cell death in circumstances of myocardial I/RI, and autophagy is enhanced in the myocardium of diabetic subjects at certain stages of the disease, when the diabetic hearts are more vulnerable to ischemic insult. It is unknown whether or not attenuation of autophagy may represent a mechanism whereby NAC confers cardioprotection against I/RI in diabetes.

Autophagy, which destructs long-lived or aggregated proteins and damaged organelles, is a basic function of vital life to main nutrient homeostasis, energy salvage, and degradation of old, malfunctioning organelles within a cell [9]. In addition, autophagy could provide immune protection and invade pathogens. Recent studies show that autophagy may have correlation with type II programmed cell death and can initiate cell death in different circumstances and plays different roles in neurodegenerative diseases [10], cancer [11], liver diseases [12], cardiac diseases [13], metabolic syndromes [14], ageing [15], and inflammations [16]. Previous study demonstrated that autophagy was upregulated during myocardial ischemia/reperfusion (I/R) and suggested that it may be cardioprotective during ischemia, but continuous activation of autophagy during reperfusion was detrimental [17]. Adenosine 5′-monophosphate-activated protein kinase (AMPK) is a sensor of energy molecule ATP and is activated when the ratio of ATP/ADP is decreased during exercise, hypoxia, oxidative stress, glucose deprivation, and myocardial I/R [18]. AMPK is also a major regulator/activator of autophagy [19]. On the other hand, mammalian target of rapamycin (mTOR) inhibits autophagy in the heart as reported. Activation of AMPK results in decreased mTOR activity, increased autophagy, and attenuated postischemic myocardial reperfusion injury [20, 21]. Likewise, Guo et al. reported that ischemia postconditioning reduced postischemic cardiac injury through clearing autophagosome and restoring autophagy flow [22]. However, in the diabetic myocardium, the extent of cardiac autophagy has been shown to be either enhanced or reduced depending on model and duration of the disease in which the susceptibility to ischemic insult was enhanced [23, 24]. Thus, the role of autophagy in diabetic myocardial I/RI is unclear. We hypothesize that attenuation of I/RI-induced autophagy is a major mechanism by which NAC confers cardioprotection in diabetes. Therefore, this study was designed to mainly investigate the cardioprotection of NAC in diabetic rats in relation to autophagy.

2. Materials and Methods

2.1. Induction of Diabetes. Diabetes was induced by single dose (65 mg/kg) streptozotocin (STZ) (Sigma-Aldrich, St. Louis, MO) in male Sprague-Dawley rats (250 g, 6–8 weeks) injection via tail vein as we described [25]. All rats were housed in the Laboratory Animal Service Center (University of Hong Kong) and received standard care in accordance with the principles of Animal Care of the University of Hong Kong. The experimental protocols were approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR).

2.2. Experimental Protocol. Rats were randomly divided into four groups: Ctrl: nondiabetic control; D4w: 4-week diabetes; D4w + I/R: 4 weeks' diabetic rats with ischemia/reperfusion; D4w + I/R + NAC: 4 weeks' diabetic rats treated with N-acetylcysteine (NAC) and were subjected to ischemia/reperfusion (Sigma-Aldrich, St. Louis, MO). The chemicals were dissolved in drinking water for 4 weeks' duration of treatment starting one week after induction of diabetes. We selected a dose of NAC at 1.5 g/kg/day, as our previous study has reported that NAC at the dose of 1.4–1.5 g/kg/day completely prevented hyperglycemia-induced oxidative stress after 8 weeks of STZ-induced diabetic rats [26].

I/R was achieved by occluding the left anterior descending (LAD) artery for 30 minutes followed by 2-hour reperfusion and myocardial infarction was determined using TTC (1%, 2,3,5-triphenyltetrazolium chloride) staining as described [27]. Briefly, at the end of reperfusion, the LAD was reoccluded; 5% Evans Blue was injected through the right jugular vein to mark the normal region of the left ventricle (LV). At the completion of the experiments, the rats were euthanized with overdose pentobarbital injection, and the hearts were quickly removed and cut into five 1–1.5 mm cross-sectional slices and incubated in 1% TTC in PBS at room temperature for 20 minutes. The slices were then fixed with 10% formalin overnight. TTC stained area was targeted as the region of survival area. Parts of the LV tissues were selected for Western Blot. PowerLab monitoring system (ML750 PowerLab/4sp with MLT0380 Reusable BP Transducer; AD Instruments, CO Springs, CO) was used to monitor the hemodynamics during myocardial I/R process.

2.3. Plasm and Cardiac Levels of Free 15-F2t-Isoprostane (15-F2t-IsoP). Free 15-F2t-IsoP, a specific marker of oxidative stress in vivo originally produced by the random oxidation of tissue phospholipids, was measured by using an enzyme immunoassay kit (Cayman chemical, Ann Arbor, MI) as described [28]. The absorbance from the enzymatic reaction was detected at 412 nm. The values of plasma or cardiac free 15-F2t-IsoP were expressed as pg/mL in the plasma. After 2 hours of reperfusion blood samples were collected from carotid artery with anticoagulation heparin and then centrifuged to separate plasma as we described [27].

2.4. Plasma Biochemical Analysis. Plasma tumor necrosis factor-α (TNF-α) level was determined using rat TNF-α ELISA kit (eBiosource International, Burlington, Ontario, Canada). Plasma creatinine kinase-MB (CK-MB) levels were determined using a commercially available rat ELISA kit (R&D Systems, Minneapolis, MN). Plasma interleukin-6 (IL-6) level was determined using a rat IL-6 ELISA kit (eBiosource International, Burlington, Ontario, Canada). The

Table 1: General characteristics after STZ injection at termination of study.

Parameters	C	D4w	D4w + NAC
Water intake (mL/kg/day)	126.3 ± 3.2	801.2 ± 12.9*	398.7 ± 8.3*#
Food consumption(g/kg/day)	70.1 ± 3.8	182.7 ± 14.1*	129.8 ± 12.7*#
Body weight (g)	492.5 ± 14.7	326 ± 7.9*	301 ± 11.7*
Plasma glucose (Mm)	6.1 ± 0.2	27.9 ± 1.2*	26.8 ± 1.3*

All values are expressed as mean ± SEM. $n = 6$ per group, water intake and food consumption values were the average value of 4 weeks. Body weight, plasma glucose, and heart/Body weight ratio were measured at 4 weeks after STZ injection. * $p < 0.05$ versus control # $p < 0.05$ versus D4w.

assays were performed following the manufacturer's instructions.

2.5. In Situ Apoptotic Cell Death Detection. Apoptotic cell death detection was achieved using TdT-mediated DUTP-X nick end labeling (TUNEL stain) according to the manufacturer's instruction (Roche Applied Science, Indianapolis, IN, USA). First, left ventricular tissue embedded by paraffin was sliced (5 mm thick sections) and deparaffinized. Second, the sections were permeabilized using proteinase K (30 mg/mL, 30 minutes, 37°C) and were washed in phosphate buffered saline (PBS). Then as the manufacturer's instructions described to detect the apoptosis cells, DNase I was used to induce DNA strand breaks as positive control and TdT was omitted from the reaction mixture as negative control.

2.6. Western Blot Analysis. Proteins from frozen LV tissues were homogenized in 1x lysis buffer from Cell Signaling Technology (Beverly, MA) and centrifuged at speed of 13200 *g* for 30 minutes. The supernatant was collected as total myocardial protein. The supernatant was collected as total myocardial protein. The concentrations of protein were then determined using the Bradford protein assay.

Equal protein amounts from rat heart homogenate were resolved by 7.5–12.5% SDS-PAGE and subsequently transferred to polyvinylidene nitrocellulose membranes and processed as previously described [28]. Primary antibodies against AMPKα, phosphorylation AMPKα, Akt, phosphorylation Akt (Ser-473), LC3, P62, mTOR, phosphorylation PTEN, Bax, Bcl-2, caspase 3, cleaved-caspase-3, and GAPDH were purchased from Cell Signaling Technology (Beverly, MA). Protein bands were detected by a standard ECL method and images were measured by a densitometer.

2.7. Statistical Analysis. All values are expressed as means ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was used for statistical analyses (GraphPad Prism, USA) of data obtained within the same group and between groups, respectively, followed by Tukey's test for multiple comparisons of group means. p values less than 0.05 were considered to indicate statistically significant differences.

3. Results

3.1. The Effects of NAC on General Characters, Postischemic Myocardial Infract Size (IS), and Heart Function in Diabetic Rats. First, we observed the effect of NAC on general characters in diabetic rats. As shown in Table 1, in STZ-induced diabetic rats, plasma glucose, water intake, and food consumption were significantly increased compared to nondiabetic rats (all $p < 0.05$). After NAC treatment, food consumption and water intake were significantly reduced compared to diabetic group (all $p < 0.05$), but NAC had no significant effect on plasma glucose in diabetic rats ($p > 0.05$). Body weight in diabetic rats was significantly reduced, and NAC had no significant impact on the body weight.

As shown in Figure 1(a), NAC significantly reduced the postischemic myocardial infarct size (IS) in diabetic rats ($p < 0.01$, NAC + D4w + I/R versus D4w + I/R). And postischemic plasma CK-MB level after 2 hours' reperfusion was significantly higher compared to sham operated diabetic group ($p < 0.05$ D4w + I/R versus D4W). NAC significantly reduced postischemic CK-MB release, in accordance with lower IS ($p < 0.05$).

As shown in Table 2, baseline hemodynamics dates were similar among groups. Heart rate (HR) at baseline was not different among the 4 groups. Coronary artery occlusion (ischemia) reduced mean arterial pressure (MAP) and rate-pressure product (RPP) in all groups in comparison with baseline MAP. No significant differences in HR or RPP were observed between groups during ischemia and reperfusion. NAC treatment facilitated recovery of MAP after reperfusion as compared to the diabetic untreated group.

3.2. Effects of NAC on Plasma 15-F2t-Isoprostane (15-F2t-IsoP), Interleukin-6 (IL-6), and Tumor Necrosis Factor-α (TNF-α) Levels. We determined plasma 15-F2t-IsoP (specific marker of oxidative stress), IL-6, and TNF-α levels in control and diabetic rats with or without NAC treatment. As shown in Figures 2(a), 2(b), and 2(c), plasma IL-6 and TNF-α levels were increased in the rats with diabetes along with significant increase of 15-2t-IsoP (all $p < 0.05$ D4w versus nondiabetic group), and these were all further exacerbated by myocardial I/RI ($p < 0.05$, D4w + I/R versus D4w). NAC treatment significantly reduced but did not prevent the increase of plasma IL-6 level in diabetic rats. By contrast, NAC significantly decreased plasma TNF-α and 15-F2t-IsoP level compared to diabetic rats subjected to I/R (all $p < 0.05$).

3.3. Effects of NAC on Postischemic Myocardial Apoptosis, Bax/Bcl-2, and Caspase-3 Expression. As Figure 3(a) showed, STZ-induced diabetic rats had a significant larger number of TUNEL-staining positive cells compared to nondiabetic group ($p < 0.05$) with concomitant increases in the ratio of Bax/Bcl-2 and cleaved-caspase-3, and myocardial I/R further

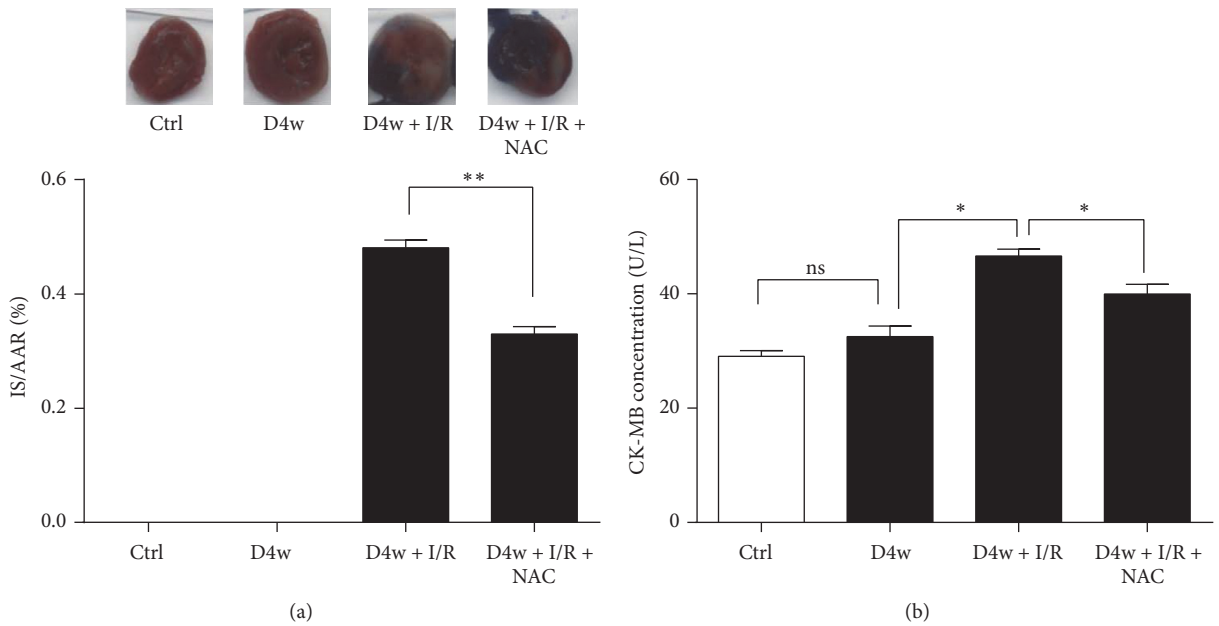

Figure 1: The effects of NAC on heart function and infract size (IS) in diabetic rats. (a) Infarct size (IS) is expressed as a percentage of the area at risk (AAR). (b) CK-MB release. Ischemia reperfusion (I/R) was achieved by 30-minute ischemia followed by 2-hour reperfusion in diabetic rats with or without NAC. Ctrl: nondiabetic control; D4w: 4-week diabetes; D4w + I/R: 4-week diabetic rats with ischemia/reperfusion; D4w + I/R + NAC: 4-week diabetic rats treated with N-acetylcysteine (NAC) and were subjected to ischemia/reperfusion. Dates are expressed as mean ± SEM ($n = 6$ per group). $p < 0.05$ versus D group before ischemia; $^{*}p < 0.05$, $^{**}p < 0.01$, and ns: $p > 0.05$ (no statistical significance).

Table 2: Hemodynamics at baseline, at 2 hours of reperfusion in nondiabetic or diabetic rats with or without NAC treatment.

	C	D	D + I/R	D + NAC + I/R
Baseline				
HR (bpm)	268 ± 9	300 ± 9	298 ± 10	315 ± 13
MAP (mmHg)	118 ± 5	110 ± 5	113 ± 4	114 ± 3
RPP (mmHg min-1/1000)	33 ± 3	32 ± 4	34 ± 3	33 ± 3
2-hour reperfusion				
HR (bpm)	—	—	275 ± 6	300 ± 6
MAP (mmHg)	—	—	$70 \pm 4^{*}$	$75 \pm 4^{*\#}$
RPP (mmHg min-1/1000)	—	—	$22 \pm 2^{*}$	$24 \pm 3^{*}$

HR: heart rate; MAP: mean arterial pressure; RPP: rate-pressure product. HR, MAP, and RPP were measured at 2 hours of reperfusion. All values are expressed as mean ± SEM ($n = 6$ per group). $^{*}p < 0.05$ versus their corresponding baseline; $^{\#}p < 0.05$ versus D + I/R.

increased the number of apoptotic cells, while NAC treatment significantly attenuated the apoptotic cells induced by I/R ($p < 0.05$). NAC significantly attenuated the increase in the ratio of Bax/Bcl-2 and prevented diabetes and I/RI-induced increase in cleaved-caspase-3 expression (all $p < 0.05$).

3.4. Effects of NAC on Diabetes Induced Myocardial Autophagy and Its Related Proteins. Autophagy has been shown to play major roles in I/RI. As Figures 4(a) and 4(b) showed, total and phosphorylated AMPKα (p-AMPKα), a strong initiator of autophagy, were significantly increased in the myocardium of diabetic rats compared to that in the nondiabetic rats ($p < 0.05$). I/R further increased p-AMPKα ($p < 0.05$) but did not have significant effect on AMPKα. The ratio of LC3 II/I represents the extent of autophagy. As Figures 4(c) and 4(d) showed, STZ-induced diabetic rats had significant higher ratio of LC3 II/I compared to nondiabetic group ($p < 0.05$). P62, which functions to clear the autophagosome in order to keep hemostasis, was also increased significantly in the myocardium of diabetic rats ($p < 0.05$, D4w versus nondiabetic ones). I/R did not further significantly increase LC3 II/I and P62. NAC treatment completely prevented diabetes induced increases of LC3 II/I and p62 proteins expression both before and after I/R as compared to D4w + IR ($p < 0.05$). In diabetic group, myocardial mTOR protein expression significantly increased (Figure 4(e)) in parallel with the significant increase in p-AMPKα. However, during I/R, myocardial mTOR moderately and significantly reduced in response to I/R induced further increase in p-AMPKα (all $p < 0.05$, D4w + I/R versus D4w). NAC treatment prevented

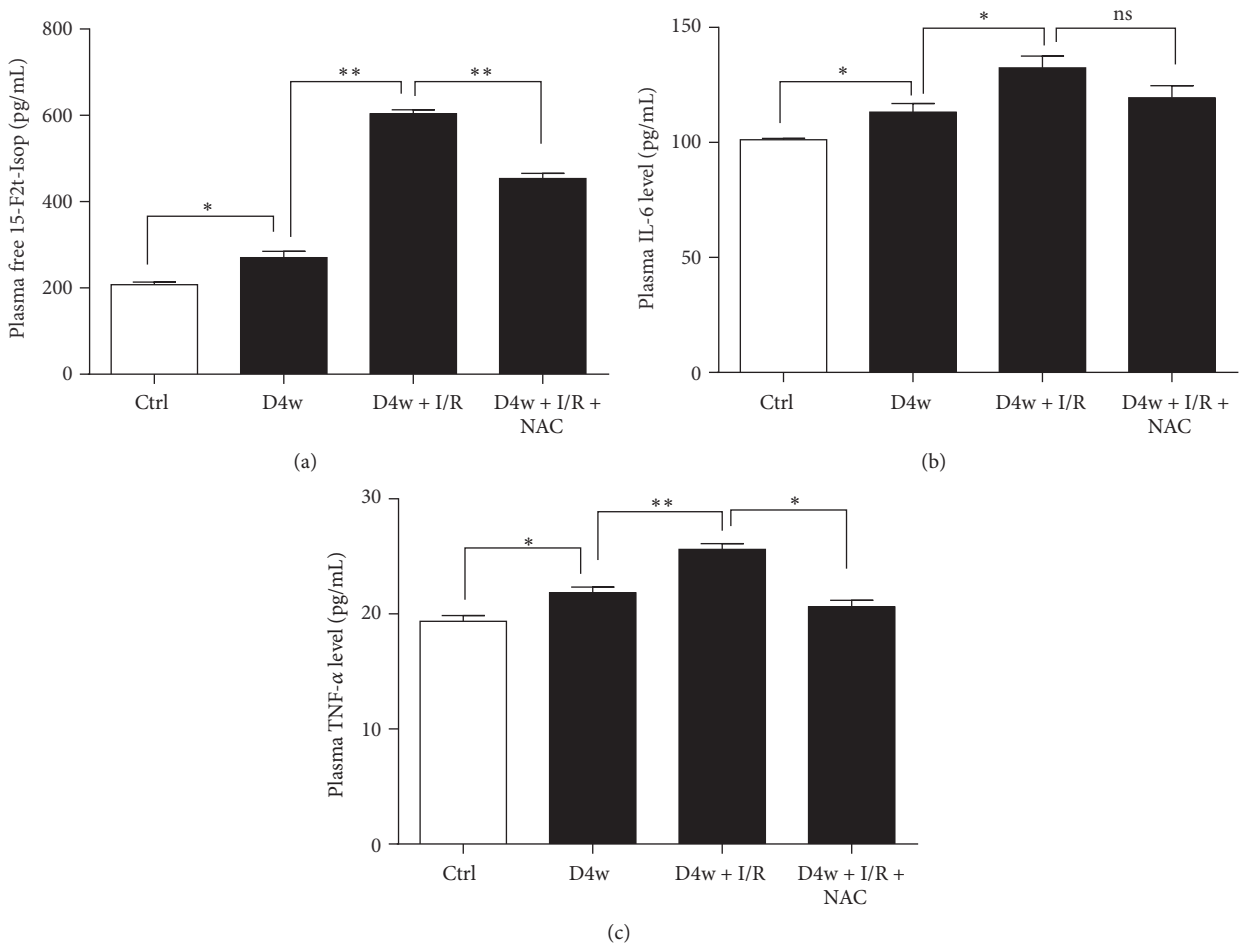

Figure 2: Effects of NAC on plasma 15-F2t-IsoP, IL-6, and TNF-α releasing. (a) Plasma level of IL-6, (b) plasma level of TNF-α, and (c) plasma level of 15-F2t-IsoP. Ischemia reperfusion (I/R) was achieved by 30-minute ischemia followed by 2-hour reperfusion in diabetic rats with or without NAC. Ctrl: nondiabetic control; D4w: 4-week diabetes; D4w + I/R: 4-week diabetic rats with ischemia/reperfusion; D4w + I/R + NAC: 4-week diabetic rats treated with N-acetylcysteine (NAC) and were subjected to ischemia/reperfusion. Dates are expressed as mean ± SEM ($n = 6$ per group), $^{*}p < 0.05$ $^{**}p < 0.01$.

diabetes and I/R induced increases in mTOR ($p < 0.05$, D4w + I/R + NAC versus D4w + I/R).

3.5. Changes of PTEN, Akt, and eNOS after NAC Treatment. PTEN has been shown to play important roles in myocardial I/R; suppression of PTEN could reduce I/RI manifested as reduced infract size, increased Akt and eNOS activation, and improved heart function [29, 30]. In accordance with the previous studies, cardiac phosphorylated PTEN (p-PTEN) protein expression (Figure 5(a)) was increased significantly by hyperglycemia ($p < 0.05$, D4w versus nondiabetic rats), and I/R further increased p-PTEN expression in diabetes. Compared to nondiabetic group, cardiac phosphorylated Akt (p-Akt) and eNOS (Figures 5(b) and 5(c)) were significantly reduced in diabetic group that was concomitant with significant increase in cardiac p-PTEN. NAC treatment completely prevented diabetes and I/R induced increases of p-PTEN and significantly attenuated the reduction of p-Akt and p-eNOS induced by I/R (all $p < 0.05$, D4w + I/R + NAC versus D4w + I/R).

4. Discussion

Hyperglycemia was the major character of diabetes along with enhanced oxidative stress. In the current study, 15-F2t-IsoP level was significantly increased in 4 weeks' diabetic rats, and NAC treatment reduced the 15-F2t-IsoP level along with attenuating I/RI. These findings are in line with findings of recent studies by us and other groups showing that antioxidant treatment with NAC can attenuate myocardial I/RI in diabetes. The novel finding of the current study is that autophagy is increased in the myocardium of 4 weeks' diabetic rats which may be the major mechanism that rendered the diabetic heart more vulnerable to ischemic insult and that NAC treatment attenuates myocardial I/RI in diabetes primarily via inhibiting and preventing excessive autophagy.

In the heart, autophagy plays critical roles in many cardiovascular diseases in response to pathological stimuli, including cardiac hypertrophy, heart failure, and myocardial I/R. Autophagy is indicated to be promoted as a response to the ATP depletion and to the ROS accumulation [31, 32].

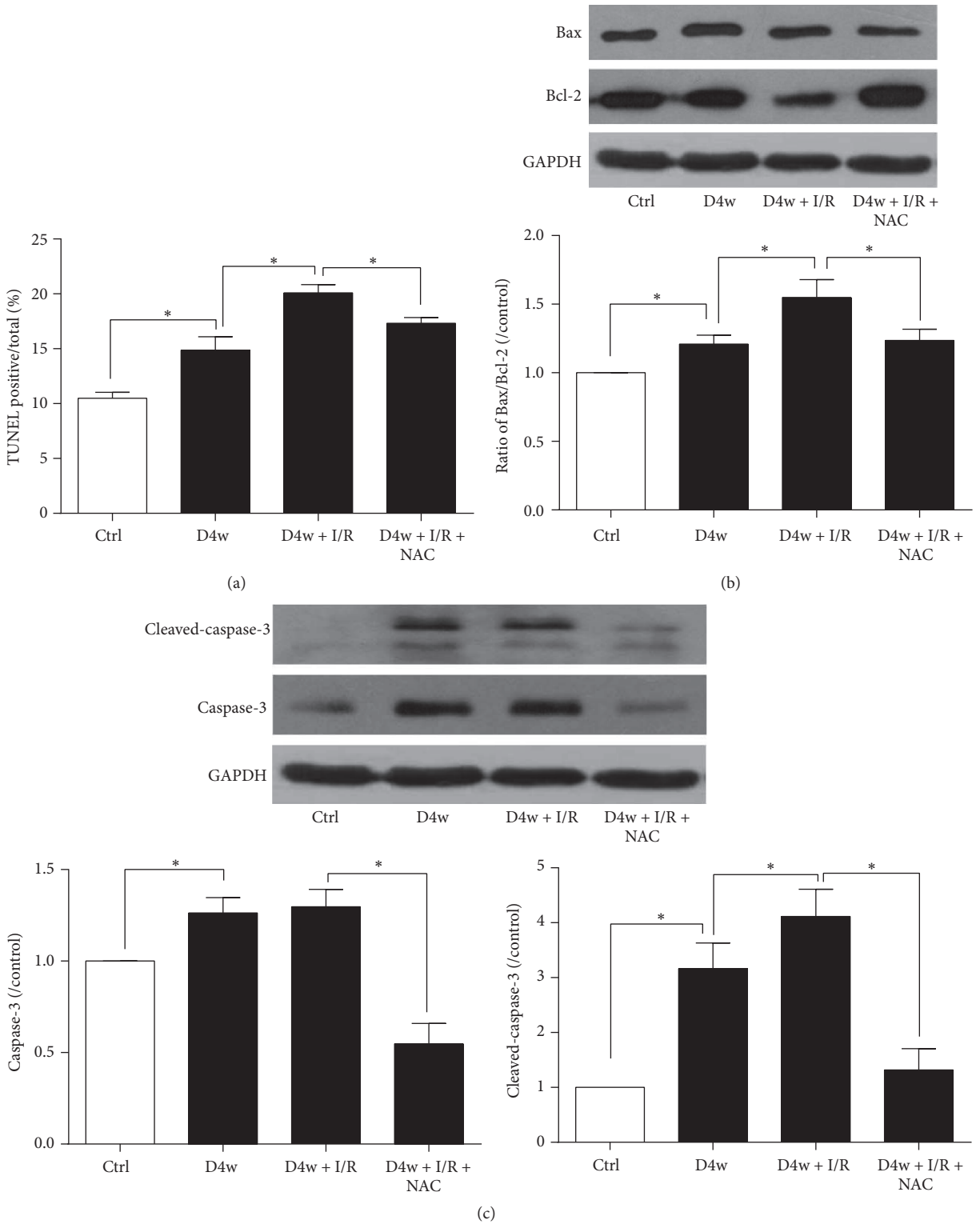

Figure 3: Effects of NAC on apoptosis. (a) Myocardial cell apoptosis assessed by TUNEL, (b) ratio of Bax/Bcl-2, and (c) expression of cleaved-caspase-3. Ischemia reperfusion (I/R) was achieved by 30-minute ischemia followed by 2-hour reperfusion in diabetic rats with or without NAC. Ctrl: nondiabetic control; D4w: 4-week diabetes; D4w + I/R: 4 weeks' diabetic rats with ischemia/reperfusion; D4w + I/R + NAC: 4 weeks' diabetic rats treated with N-acetylcysteine (NAC) and were subjected to ischemia/reperfusion. Dates are expressed as mean ± SEM ($n = 6$ per group), $^{*}p < 0.05$.

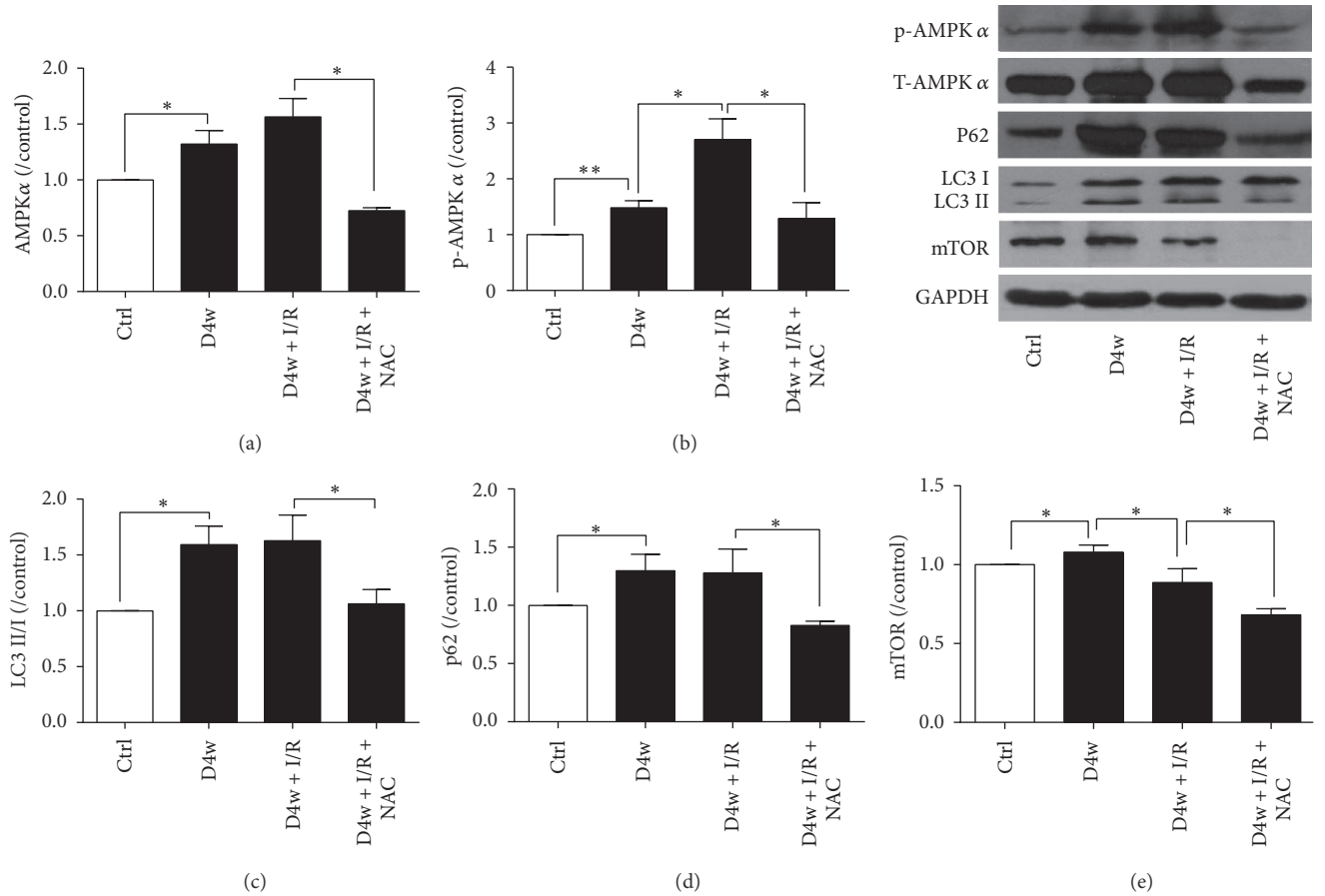

Figure 4: Effects of NAC on autophagy pathway. (a) Protein AMPK expression; (b) p-AMPK; (c) ratio of LC3 II/I; (d) protein P62 expression; (e) protein mTOR expression. Ischemia reperfusion (I/R) was achieved by 30-minute ischemia followed by 2-hour reperfusion in diabetic rats with or without NAC. Ctrl: nondiabetic control; D4w: 4-week diabetes; D4w + I/R: 4 weeks' diabetic rats with ischemia/reperfusion; D4w + I/R + NAC: 4 weeks' diabetic rats treated with N-acetylcysteine (NAC) and were subjected to ischemia/reperfusion. Dates are expressed as mean ± SEM ($n = 6$ per group), $^{*}p < 0.05$, and $^{**}p < 0.01$.

In diabetic mice, with autophagy-associated-gene (ATG5) knockout, autophagy was impaired, leading to mitochondria dysfunction and ROS overproduction [33], suggesting that maintaining normal function of autophagy is critical for diabetic heart functioning. In our previous study, we found antioxidant NAC treatment could reduce ROS level and I/RI in diabetic rats [8], and in the current study we found that NAC could prevent diabetes and I/R induced excessive autophagy and restore the normal autophagy function in diabetic rats subjected to I/R and subsequently reducing I/RI. The extent of cardiac autophagy has been reported to be either reduced at [34] or enhanced [35] in diabetes during different stages of the disease, but the impact of autophagy on diabetic heart vulnerability to ischemic insult is largely unknown. While moderate increase of autophagy during myocardial ischemia has been shown to be beneficial, excessive autophagy during reperfusion is harmful [24]. Mitochondria serve as the power of a cell, especially in cardiac myocytes which have a large energy requirement of energy. Mitochondrial permeability transition pore (mPTP) plays a key role in myocardial I/RI. The increase of ROS during I/RI enhances the likelihood of mPTP opening upon reperfusion [4]. In this study, excessive autophagy was found in 4 weeks' diabetic rats with myocardial I/R, which was accompanied with high levels of 15-F2t-IsoP, IL-6, TNF-α, and apoptosis, and NAC treatment for 4 weeks attenuated all these changes, which should have reduced mPTP opening, although further study is needed to confirm it. And, the potential causal relationship between diabetes induced excessive autophagy, apoptosis, inflammation, and oxidative stress in the context of myocardial I/RI has yet to be determined.

Autophagy is not only associated with cell survival but has also tight relationship with cell death. As reported, autophagic cell death is caspase independent and is clearly distinct from apoptotic cell death but they can occur in mixed forms with autophagic and caspase related apoptotic features. Our results showed that NAC could reduce I/R induced cell apoptosis, manifested by reducing positive apoptosis cells, the ratio of Bax/Bcl-2, and expression of cleaved-caspase-3 but has more profound effect on autophagy because it could attenuate autophagy to a greater extent. This suggests that, compared to apoptosis, autophagy played major roles in diabetic I/RI, and reducing excessive autophagy may be a potential therapy for I/RI in diabetes.

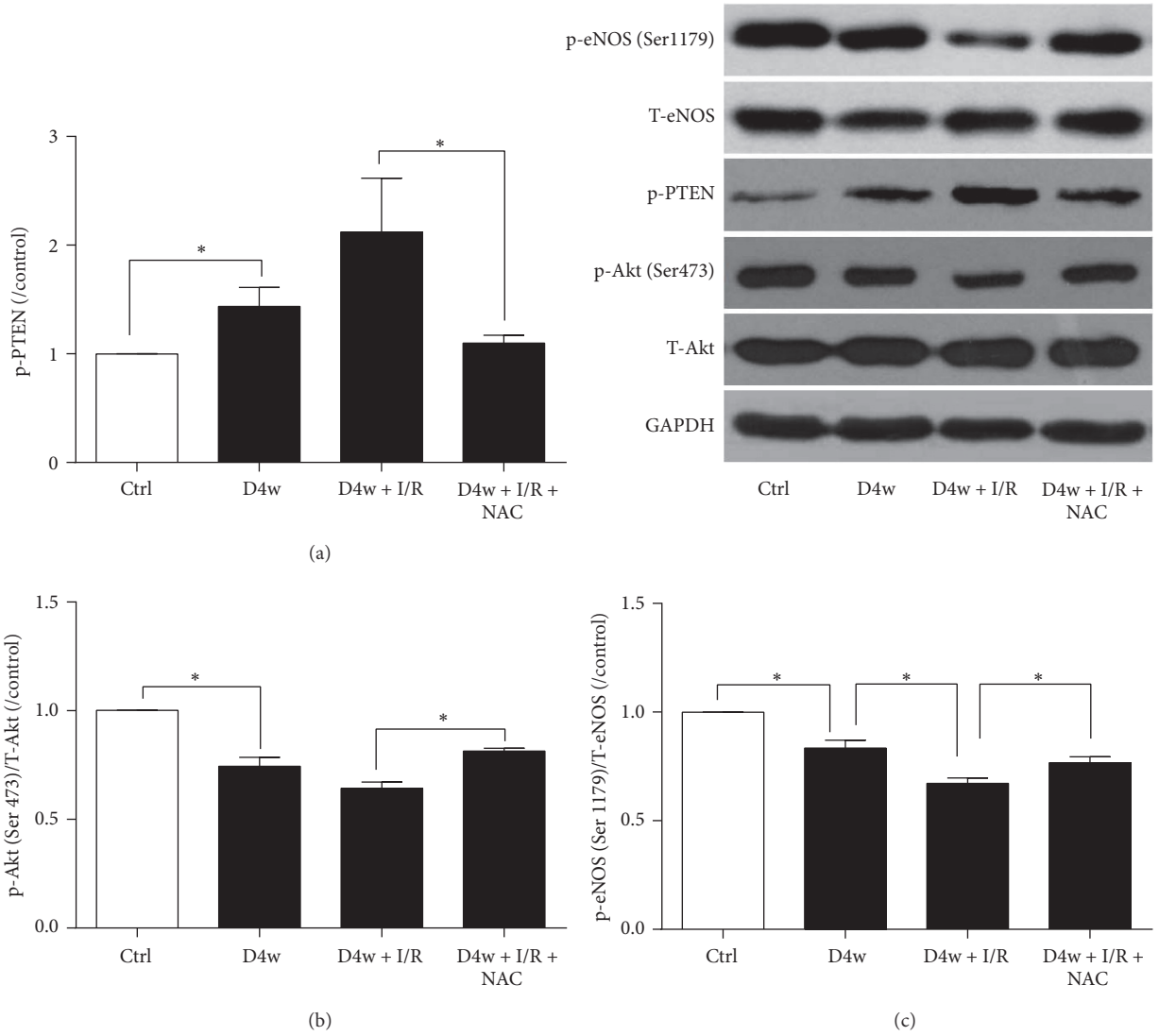

Figure 5: Changes of PTEN, Akt, and eNOS after NAC treatment. (a) Protein p-PTEN expression; (b) protein p-Akt expression; (c) protein p-eNOS expression. Ischemia reperfusion (I/R) was achieved by 30-minute ischemia followed by 2-hour reperfusion in diabetic rats with or without NAC. Ctrl: nondiabetic control; D4w: 4-week diabetes; D4w + I/R: 4 weeks' diabetic rats with ischemia/reperfusion; D4w + I/R + NAC: 4 weeks' diabetic rats treated with N-acetylcysteine (NAC) and were subjected to ischemia/reperfusion. Dates are expressed as mean ± SEM ($n = 6$ per group), $^{*}p < 0.05$.

AMPK is a serine-threonine kinase which functions primarily as a metabolic sensor to coordinate anabolic and catabolic activities in the cell to maintain the cellular energy homeostasis via the phosphorylation of multiple proteins involved in metabolic pathways [36]. It has effects on many signaling pathways, such as autophagy and apoptosis [37, 38]. Our results showed that, in early diabetic rats, p-AMPKα was significantly increased which was induced by either diabetes or diabetes with I/R and that the increase of cardiac mTOR in diabetes was not sufficient to combat p-AMPKα to stimulate autophagy, and this led to excessive autophagy as reflected by significant increases in the ratio of LC3 II/I and protein P62 expression and exacerbated postischemic I/RI. Treatment with NAC completely prevented diabetes I/R induced increase in p-AMPKα and reduced the ratio LC3 II/I and protein P62 expression to levels comparable to normal control and concomitantly reduced mTOR. The fact that I/R in diabetes did not further increase the extent of autophagy despite I/R induced further significant increase of p-AMPKα and concomitant moderate but significant reduction in mTOR may suggest that excessive cardiac autophagy already occurred in diabetes is the major contributor of myocardial I/RI. The finding that NAC could reduce the activation of cardiac p-AMPKα is in line with our previous study [28]. In the current study, NAC restored the autophagy flow, prevented diabetes and I/R induced increases of p-PTEN, and attenuated the reduction of p-Akt and p-eNOS induced by I/R to confer cardioprotection effect in diabetic rats. Inhibition of p-PTEN has been shown to attenuate myocardial I/RI in diabetic rats [39]. However, the potential interplay between

PTEN and autophagy and their regulating molecules such as mTOR in the context of diabetic myocardial I/RI has not been reported. Findings from our current study may serve to stimulate further in-depth studies in this interesting and important area to foster the development of effective therapies in combating diabetic myocardial I/RI.

In summary, we found that autophagy was excessive in 4 weeks' diabetes and this was detrimental to I/RI. Antioxidant treatment with NAC could suppress autophagy in 4 weeks' diabetic rats and reduce I/RI. NAC confers cardioprotection against diabetic heart I/RI primarily through inhibiting excessive autophagy formation following reducing oxidant stress in diabetes.

4.1. Clinical Perspectives. Patients with diabetes are more vulnerable to myocardial I/RI. The present study was designed to investigate the role of autophagy on NAC conferring cardioprotection effects in diabetic myocardial I/R injury. Our results showed that autophagy was excessive in 4 weeks' diabetes and this was detrimental to I/RI. Antioxidant treatment with NAC could suppress autophagy in 4 weeks' diabetic rats and reduce I/RI. It is strongly suggested that targeting inhibition of excessive autophagy may be a potential therapeutic strategy for the treatment of diabetes-associated cardiac disease in patients. Patients with acute myocardial infarction usually had increased levels of oxidative stress that were associated with a reduction in enzymatic antioxidant reserve in particular in patients with diabetes, while conditional treatments such as glucose-insulin-potassium solution did not improve these abnormalities among patients undergoing primary angioplasty [40]. Findings from our current study may suggest antioxidant treatment with NAC as a potential adjunct therapy.

Competing Interests

No conflict of interests is declared by the authors.

Authors' Contributions

Zhengyuan Xia, Zhongjun Zhang, and Chunyan Wang conceived and designed the experiments. Sheng Wang, Chunyan Wang, Zhengyuan Xia, Haobo Li, Tingting Wang, and Yi He performed the experiments. Chunyan Wang, Zhongjun Zhang, Fuxia Yan, and Zhengyuan Xia analyzed the data. Sheng Wang contributed reagents/materials/analysis tools. Sheng Wang, Chunyan Wang, Zhongjun Zhang, and Zhengyuan Xia wrote the paper. Sheng Wang and Chunyan Wang equally contributed to this study.

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Review Article

Interleukin-6 "Trans-Signaling" and Ischemic Vascular Disease: The Important Role of Soluble gp130

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Inflammation plays a major role in the onset of cardiovascular disease (CVD). Interleukine-6 (IL-6) is a multifunctional cytokine involved both in the beneficial acute inflammatory response and in the detrimental chronic low-grade systemic inflammation. Large genetic human studies, using Mendelian randomization approaches, have clearly showed that IL-6 pathway is causally involved in the onset of myocardial infarction. At the same time, IL-6 pathway is divided into two arms: classic signaling (effective in hepatocytes and leukocytes) and trans-signaling (with ubiquitous activity). Trans-signaling is known to be inhibited by the circulating soluble glycoprotein 130 (sgp130). In animal and in vitro models, trans-signaling inhibition with sgp130 antibody clearly shows a beneficial effect on inflammatory disease and atherosclerosis. Conversely, epidemiological data report inconsistent results between sgp130 levels and CV risk factors as well as CV outcome. We have reviewed the literature to understand the role of sgp130 and to find the evidence in favor of or against a possible clinical application of sgp130 treatment in the prevention of cardiovascular disease.

1. Introduction

Cardiovascular disease (CVD) is one of the leading causes of morbidity and mortality all around the world. Over the last decades, the role of inflammation in atherosclerosis has been widely recognized and studied [1]. Identification of the detailed pathways that link inflammation to atherosclerosis and CVD provides an auspicious ground to find new possible therapeutic targets. Since the last century, plasma C-reactive protein (CRP) levels have attracted great attention, showing robust results as a marker of systemic inflammation associated with cardiovascular risk [2]. We have recently reported that also among elderly population low-grade systemic inflammation, as identified by hsCRP levels, was associated with increased CV risk [3]. Conversely, while hsCRP is clearly a reliable marker to identify subjects at higher CV risk, it does not seem to be an effector of the inflammation-driven atherogenesis. Genetic studies have addressed this issue with the use of Mendelian randomization approach [4]. In this type of analysis, several authors have argued against a causal association of CRP with coronary heart disease due to the lack of consistency between the effect of CRP genetic variants on CVD and CRP levels [4–6]. Plasma CRP is produced primarily in the liver, as a response to inflammatory stimulation by cytokines, such as Interleukine-6 (IL-6). As reported by different studies, IL-6 represents an upstream inflammatory cytokine that seems to be responsible for the chronic-inflammation-related atherogenesis [7, 8]. The causal role of this pathway has been nicely shown in studies involving the principles of Mendelian randomization [9, 10].

As shown in Figure 1, IL-6 pathway could be differentiated into two axes, with different cell targets and divergent downstream effects. In the classic pathway, IL-6 binds the membrane-bound IL-6 receptor (IL-6R), located on the surface of hepatocytes and some leukocytes, and activates the IL-6 classic signaling transduction cascade with the homodimerization of the membrane-bound β-receptor glycoprotein 130 (gp130). In the "trans-signaling" axis, circulating IL-6 forms a

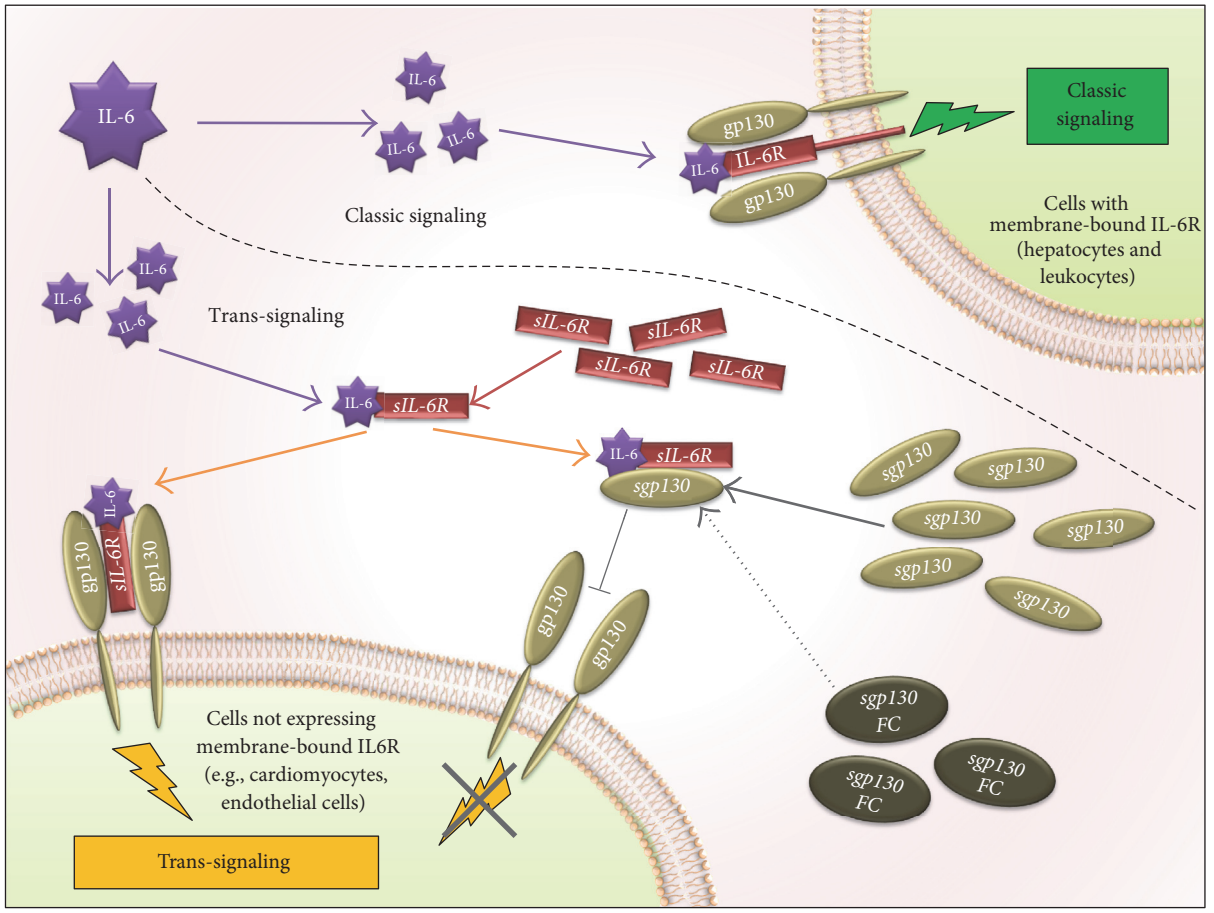

Figure 1: IL-6 signal transduction via classic and trans-signaling. The upper part of the figure depicts IL-6 signaling in cells expressing the membrane-bound receptor for IL-6 (IL-6R). In these cells (e.g., hepatocytes and several white blood cells), circulating IL-6 binds directly to IL-6R that forms a signaling complex with the membrane-bound glycoprotein 130 (gp130); this pathway is known as classic signaling. The bottom part depicts the IL-6 signaling in those cells that do not express the membrane-bound IL-6R. In these cells, membrane-bound gp130 (ubiquitously expressed) is activated by the circulating IL-6/sIL-6R complex (composed of IL-6 and the circulating soluble portion of IL-6R, sIL-6R). This pathway, known as trans-signaling, could be inhibited by the circulating soluble portion of gp130 (sgp130), which, by means of binding the circulating IL-6/sIL-6R complex, blocks the activation of the membrane-bound gp130. sgp130fc is a recombinant fusion protein of soluble gp130 and human IgG1 Fc that blocks IL-6 trans-signaling mimicking sgp130 functions.

heterodimer with the soluble form of IL-6 receptor, IL-6/sIL-6R, that could transduce a proinflammatory cascade in virtually any cell types through direct binding with membrane-bound gp130. The soluble form of the gp130 (sgp130) could instead inhibit the latter axis, through specific binding with the IL-6/sIL-6R heterodimer (interfering with its ability to bind the membrane gp130). Recently, new basic and clinical studies have highlighted the probable determinant role of the IL-6 trans-signaling pathway in the inflammatory-driven atherogenesis process. Our aim was to evaluate the current "state of the art" providing a comprehensive review of the relationship between CVD and IL-6 trans-signaling. Based on this review, we further speculate on the possible use of drugs targeting this pathway in the treatment of CVD.

2. IL-6 Classic and Trans-Signaling Effects

IL-6 is a cytokine with a multifactorial function and induces both pro- and anti-inflammatory responses [11]. It appears to have ubiquitous functions in several physiological and pathological processes [11, 12]. IL-6 has two different pathways for its induction of intracellular signaling: classic signaling (active primarily in hepatocytes and lymphocytes) and trans-signaling (with ubiquitous activity). The downstream effect of these signaling axes shows divergent functions [12]. Consistent data suggest that the classic signaling (through direct binding of membrane-bound IL-6R) is mainly responsible for the beneficial regenerative and antibacterial effects of IL-6 [13, 14], while the trans-signaling (through "IL-6/soluble- IL-6R heterodimer" bound to membrane-bound gp130) seems to account for the majority of the deleterious effect of IL-6 [15, 16]. While this simplistic view is nonexhaustive for the complex pathway of IL-6, it gives a glimpse of the reason why trans-signaling is considered so important. Furthermore, since sgp130 (the soluble form of gp130) is known to inhibit the IL-6/sIL-6R induced trans-signaling [17], it represents an ideal pharmacological target for IL-6 signaling.

Animal model and in vitro studies have reported the beneficial effect, in several inflammatory and degenerative disease models, of specific inhibition of trans-signaling with an fc-dimerized version of sgp130 (sgp130fc) [18–23] (as depicted in Figure 1). This process has been consistently reported also in cardiometabolic disease. Kraakman and colleagues showed that specific inhibition of trans-signaling, with sgp130fc protein, prevents the recruitment of macrophages in adipose tissue induced by high fat diet (ATM recruitment). On the contrary, in the same study, the complete blockade of IL-6 (both classic and trans-signaling) exacerbates obesity/induced weight gain, liver steatosis, or insulin resistance [24].

Schuett et al. have shown the protective effect of sgp130 in an animal model of atherosclerosis. Treatment with sgp130fc attenuates the atherosclerotic lesion progression in $LDL^{-/-}$ mice by decreasing endothelial activation, smooth muscle cell infiltration, and monocyte recruitment [25]. Furthermore, these authors assessed the therapeutic relevance of sgp130fc in a model of preexisting atherosclerosis, showing a reduction of thoracoabdominal lipid deposition and of aortic root lesion size with this treatment. In addition, Schuett et al. confirmed that sgp130fc did not influence hepatic effects of IL-6 (suggesting preserved IL-6 classic signaling). This specific and trans-signaling-targeted effect of the sgp130fc is of great relevance since other studies have shown that overall IL-6 signaling has also a beneficial cardiometabolic function, and complete blockade of IL-6 could be counterproductive [26–28].

3. Trans-Signaling and Cardiovascular Disease in Human

3.1. Epidemiological Studies on sgp130 Levels. While animal models provided consistent results for the beneficial consequences of the sgp130fc-induced blockade of the IL-6 trans-signaling, in human studies, the association of sgp130 levels and cardiovascular disease appears less straightforward. Indeed, while some studies reported an inverse association between sgp130 levels and CVD, others reported null or positive association.

Schuett et al. confirmed the translational relevance of the beneficial effect of sgp130fc treatment showing that sgp130 levels were lower among 50 patients with coronary artery disease (CAD) as compared to controls [25]. Furthermore, among patients with CAD, sgp130 levels were inversely associated with extension of disease. Anderson et al., in a similar sample size, found that sgp130 had an inverse association with previous myocardial infarction (MI), although there were no differences between patients with acute MI and CAD. Interestingly, sgp130 levels had a positive correlation with the peak of troponin I [29]. In a much larger population-based case-control study, involving 664 cases and 1062 controls, very high levels of sgp130 were associated with a 30% reduction in the incidence of myocardial infarction (OR: 0.7; 95% CI: 0.5–0.9) [30].

On the contrary, different studies showed a negative prognostic value of sgp130 among those patients with a history of MI [31] and in particular among subjects with heart failure (HF) [32, 33]. Indeed, serum levels of sgp130 were reported to be higher among patients suffering from chronic heart failure [34, 35] and, most of all, as reported by Askevold et al., to be associated with CV and total mortality in elderly patients with HF of ischemic cause. In this study, subjects with high levels of sgp130 (those in the 5th quintile versus all the others) had a significant 38% increase in CV mortality, a 47% increase in all-cause mortality, and an 85% increase in death from worsening of HF [32].

A possible explanation for these apparently counterintuitive results is that, in the context of chronic ischemic disease and vascular remodeling, higher sgp130 levels are representative of a compensatory response to higher activation of the IL-6 signaling, with increased gp130 expression. In support of this hypothesis, Inta et al. reported that sgp130 levels correlated with blood pressure and carotid intima-media thickness in stroke patients and that these increased levels may reflect the vascular remodeling response to arterial hypertension, as suggested by the increased gp130 mRNA expression in the aortic wall of spontaneous hypertensive rats [36]. Furthermore, we have recently found that also among community dwelling elderly individuals there was a significant association between sgp130 levels and metabolic syndrome; nevertheless, this association seemed to be mediated by insulin resistance [37].

Thus, it is possible that in these groups of patients higher sgp130 represents a marker of higher fragility more than being a cause of adverse outcome.

3.2. Genetic Variants in IL-6R and gp130 and Cardiovascular Disease. By using the principle of the Mendelian randomization, it is possible to address the issue of causality. The general principle of these studies is that lifelong genetically determined exposure to a marker of CV risk factor should induce higher prevalence of CVD only if this risk factor is a causal mediator of the disease.

In two independent large-scale human genetic studies, a functional genetic variant (Asp358Ala) located in the gene coding the IL-6R has been shown to be associated with lower coronary heart disease (CHD) [9, 10]. This nonsynonymous variant (358Ala), located in the cleavage site of IL-6R, confers increased proteolytic conversion rates by ADAM proteases (ADAM10 and ADAM17) [38], resulting in higher circulating levels of soluble IL-6 receptor and lower downstream transduction of IL-6 signals. As a consequence, carriers of the alternative allele, those with lower risk of CHD, have a 2-fold increase in the circulating levels of soluble IL-6R and reduced downstream IL-6 signaling as demonstrated by the lower levels of hsCRP and fibrinogen. A further consequence of this functional variant is positive feedback with paradoxical increase in IL-6 levels. Thus, from these studies, it is possible to confirm the causal association between IL-6 signaling and CHD but it is not possible to depict whether the reduced transduction of the IL-6 signal involved only the classic signaling (as suggested by the hsCRP and fibrinogen lower levels) and/or the trans-signaling too.

Given the higher levels of IL-6 and sIL-6R associated with the 358Ala variants, one would expect that trans-signaling

should be increased as well. Conversely, the opposite scenario is also possible; indeed, it must be considered that the increase in levels of the soluble IL-6R could potentiate the antagonistic activity of sgp130 on IL-6 response [39]. As mentioned also by Scheller and Rose-John [40], the decoy receptor sgp130 has a much higher concentration (≈200 μg/L) than soluble IL-6R (≈50 μg/L) and IL-6 (≈2 ng/L) [12, 37]. Thus, it is probable that among carriers of the alternative allele (with 358Ala) the higher soluble IL-6R levels improve the buffer activity of sgp130 with reduced ubiquitous IL-6 trans-signaling (as well as the reduced classic signaling in hepatocytes and leukocytes [41]).

3.3. gp130 Genetic Variants and Cardiovascular Disease. Further evidence for the role of sgp130 in the onset of cardiovascular disease has been reported in studies on genetic variants located in the gene coding gp130 *(IL6ST)*.

Lucthefeld et al., in a haplotype-based analysis, identified that genetic variability in the *IL6ST* gene was associated with CAD and MI in two independent populations [42]. Interestingly, in this study, a highly significant association was detected with the atherosclerosis of the ostium of the coronary arteries, which has an important clinical relevance for the coronary flow.

Bernick et al. then analyzed one of the nonsynonymous single nucleotide polymorphisms studied in this paper (Gly148Arg, rs3729960) [43]. This functional SNP is known to change the stability of the glycoprotein and influence the responsiveness to IL-6, as shown by the slightly reduced transduction of the signals associated with the 148Arg allele [43]. Most interestingly, this variant was associated with a 46% decreased risk of myocardial infarction, confirming the previous report by Lucthefeld et al. Recently, Wonnerth et al. have shown that carriers of the 148Arg allele had higher circulating levels of soluble gp130 (sgp130); interestingly, they were able to replicate these results in two different cohorts [44]. Even though this data suggests that the lower risk of MI in 148Arg carriers could be mediated by higher sgp130 circulating levels, this could not be proven at this point. Indeed, cells transfected with the 148Arg allele showed lower activity of the membrane-bound receptor, and furthermore this amino acid change is located in the cytokine binding site; thus, it is not possible to exclude an altered interaction with the IL-6/sIL-6R heterodimer.

Finally, it is important to notice that this and other variants in the IL6ST gene have been associated with increased prevalence of metabolic syndrome and higher BMI [44, 45]. These results confirmed the complexity of this pathway in the onset of cardiometabolic disease; nevertheless, animal models suggest that only the complete blockade of IL-6 (both classic and trans-signaling) exacerbates obesity and insulin resistance [24], while this effect was not present in specific inhibition of the trans-signaling.

4. Conclusion

Current IL-6 blockade treatments, used in specific inflammatory diseases, such as rheumatoid arthritis, are nonspecific (targeting both IL-6 classic and trans-signaling) and are associated with increased infection and metabolic disturbances. Development of new treatments (e.g., sgp130fc) aiming at specific inhibition of IL-6 trans-signaling seems to be a promising avenue also for the treatment and prevention of cardiovascular disease. Nonetheless, given the complexity of the IL-6 cascade, further studies to confirm this hypothesis are warranted. Specifically, human genetic studies, conducted in large and different cohorts, could provide interesting validation of this hypothesis; furthermore, these studies could identify specific subjects who may benefit more from this possible treatment.

Competing Interests

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

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Research Article

High Levels of Hemoglobin Promote Carotid Adventitial Vasa Vasorum Neoangiogenesis in Chronic Kidney Disease

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Chronic kidney disease (CKD) patients, characterized by traditional and nontraditional risk factors, are prone to develop atheromatosis and thus cardiovascular events and mortality. The angiogenesis of the adventitial vasa vasorum (aVV) surrounding the carotid has been described as the atheromatosis initiator. Therefore, the aim of the study was to (1) evaluate if the carotid aVV in CKD patients increases in comparison to its physiological value of healthy patients; (2) explore which traditional or nontraditional risk factor including inflammation, bone and mineral metabolism, and anemia could be related to the aVV angiogenesis. CKD patients without previous cardiovascular events (44, stages 3-4; 37, stage 5D) and 65 healthy subjects were compared. The carotid aVV and the intima-media thickness (cIMT) were evaluated by ultrasound. CKD patients at stages 3-4 showed higher aVV of the right carotid artery even after adjusting for age. Importantly, a multiple linear regression model showed hemoglobin levels > 12.5 g/dL as the factor for an estimated higher aVV of the right carotid artery. In conclusion, the association of hemoglobin with higher aVV could suggest the role of high hemoglobin in the higher incidence of adverse cardiovascular outcomes in CKD patients.

1. Introduction

Chronic kidney disease (CKD) has been reported as a major risk factor for cardiovascular (CV) disease [1]. Indeed, CKD patients at all stages demonstrate a marked increase in the incidence of CV events and CV disease mortality in comparison with age- and sex-matched subjects of the general population [2] and, in particular, in dialysis patients, CV mortality rate is 10 times higher than that for the general population [3, 4]. This high incidence is due to the fact that not only are patients with CKD affected by traditional risk factors including hypertension, diabetes, dyslipidemia, and smoking [5], but they are also affected by nontraditional risk factors including inflammation, altered bone and mineral metabolism, anemia, and albuminuria. Both traditional [6, 7] and nontraditional risk factors [8–11] are associated with the impairment of the endothelial function and the inflammatory response [12], key steps in the development of atherosclerosis, plaque progression, and a higher risk of CV events.

Challenging the common belief that subclinical endothelial dysfunction has been considered the earliest step in the chronic inflammation of the vessel wall, studies by Herrmann

et al. demonstrated conclusively that, during pathological conditions, increases in the density of adventitial vasa vasorum (VV), the plexus of physiological microvessels surrounding the adventitial layer, precede endothelial dysfunction [13].

Inflammation has been strongly related to VV neoangiogenesis [14]. Indeed, inflammation is associated with the recruitment of circulating inflammatory cells as neutrophils, macrophages, and lymphocytes which secrete proangiogenic factors including cytokines and the well characterized signaling molecule Vascular Endothelial Growth Factor (VEGF) [15, 16]. Together with inflammation, elevated levels of parathyroid hormone (PTH), lower levels of vitamin D, and anemia, all common features of CKD, have been recognized as proangiogenic stimuli. Specifically, recent experimental animal studies associated PTH treatment with increased angiogenesis in both the ischemic brain and the infarcted heart [17, 18]. Equally important in the angiogenesis process is the evidence of the involvement of vitamin D. Oikawa et al. showed that active vitamin D, 1α,25-dihydroxyvitamin D_3 (1,25D), was highly effective in inhibiting angiogenesis in a dose-dependent manner in chick embryo chorioallantoic membranes [19]. Consequently, while several lines of evidence support the efficacy of 1,25D to suppress neoangiogenesis through strong anti-VEGF properties [20–22], others demonstrated that calcitriol exerts antiangiogenic properties in a VEGF-independent manner. Indeed, in the retina, calcitriol suppresses neovascularization through the inhibition of the endothelial cells proliferation and sprouting [23]. Additionally, a third feature of CKD that could be involved in angiogenesis and therefore in the increases of the adventitial VV is anemia. In a study of 59 patients with nonmalignant cancers, 23 patients with renal anemia exhibited significantly higher levels of VEGF in comparison to those of normemic patients [24]. Furthermore, in a CKD population study including patients at stages 3b-4, the adjusted linear inverse association of the flow-mediated dilatation with hemoglobin (Hb) in a range between 8.5 and 14.5 g/dL, demonstrated that (Hb) per se affects the endothelial function regulation which could likely determine the VV neovascularization [25, 26].

Since, to our knowledge, there are no reports describing the adventitial VV density in patients with renal disease nor the pathophysiology of adventitial VV density in this population, the current study was designed to compare the carotid adventitial VV density in CKD patients with the physiological carotid adventitial VV density in healthy volunteers with none of the classical risk factors. Moreover, since, inflammation, high levels of PTH, vitamin D deficiency, and anemia are generally observed features of CKD, we examined the correlation of these variables, including VEGF, with the carotid adventitial VV content in order to evaluate their role in increasing the carotid adventitial VV, therefore aggravating CV disease (CVD), in both dialysis and nondialysis-dependent CKD patients.

2. Material and Methods

2.1. Study Subjects. In this single-centre, cross-sectional study, 81 patients with CKD (53% males; median age 60 [53.0; 66.0]) were enrolled as the case group according to a previous selection of 104 CKD patients: patients were withdrawn from the study because of poor or overly strong echogenicity or because of poor or inaccessible venous access. Further exclusion criteria included the following: (1) they experienced previous cardiovascular events such as coronary heart disease, cerebrovascular disease, and peripheral vascular disease; (2) they met the exclusion criteria for the administration of the contrast agent including (a) recent cardiac instability, (b) recent (<7 days) coronary intervention, (c) class III or IV heart failure, (d) severe pulmonary hypertension, and (e) allergic reaction to sulphur hexafluoride, the gas contained in the contrast agent. Specifically, 44 patients belonged to CKD stages 3-4, while 37 belonged to CKD stage 5D (28 hemodialysis patients and 9 peritoneal dialysis patients). Sixty-five subjects with none of the classical risk factors for cardiovascular disease (46% male; median age 49 [41; 57]) were enrolled as control group. The age range was between 30 and 70 years.

The study protocol was approved by the ethical committee at the University Hospital Arnau de Vilanova (HUAV, Lleida, Spain). All subjects signed an informed consent prior to morphometric parameter acquisition and blood drawing after overnight fasting.

2.2. Morphometric and Biochemical Parameters. Morphometric body parameters such as weight (kilograms) and height (meters) were measured with a digital weight scale equipped with a stadiometer. Body mass index (BMI) was calculated as weight divided by the square of height. The waist circumference (WC) was measured in the umbilicus level (cm). While patients were seated and had rested for ten minutes, systolic and diastolic blood pressure (SBP and DBP) were obtained with an automated oscillometer (Omron HEM-705CP) and calculated as the average of three independent measurements. Serum levels of glucose, total cholesterol (cholesterol), HDL and LDL cholesterol, triglycerides (TG), and ultrasensitive C-reactive protein (CRP), creatinine clearance (CC), calcium (Ca), phosphorous (P), PTH, albumin (Alb), ferritin, hemoglobin (Hb), fibrinogen, and leukocytes were obtained using the standard methods of the laboratory of Clinical Biochemistry at the HUAV. For serum VEGF, the Human VEGF ELISA Kit was used (R&D System, DVE00). Serum levels of 25-hydroxyvitamin D (25D) were measured using the Chemiluminescence Immunoassay on the LIAISON XL Analyzer (DiaSorin), while serum 1,25D levels were determined using a radioreceptor assay (Gamma-B dihydroxyvitamin D, IDS Hybritec®).

2.3. Standard and Contrast-Enhanced Carotid Ultrasound. All subjects underwent a B-mode ultrasound examination of the extracranial carotid arteries (CA). For the measurements of carotid intima-media thickness (cIMT; mm) of the far wall of the common carotid artery (CCA) 1 cm proximal to the bifurcation and for the evaluation of atheromatous plaque presence, a prior axial exploration was followed by a longitudinal exploration. Carotid plaque was defined as a cIMT > 1.5 mm or as a focal thickening overpassing into the arterial lumen by at least 50% of the surrounding cIMT value

[27, 28]. Then, subjects underwent the contrast-enhanced ultrasound (CEUS) imaging procedure using the contrast agent SonoVue (Bracco Spa, Milan, Italy). The contrast agent was prepared as recommended on the manufacturing data sheet, and, for each CA explored, a 2.5 mL contrast agent bolus, followed by 10 mL of a saline, was injected in the antecubital vein (20-gauge needle). This chosen dose was the lowest dose adequate to reach a strong and clear signal that suffice to obtain statistically significant differences between the adventitial VV signal in control subjects and CKD patients based on the previous differences obtained between type 2 diabetic patients with or without retinopathy and control subjects [29]. The CEUS imaging was performed with a Siemens Sequoia 512 using the 15L8W linear array probe (7 Mhz) with a low mechanical index of 0.18. This device is equipped with Cadence contrast pulse sequencing (CPS) technology able to determine a high sensitivity and specificity of contrast agent detection. All the videos were analyzed using the Siemens software Syngo. Adventitial VV content was measured as previously described [30]. Videos in which signals (clipping artefacts) were observed in the far arterial wall before contrast injection were excluded from reading. Moreover, additional reasons for reading exclusion were as follows: (1) the contrast agent which disappeared rapidly, thus impeding proper visualization or (2) the presence of an ultrasound shadow that impeded the reading in the area under analysis.

Since the optimal cIMT measurement is obtained on the far wall and because our applied method elicits strong clipping artefacts in the near wall [31], in accordance with other reports exploring the adventitial and intraplaque VV [32–34], the VV signal was measured in the far wall as well. Importantly, although the far wall could be liable to imaging artefacts due to nonlinear propagation [35], there is a licit expectation that these artefacts are similar for healthy controls and CKD patients.

2.4. Statistical Analysis. Descriptive statistics of mean (standard deviation) or median [interquartile range] were estimated for quantitative variables with a normal or nonnormal distribution, respectively, while, for qualitative variables, absolute and relative frequencies were used. Normal distribution was analyzed using the Shapiro-Wilks test.

The significance of the differences in quantitative variables between groups (control, CKD stages 3-4, and CKD stage 5D) was assessed by analysis of variance or Kruskal-Wallis test depending on their normal distribution. The significance of the differences in qualitative variables between the three studied groups was assessed by Chi-squared test or Fisher's exact test in case of any expected frequency of the corresponding contingency table lower than 5. In case of significant differences, multiple testing adjusted pairwise comparisons were performed using the Tukey or Benjamini-Hochberg method according to a normal or not normal distribution. Linear regression models were fitted to each measure of cIMT and carotid adventitial VV in order to assess differences between groups considering or not age as angiogenic factor. The most adequate Box-Cox transformation was applied in case of nonnormally distributed residuals from multivariable regression models. Within the CKD population, monotonic relationships of the right and left carotid adventitial VV with quantitative characteristics were assessed by using Spearman's rank correlation coefficients. In case of significant association, age-adjusted linear regression models were fitted. Associations of the right and the left carotid adventitial VV with dichotomous characteristics of each CKD studied stage were assessed by Mann–Whitney U test (Kruskal-Wallis test for smoking status). An adjusted linear regression model was fitted to the right carotid adventitial VV mean, including the assessment of the significant interactions. Only interactions or main effects with a significant contribution to the final model according to the likelihood ratio test were included. All the studied variables (see Tables 1 and 5) were assessed for significant contribution into the model. A significance level of 0.05 was fixed previously to the statistical analysis of the study data with the freeware statistical software R [36]. A figure to illustrate the estimated right carotid adventitial VV mean and its confidence intervals was performed to show the relationship with hemoglobin, once adjusted by all the significant variables and their interactions.

3. Results

Baseline characteristics of the study populations, controls (C) and patients with chronic kidney disease at stages 3-4 and 5D, are shown in Table 1. According to disease characteristics, subjects affected by CKD were significantly older than subjects of the control group ($p = 0.001$, CKD 3-4 versus C; $p < 0.001$, CKD 5D versus C). Moreover, as expected from the inclusion criteria of the selected control population with none of the classical risk factors for cardiovascular disease and according to the disease characteristics, body mass index (BMI, $p = 0.013$, CKD 3-4 versus C), waist circumference (WC, $p < 0.001$, CKD 3-4 versus C; $p = 0.017$, CKD 5D versus C), serum glucose ($p = 0.004$, CKD 3-4 versus C), triglycerides (TG, $p < 0.001$, both CKD groups versus C), systolic blood pressure (SBP, $p < 0.001$, both CKD groups versus C), and diastolic blood pressure (DBP, $p < 0.001$, CKD 3-4 versus C), as well as serum C-reactive protein (CRP, $p < 0.001$, CKD 5D versus C), were significantly higher in patients with CKD. Additionally, serum 25-hydroxyvitamin D (25D) levels and LDL cholesterol were lower in the CKD 5D group than in the control group ($p < 0.001$ and $p = 0.018$, resp.). On the other hand, serum VEGF levels were significantly higher in both CKD groups than in control subjects ($p = 0.004$ and $p = 0.013$). Finally, dialysis patients showed, in comparison with nondialysis CKD patients, a significantly higher CRP ($p = 0.001$) and lower levels of 25D ($p < 0.001$).

The analysis of the cIMT and of the carotid adventitial VV showed that, after adjusting by the natural atherogenic factor age, CKD 5D patients had a higher cIMT only in the right carotid artery (Table 2: $p = 0.002$ and $p = 0.043$, CKD 5D versus C and CKD 3-4, resp.), while the adventitial VV were significantly higher only in the right carotid artery of CKD patients at stages 3-4 (Table 2: $p = 0.007$, CKD 3-4 versus C)

Tables 3 and 4 show the unadjusted associations between the adventitial VV and biochemical/anthropometric and

Table 1: Baseline characteristics of the study populations.

	Control ($n = 65$)	CKD 3-4 ($n = 44$)	CKD 5D ($n = 37$)	p overall	p (C versus CKD 3-4)	p (C versus 5D)	p (CKD 3-4 versus 5D)
Age (years)	49 [41.0; 57.0]	59.5 [52.8; 65.0]	60.0 [53.0; 68.0]	*<0.001*	0.001	<0.001	0.214
Sex							
Man	30 (46.2%)	27 (61.4%)	16 (43.2%)	0.189#			
Women	35 (53.8%)	17 (38.6%)	21 (56.8%)				
BMI (Kg/m^2)	24.8 [22.9, 26.3]	27.8 [24.0; 30.7]	25.9 [23.7; 27.9]	*0.010*	0.013	0.130	0.151
WC (cm)*	88.6 ± 9.29	97.3 ± 12.4	94.8 ± 10.0	*<0.001*	<0.001	0.017	0.533
Glucose (mg/dL)	91.0 [87.0; 96.0]	100 [90.0; 113]	93.0 [84.0; 102]	*0.009*	0.004	0.928	0.088
Total cholesterol (mg/dL)	183 [162; 195]	181 [165; 206]	172 [148; 198]	*0.353#*			
HDL cholesterol (mg/dL)	54.0 [45.0; 63.0]	50.0 [44.0; 61.0]	48.0 [41.0; 53.0]	*0.087#*			
LDL cholesterol (mg/dL)*	111 ± 23.90	109 ± 28.9	95.3 ± 30.8	*0.022*	0.875	0.018	0.095
TG (mg/dL)	58 [48.0; 76]	128 [80.0; 153]	108 [84; 168]	*<0.001*	<0.001	<0.001	0.857
SBP (mmHg)	121 [113; 132]	143 [134; 158]	140 [130; 158]	*<0.001*	<0.001	<0.001	0.665
DBP (mmHg)*	74.7 ± 7.95	83.3 ± 10.6	78.1 ± 12.6	*<0.001*	<0.001	0.254	0.061
CRP (mg/L)	0.82 [0.50; 1.96]	1.38 [0.78; 3.65]	4.13 [2.27; 9.80]	*<0.001*	0.099	<0.001	0.001
25D (ng/mL)	25.7 [20.2; 30.4]	28.1 [20.4; 33.0]	12.7 [8.80; 20.7]	*<0.001*	0.556	<0.001	<0.001
VEGF (pg/mL)	233 [112; 372]	398 [230; 599]	373 [219; 507]	*0.002*	0.004	0.013	0.595

*Identified variables with normal distribution. Values of these variables are described with mean ± standard deviation. For variables with significant deviations from normal distribution, values are provided as median [IQR]. #Adjusted p values in multiple comparisons are not performed for variables with nonsignificant differences according to the overall test (p overall). BMI: body mass index; WC: waist circumference; TG: triglycerides; SBP: systolic blood pressure; DBP: diastolic blood pressure; CRP: C-reactive protein; 25D: 25-hydroxyvitamin D; VEGF: Vascular Endothelial Growth Factor.

Table 2: Carotid IMT and carotid adventitial VV values in control subjects and patients affected by chronic kidney disease.

		Control ($n = 65$)	CKD 3-4 ($n = 44$)	CKD 5D ($n = 37$)	LRT p	p (C versus CKD 3-4)	p (C versus 5D)	p (CKD 3-4 versus 5D)
A	Right cIMT*	0.63 ± 0.11	0.71 ± 0.15	0.78 ± 0.14	<0.001[a] (0.006[ab])	0.005[a] (0.248[ab])	<0.001[a] (0.002[ab])	0.028[a] (0.043[ab])
	Left cIMT*	0.66 ± 0.13	0.72 ± 0.15	0.79 ± 0.16	<0.001 (0.055[b])	0.030	<0.001	0.053
B	Right VV	0.59 [0.44; 0.70]	0.69 [0.56; 0.80]	0.62 [0.52; 0.73]	0.011[a] (0.022[ab])	0.003[a] (0.007[ab])	0.227[a] (0.295[ab])	0.147[a] (0.147[ab])
	Left VV	0.56 [0.49; 0.66]	0.58 [0.50; 0.68]	0.53 [0.42; 0.66]	0.606[a#] (0.487[ab])			

*Identified variables with normal distribution. Descriptive values are expressed as mean ± SD or median [IQR] depending on their normal distribution according to Shapiro-Wilks test. #Adjusted p values in multiple comparisons are not performed for variables with nonsignificant differences according to the overall test (p overall). The column "LRT p" refers to the likelihood ratio test p value measuring the overall differences among groups by comparing the models with and without group identification. [a]Box-Cox transformation of the dependent variable to get normally distributed residuals from the multivariate linear regression analysis. [b]Age adjusted p value in linear regression analysis.

clinical variables known to be potentially involved in the development of atheromatosis. As shown in Table 3, in CKD patients at stages 3-4, the adventitial VV of both carotid arteries did not correlate with any of the studied variables. On the other hand, in patients at CKD stage 5D, the right adventitial VV positively and monotonically associated with serum glucose and HDL cholesterol ($p = 0.0089$ and $p = 0.0073$, resp.), while the left adventitial VV negatively associated with serum calcium ($p = 0.0072$) and positively associated with albumin ($p = 0.0392$). Nevertheless, when an age-adjusted linear regression model was applied, significantly higher right adventitial VV were identified only for the second and the third tertile of glucose levels (90–104 and 105–227 mL/dL; $p = 0.0241$ and $p = 0.0099$, resp.). This association is consistent with the right adventitial VV significant association with diabetes in dialysis patients (Table 4: $p = 0.038$).

Table 3: Association between adventitial VV and biochemical and anthropometric variables in CKD patients.

	CKD 3-4				CKD 5D			
	Right VV		Left VV		Right VV		Left VV	
	Correlation	p value	Correlation	p value	Correlation	p value	Correlation	p value
Age (years)	0.0666	0.6913	−0.1730	0.3279	−0.0788	0.6790	0.2077	0.2708
WC (cm)	0.1106	0.5084	−0.0870	0.6245	−0.0627	0.7466	−0.1939	0.3064
BMI (Kg/m^2)	0.1226	0.4635	−0.0212	0.9051	0.2107	0.2636	−0.1618	0.3931
Glucose (mg/dL)	0.2134	0.2183	−0.2012	0.2777	**0.4691**	**0.0089**	0.0726	0.7029
Cholesterol (mg/dL)	0.0507	0.7722	0.1797	0.3335	0.3547	0.0545	−0.2098	0.2658
HDL cholesterol (mg/dL)	−0.2872	0.0943	−0.1220	0.5133	**0.5044**	**0.0073**	0.1116	0.5871
LDL cholesterol (mg/dL)*	0.1696	0.3301	0.0217	0.9076	0.2486	0.2111	−0.1562	0.4461
TG (mg/dL)	0.1711	0.3259	0.3021	0.0986	0.0974	0.6088	−0.1953	0.3009
SBP (mmHg)	0.0165	0.9215	−0.3020	0.0826	−0.1072	0.5945	−0.1881	0.3378
DBP (mmHg)*	−0.1329	0.4262	−0.2240	0.2029	−0.0542	0.7885	−0.3296	0.0868
CRP (mg/L)	0.2230	0.3169	0.1123	0.6471	−0.0024	0.9898	0.0265	0.8898
Ca (mg/dL)*	0.0117	0.9468	0.0306	0.8702	0.0031	0.9869	**−0.4805**	**0.0072**
P (mg/dL)	−0.0686	0.6943	−0.0157	0.9331	−0.2310	0.2193	−0.1963	0.2986
PTH (pg/mL)	−0.3066	0.1649	−0.1738	0.4768	−0.1056	0.6000	−0.2124	0.2767
25D (ng/mL)	0.0902	0.6175	0.0874	0.6519	0.1395	0.4621	0.1600	0.3984
1,25D (pg/mL)	0.0089	0.9656	0.1404	0.5439	−0.2132	0.2667	0.0175	0.9311
Albumin (mg/dL)*	−0.0547	0.7586	0.0710	0.7092	0.1052	0.5802	**0.3785**	**0.0392**
Ferritin (mg/dL)	0.0378	0.8680	−0.2115	0.3847	−0.2234	0.2354	−0.0476	0.8096
Hemoglobin (g/dL)*	0.0572	0.7518	−0.0715	0.7123	−0.3118	0.0935	0.0661	0.7286
Fibrinogen (g/L)	0.3145	0.0966	−0.0017	0.9934	0.0913	0.6439	0.1483	0.4515
Leukocytes (10^6/L)	0.2373	0.1835	−0.2228	0.2454	0.1861	0.3429	0.0889	0.6658
VEGF (pg/mL)	0.0645	0.7037	−0.3152	0.0740	0.2779	0.1370	0.2934	0.1224

*Identified variables with normal distribution (Pearson's correlation). For variables with significant deviations from normal distribution, Spearman's correlation was applied. Statistically significant p values are indicated in bold. WC: waist circumference; BMI: body mass index; TG: triglycerides; SBP: systolic blood pressure; DBP: diastolic blood pressure; CRP: C-reactive protein; Ca: serum calcium; P: serum phosphorus; PTH: parathyroid hormone; 25D: 25-hydroxyvitamin D; 1,25D: 1α,25-dihydroxyvitamin D; VEGF: Vascular Endothelial Growth Factor.

Since this was the main goal of the study, we further explored if there were differences between the two CKD groups in any of the characteristic variables of the disease (Table 5). As a consequence of the disease stages, phosphorus (P), ferritin, and PTH were higher in dialysis patients ($p < 0.001$; $p = 0.001$; $p < 0.001$, resp.). On the other hand, serum calcium (Ca), 1,25D, and albumin were lower in dialysis patients ($p = 0.001$; $p < 0.001$ and $p < 0.006$, resp.). As expected, CKD patients on dialysis, due to the high incidence of anemia, required more treatment with erythropoiesis stimulating agents (ESA) and/or iron than nondialysis patients ($p < 0.001$ and $p = 0.001$, resp.), while the level of hemoglobin was lower than that in the nondialysis patients ($p < 0.001$).

Finally, an adjusted linear regression model was fitted in order to explore how the variables involved in angiogenesis and CKD stage could contribute to the increase in the adventitial VV content (Table 6). Specifically, significant differences in the carotid adventitial VV were determined by the hemoglobin levels depending on whether or not patients were on dialysis. As shown in Figure 1, the adjusted mean right VV (RVV) was estimated to be statistically higher in CKD nondialysis patients with hemoglobin levels of 12.5 mg/dL or above than in CKD nondialysis patients with hemoglobin levels below 12.5 mg/dL (0.91 versus 0.54; $p = 0.0104$). Furthermore, for hemoglobin levels of 12.5 mg/dL or above, CKD nondialysis patients showed an estimated mean RVV higher than that for CKD dialysis patients (0.91 versus 0.66; $p = 0.0008$).

In addition, two more variables significantly contributed to the model: 1,25D and P (Table 6). Specifically, the levels of 1,25D were related to the right carotid adventitial VV only for those patients with low P levels (below its median value). Consequently, the estimated mean RVV was statistically different for CKD patients with low 1,25D and P below 4 mg/dL compared to that of the patients with P levels above 4 mg/dL (0.54 versus 0.36; $p = 0.039$).

Furthermore, since ESA treatment is a recognized angiogenic factor and it is associated with being on dialysis, the difference between ESA users and nonusers in both CKD

Table 4: Association between adventitial VV and clinical dichotomous variables in CKD patients.

	Right VV				Left VV			
	CKD 3-4		CKD 5D		CKD 3-4		CKD 5D	
	Median [IQR]	p value	Median [IQR]	p value	Median [IQR]	p value	Median [IQR]	p value
Sex								
Man	0.69 [0.58; 0.81]	0.586	0.65 [0.54; 0.74]	0.645	0.60 [0.55; 0.66]	0.275	0.52 [0.44; 0.78]	0.708
Woman	0.68 [0.48; 0.78]		0.61 [0.51; 0.70]		0.56 [0.46; 0.65]		0.55 [0.42; 0.61]	
Diabetes								
No	0.65 [0.53; 0.79]	0.397	0.56 [0.51; 0.69]	**0.038**	0.59 [0.50; 0.66]	0.881	0.52 [0.43; 0.64]	0.604
Yes	0.73 [0.65; 0.88]		0.70 [0.68; 0.75]		0.57 [0.53; 0.65]		0.57 [0.46; 0.65]	
Hypertension								
No	0.86 [0.60; 0.90]	0.188	0.66 [0.55; 0.81]	0.534	0.69 [0.58; 0.79]	0.661	0.50 [0.47; 0.76]	0.604
Yes	0.67 [0.51; 0.78]		0.59 [0.53; 0.71]		0.58 [0.51; 0.65]		0.54 [0.42; 0.64]	
Dyslipidemia								
No	0.66 [0.58; 0.78]	0.921	0.57 [0.54; 0.75]	0.698	0.65 [0.56; 0.74]	0.155	0.55 [0.48; 0.60]	0.860
Yes	0.70 [0.50; 0.81]		0.63 [0.51; 0.69]		0.57 [0.48; 0.61]		0.50 [0.42; 0.80]	
Smoking status								
Nonsmoker	0.70 [0.56; 0.80]		0.63 [0.52; 0.76]		0.56 [0.49; 0.61]		0.55 [0.42; 0.67]	
Former smoker	0.53 [0.46; 0.64]	0.062	0.70 [0.54; 0.74]	0.460	0.68 [0.62; 0.79]	0.068	0.53 [0.48; 0.71]	0.450
Smoker	0.79 [0.71; 0.84]		0.56 [0.53; 0.57]		0.64 [0.59; 0.74]		0.47 [0.44; 0.49]	

Differences in the right or the left adventitial VV distribution depending on sex, diabetes, hypertension, dyslipidemia, and smoking in nondialysis and dialysis patients.

populations was explored: no differences in the right VV associated with the use of ESA. Moreover, no significant interaction between ESA use and hemoglobin levels associated with the right carotid adventitial VV (data not shown).

4. Discussion

This study demonstrated that only the right carotid artery (CA) is characterized by higher cIMT and adventitial VV in CKD patients than in control subjects. In addition, of high relevance from the nephrologist's point of view, this study identified hemoglobin as the factor that, at levels of 12.5 g/dL or above, determines the highest estimated adventitial VV of the right carotid artery in CKD patients at stages 3-4.

Specifically, in our study population, after adjusting for age, a natural atherogenic factor, the highest cIMT, was observed only in the right carotid artery of CKD patients undergoing dialysis, while the highest adventitial VV content was observed in CKD patients at stages 3-4 suggesting the carotid adventitial VV neoangiogenesis as the earliest step in the intima-media thickening. The difference in cIMT observed in the right carotid artery, but not in the left, is in accordance with several clinical studies in which asymmetrical differences between the left and the right cIMT are modulated by altered biochemical and hemodynamic parameters [37, 38]. For instance, Chaubey et al.'s findings demonstrated that the difference in cIMT, higher in the left than in the right carotid artery in normotensive subjects, is attenuated when considering subjects with a mean blood pressure higher than 90 mmHg. Moreover, divergent values of the right and the left cIMT could be explained by the fact that the left cIMT thickens every ten years after the age of 35, while the same trend occurs in the right cIMT ten years later and it is correlated with hemodynamic parameters as observed by Luo and colleagues [39]. Importantly, our results demonstrated that, at earlier stages of CKD, the adventitial VV content is also higher only in the right carotid artery in comparison with that in the control population, probably due to similar hemodynamic changes such as shear stress. Previous studies in healthy individuals without classical risk factors for atheromatosis demonstrated that the left carotid adventitial VV correlated with age and the left cIMT, explaining in part the earliest appearance of atheromatosis lesions in the left carotid artery [30, 40]. Taken together, these results underline the importance of differentiating between the left and the right carotid artery not only in control subjects or in the general population but also in patients with known cardiovascular risk factors which could determine heterogeneous atheromatosis in different territories of the vascular bed. Indeed, although the differences in the two carotid arteries are scarcely investigated, some works reported how local factors, mainly the geometry of the carotid artery, may play a role in the heterogeneous atherosclerotic lesion localization due to different blood flow patterns that influence changes in the shear stress [41, 42].

Unfortunately, the role of the carotid adventitial VV in CKD patients and the factors that modulate its content are still unknown. Consequently, the aim of this study was to explore not only the content of adventitial VV but also how angiogenic factors that are modified in CKD could have been involved in its increase. Therefore, since cIMT and adventitial VV were higher in the right carotid artery of the overall CKD population in parallel with higher levels of inflammation, TG, glucose, and blood pressure, as well as with lower levels of vitamin D, we evaluated the relationship between these variables and VV content in both dialysis and nondialysis

Table 5: Clinical and biochemical differences between nondialysis and dialysis CKD patients.

	CKD 3-4 (n = 44)	CKD 5D (n = 37)	p overall
Diabetes			
No	35 (79.5%)	30 (81.1%)	1.000
Yes	9 (20.5%)	7 (18.9%)	
Hypertension			
No	5 (11.4%)	7 (18.9%)	0.522
Yes	39 (88.6%)	30 (81.1%)	
Dyslipidemia			
No	11 (25.0%)	14 (37.8%)	0.315
Yes	33 (75.0%)	23 (62.2%)	
Etiology			
Diabetes	4 (9.09%)	4 (10.8%)	
Glomerular	6 (13.6%)	12 (32.4%)	
Interstitial	9 (20.5%)	9 (24.3%)	0.296
Polycystic	7 (15.9%)	4 (10.8%)	
Vascular	8 (18.2%)	3 (8.11%)	
Unknown	10 (22.7%)	5 (13.5%)	
Plaques			
No	21 (47.7%)	13 (35.1%)	0.359
Yes	23 (52.3%)	24 (64.9%)	
Right plaques			
No	25 (56.8%)	17 (45.9%)	0.452
Yes	19 (43.2%)	20 (54.1%)	
Left plaques			
No	27 (61.4%)	21 (56.8%)	0.847
Yes	17 (38.6 %)	16 (43.2%)	
Smoking			
Nonsmoker	15 (50%)	23 (62.2%)	
Former smoker	7 (23.3%)	8 (21.6%)	0.517
Smoker	8 (26.7%)	6 (16.2%)	
Patients on ESA treatment			
No	42 (95.5%)	5 (14.7%)	**<0.001**
Yes	2 (4.5%)	29 (85.3%)	
NESP dose (μg/kg/week)	0.64 [0.62; 0.66]	0.33 [0.17; 0.57]	0.198
Patients on iron treatment			
No	39 (88.6%)	17 (51.5%)	
Yes	5 (11.4%)	16 (48.5%)	**0.001**
Dose (g/month)	105 [100; 105]	102 [100; 250]	
Ca (mg/dL)*	9.22 ± 0.41	8.84 ± 0.56	**0.001**
P (mg/dL)	3.62 [3.22; 4.29]	4.16 [3.71; 5.38]	**<0.001**
PTH (pg/mL)	8.80 [6.80; 13.8]	29.7 [17.1; 47.7]	**<0.001**
1,25D (pg/mL)	38.5 [21.0; 45.5]	5.90 [5.90; 10.2]	**<0.001**
Albumin (mg/dL)*	4.46 ± 0.32	4.25 ± 0.32	**0.006**
Ferritin (mg/dL)	156 [59.2; 195]	293 [143; 468]	**0.001**
Hemoglobin (g/dL)*	13.8 ± 1.79	12.4 ± 1.15	**<0.001**
Fibrinogen (g/L)	4.25 [3.82; 4.68]	4.40 [4.10; 5.10]	0.232
Leukocytes (10^6/L)	6.29 [5.32; 8.58]	5.77 [4.46; 6.98]	0.056
REGICORE	4.00 [3.00; 5.50]	2.50 [1.75; 5.25]	0.283
Score	1.00 [1.00; 2.00]	1.00 [0.00; 2.00]	0.374

*Identified variables with normal distribution. Values of these variables are described with mean ± standard deviation. For variables with significant deviations from normal distribution, values are provided as median [IQR]. Statistically significant values are indicated in bold. NESP: darbepoetin; Ca: serum calcium; P: serum phosphorus; PTH: parathyroid hormone; 1,25D: 1α,25-dihydroxyvitamin D; REGICORE: REgistre GIroní del COR (cardiovascular risk chart).

Table 6: Adjusted association between RVV and dialysis in CKD patients.

Coefficients	Estimate	Std. error	Pr (>t)
(Intercept)	0.53955	0.14587	0.000623
Hb (g/dL) [11.5, 12.5)	0.11525	0.14998	0.446544
Hb (g/dL) ≥ 12.5	0.36840	0.13735	0.010411
Dialysis	0.17840	0.14382	0.221679
Hb (g/dL) [11.5, 12.5): dialysis	−0.04890	0.17228	0.777940
Hb (g/dL) ≥ 12.5: dialysis	−0.42202	0.15385	0.008912
1,25D (pg/mL) [12.1, 66.0]	−0.17625	0.08000	0.033131
P (mg/dL) [4.00, 7.31]	−0.10963	0.06917	0.120476
1,25D (pg/mL) [12.1, 66.0]: P (mg/dL) [4.00, 7.31]	0.24083	0.10556	0.027658

Multiple R-squared: 0.3592. Hb was recoded into three levels according to values 11.5 and 12.5 based on its relationship with RVV. Levels of 1,25D were recoded into two levels according to their median in the subsample without missing values in the variables of the model. Hb: hemoglobin; 1,25D: 1α,25-dihydroxyvitamin D; P: serum phosphorus.

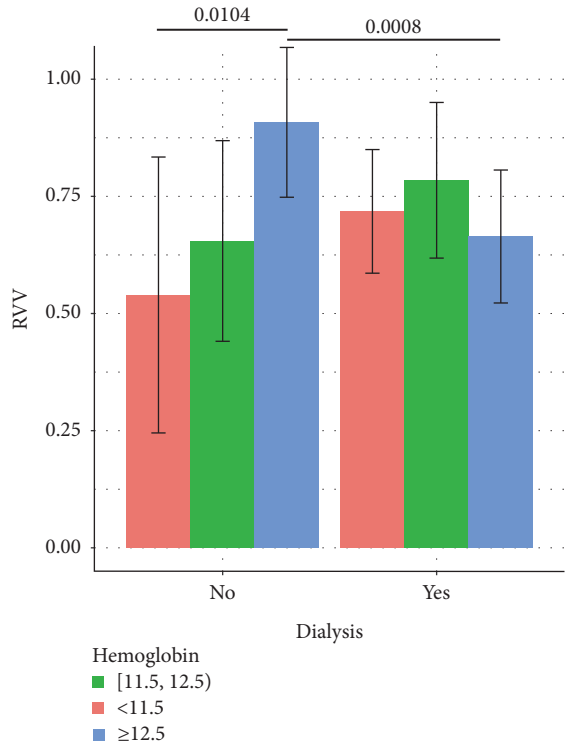

Figure 1: Effect of hemoglobin and dialysis on the right carotid adventitial VV. Hemoglobin at levels of 12.5 g/dL or above determines higher levels of the right carotid adventitial VV (RVV). Bars and error bars represent the mean and the 95% CI of the RVV.

patients. The unadjusted analysis revealed that while the VV of CKD patients at stages 3-4 did not correlate with any of the studied biochemical or anthropometric parameters, all of which are highly involved in the development of atheromatosis, the VV of patients on dialysis was positively associated only with serum glucose levels at the second and third tertile (90–104 and 105–227 mg/dL) and positively associated with diabetes. This result is in accordance with the increase of carotid adventitial VV in type 2 diabetic patients with hyperglycemia even in absence of retinopathy [29]. The lack of association of VV with glucose in CKD patients at stages 3-4 characterized by higher glucose levels suggests that VV increase is driven by distinct factors according to the stage of the disease. Indeed, previous studies have demonstrated that while, in the general population and CKD patients at stage 3, there was a significant interaction between smoking and TG which were independently associated with atheromatosis [43, 44], in patients at advanced stages, this association was lost. Similar results, depending on the stages of the disease and the interaction between biochemical and life habit risk factors, were observed for CRP which only at CKD stages 4-5 was associated with smoking, high phosphate, and atheromatosis only at the highest tertile [44, 45]. Moreover, these results are in agreement with Sampson and colleagues' study that proved no remarkable variations of VV content with respect to the range of measurements of systolic and diastolic blood pressure [33]. On the contrary, our results do not coincide with experimental evidence that supports a role of LPS-induced inflammation and high TG in the increase of adventitial VV measured by CEUS and histological analysis in rabbits with atherosclerosis [46]. Indeed, although CKD patients are characterized by elevated CRP and TG levels, this is a transversal cohort study in which neither time nor levels of exposure of the artery wall to biochemical parameters have been taken into account. Therefore, in accordance with the demonstration that the atheromatous process is driven by specific risk factors according to the CKD stage [44], an adjusted linear regression model was used to identify the variables that could independently predict the increase of the carotid adventitial VV accordingly whether or not patients were on dialysis. This analysis demonstrated that Hb levels interact with the dialysis status. Specifically, in CKD patients at stages 3-4, hemoglobin higher than 12.5 g/dL is a predictor of an estimated higher adventitial VV of the right artery. Although a high percentage of CKD patients were under ESA treatment, especially those being on dialysis, no difference in the adventitial VV content was associated with the use of ESA. Therefore, the hemoglobin status in our CKD nondialysis study population patients could be considered a natural angiogenic mediator. This is of high relevance in nephrology, since anemia greatly influences cardiovascular outcomes. For instance, in Vlagopoulos et al.'s study, in which four community-based trials were pooled,

it was reported that, in CKD patients with anemia, the risk of CVD was 1.7-fold higher [47]. On the other hand, intervention with ESA for the correction of renal anemia might increase occurrence of CVD. In fact, in the CREATE study, the complete correction of anemia did not delay the onset of cardiovascular events [48]. Moreover, in the CHOIR study, target Hb level of 13.5 g/dL in nondialysis CKD patients were associated with increased risk for death, myocardial infarction, and cardiovascular events [48, 49]. Important to support our results is the demonstration by Yilmanz et al. that nondiabetic CKD patients at stages 3-4, not using erythropoietin-based agents, showed a reduction of the flow-mediated dilatation for hemoglobin levels higher that 11.6 g/dL. This is in contrast with the finding of the prospective and observational Dialysis Outcomes and Practice Patterns Study in which the authors showed that the natural increase of Hb to concentration >12 g/dL in hemodialysis patients did not associate with increased mortality, suggesting that high Hb levels per se are not harmful for CKD patients [50].

The interaction of 1,25D and P in the estimation of the adventitial VV of the right carotid artery is intriguing. Indeed, calcitriol is inversely correlated with the carotid adventitial VV and is statistically different only for P levels below its median value (4 mg/dL). This apparent contradiction could be due to the vitamin D supplementation, paricalcitol treatment, and also the P-binding treatment that were not taken into account in the present study.

As has been noted, this limitation of study could be related to the administration of paricalcitol which could have a direct effect on angiogenesis [51], inflammation [52] and bone mineral metabolism, and above all PTH [53], while the serum levels of active vitamin D cannot be measured except for the calcitriol form. Further studies on a larger sample size are needed to clarify the role of 1,25D and P on VV angiogenesis. In addition to the low number of patients, the present study is a cross-sectional study, the reason why there is no indication on how the factors taken into account contributed to a temporal relationship between exposure to these risk factors and the increase of VV. Therefore, a future study should be designed to evaluate the outcomes over time, also taking into account the time of exposure to treatment. Moreover, according to our results, the lack of hemoglobin levels and bone mineral metabolism in the control population impeded the evaluation of the physiological association of these parameters with neoangiogenesis in a healthy population.

Despite these limitations, this study not only confirms the strength of the use of CEUS in measuring adventitial VV as a predictor of the atheromatous process based on the fact that there is a high object evaluation of the imaging (high intraobserver reproducibility) but also is a further confirmation of the importance of separately evaluating the left and the right carotid artery. Moreover, this study, to our knowledge, is the first measuring the carotid adventitial VV in a CKD population and the first indicating that Hb levels and the interaction of 1,25D and P could be associated with the carotid adventitial VV in this population.

In conclusion, this work suggests that, in CKD patients at stages 3-4, hemoglobin levels higher than 12.5 g/dL might be a cause for the increased adventitial VV of the right CA that consequently could increase the right cIMT. Therefore, it could be speculated that the higher incidence of atheromatous disease in CKD patients could be driven by a higher incidence of microangiopathy of the common carotid wall driven by the hemoglobin status. Indeed, this could explain why Hb levels as in the CHOIR study associated with higher cardiovascular events and risk of death [49]. Consequently, as previously specified, a longitudinal study with a higher number of patients is required to evaluate the relationship of Hb levels and the carotid adventitial VV in the onset of the process of atheromatosis and cardiovascular events in CKD patients.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Maria Vittoria Arcidiacono, Mercè Borràs, and Elvira Fernández designed the study. Montserrat Belart, Ana Vilar, Marisa Martín, Lourdes Craver, Àngels Betriu, and Mercè Borràs recruited the study subjects. Maria Vittoria Arcidiacono helped in the acquisition of the B-mode and contrast-enhanced ultrasound. Maria Vittoria Arcidiacono was in charge of the cIMT and adventitial VV reading. Maria Vittoria Arcidiacono and Mercè Borràs drafted the manuscript and Montserrat Martinez-Alonso, Dídac Mauricio, José Manuel Valdivielso, and Elvira Fernández contributed to the final writing, reviewing, and editing. Montserrat Martinez-Alonso, Maria Vittoria Arcidiacono, and Mercè Borràs worked on the statistic section. All the authors read and approved the final version of the manuscript.

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Research Article

The Time Course of Markers of Neutrophil Extracellular Traps in Patients Undergoing Revascularisation for Acute Myocardial Infarction or Stable Angina Pectoris

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Background and Aims. Neutrophil extracellular traps (NETs) have been identified in acute myocardial infarction. We assessed the time profile and association with infarct size for NETs markers in ST-elevation myocardial infarction (STEMI) and stable angina pectoris (AP). *Methods*. In 20 patients with STEMI and 10 with AP undergoing percutaneous coronary intervention (PCI), blood samples were collected before PCI (only AP group) and after 3 and 12 hours, days 1, 3, 5, 7, and 14 for measurement of NETs markers. *Results*. Double-stranded deoxyribonucleic acid (dsDNA) and nucleosome levels were higher in STEMI than AP until day 3 and 12 hours ($p < 0.03$, all). DsDNA declined after day 5 in both groups ($p < 0.04$, all), while nucleosomes declined until day 3 only in the AP group ($p < 0.05$, all). DsDNA correlated with peak troponin T and creatine kinase MB (CKMB) at day 5 ($r = 0.48$, $p = 0.03$, both) and with MRI-measured infarct size at days 5 and 7 ($r = 0.61$, $p = 0.01$ and $r = 0.52$, $p = 0.04$, resp.), while nucleosomes correlated with infarct size at day 5 ($r = 0.58$, $p = 0.02$). *Conclusions*. High levels of NETs markers were observed in STEMI shortly after revascularisation and were partly associated with infarct size. The decline thereafter in both groups indicates a role for NETs in both acute and chronic atherothrombosis.

1. Introduction

Neutrophil cell activation in acute myocardial infarction (MI) has lately gained attention. Quantitative amounts of neutrophils and several neutrophil granule proteins are suggested to be predictive of infarct size, left ventricular ejection fraction (LVEF), new cardiovascular events, and death after acute MI [1–6]. A decade ago, it became evident that neutrophils upon activation are able to release parts of their nuclear content with residing neutrophil granule proteins into the extracellular space to form spindle-like networks called neutrophil extracellular traps (NETs) [7]. Although NETs initially were thought to have their main role in infectious diseases, ensuring entrapment of microorganisms in areas with high concentrations of antimicrobial proteins [7], NETs have lately been identified in coronary artery disease (CAD) [8–10].

In acute MI, high levels of circulating cell-free deoxyribonucleic acid (DNA), a surrogate marker of NETs, have been reported [11–15] and also linked to infarct size [8, 13, 15]. NETs are also suggested to be present in coronary thrombi [8, 9], in mural atherosclerotic plaques [16, 17], and in stable angina pectoris (AP) where they further seem to serve as a predictor of coronary artery severity and risk of new coronary events [10]. By the seemingly wide-ranging prothrombotic properties like platelet entrapment and activation [18], activation of the coagulation system [19, 20], and inhibition of fibrinolysis [21], NETs are potentially important players in the pathogenesis of atherothrombosis.

The dynamic profile of circulating NETs in the acute and subacute phase of ST-elevation MI (STEMI) has not been reported in detail previously. In this study, we aimed to explore the time profile of the circulating surrogate markers of NETs double-stranded deoxyribonucleic acid

(dsDNA) and nucleosomes (DNA-histone complexes) in patients with STEMI or stable AP undergoing coronary angiography with percutaneous coronary intervention (PCI). The time profiles of myeloperoxidase (MPO) and pentraxin 3 (PTX3), well-known neutrophil cell granule proteins, were also investigated in order to explore whether these proteins could be reflected in the measured markers of NETs. We further assessed whether levels of dsDNA, nucleosomes, and MPO were related to indices of myocardial injury and left ventricular function.

2. Material and Methods

2.1. Study Design. Thirty patients with CAD, 20 with STEMI, and 10 with stable AP, admitted to Oslo University Hospital (OUS) Ullevål, Norway, undergoing successful revascularisation with PCI, were included. Details of the study design have been reported previously [22]. In brief, inclusion criteria in the STEMI group were characteristic clinical symptoms, electrocardiographic ST-elevations, and angiographic verification of coronary artery occlusion, while characteristic clinical symptoms and CAD angiographically suitable for PCI were inclusion criteria in the AP group. Exclusion criteria in both groups were previous transmural infarction, cardiogenic shock, and serious comorbidity. All patients were medically treated according to current guidelines and gave written informed consent for study participation. The study protocol was approved by the Regional Committee for Medical Research Ethics and conforms to the ethical guidelines of 1975 Declaration of Helsinki.

2.2. Blood Sampling. Blood samples were collected by standard venipuncture immediately before PCI in the AP group and after 3 and 12 hours, days 1, 3, 5, 7, and 14 in both groups. All blood samples from day 1 and further were obtained in fasting state before intake of morning medication. Routine analyses were obtained by conventional methods. Serum was prepared by centrifugation within 1 hour at 2500 ×g for 10 min and EDTA plasma was prepared by centrifugation within 1 hour at 2500 ×g for 20 minutes at 4°C, both stored at −80°C until analysed.

2.3. Laboratory Analyses. Levels of dsDNA and nucleosomes were quantified in serum by use of Quant-iT Picogreen dsDNA Assay # P11496 (Invitrogen, Carlsbad, CA, USA) and ELISA Cell Death Detection kit # 11774425001 (Roche Diagnostics, Indianapolis, USA), respectively. Optical density values in the nucleosome assay were normalized to an internal positive control and expressed as arbitrary units of nucleosomes per milliliter (NU/mL). All related samples from one patient and samples from both groups were analysed on the same plate in order to reduce the significance of interassay variability. Levels of MPO were measured in EDTA plasma by ELISA (Mercodia AB, Uppsala, Sweden). Interassay coefficient of variation (CV) for the measurements of dsDNA, nucleosomes, and MPO were 2.5%, 28.7%, and 6.3%, respectively.

Myocardial injury was assessed by peak serum levels of troponin T (reference value < 0.03 μg/L) and creatine kinase MB (CKMB) (reference value < 5 μg/L) and by gadolinium late contrast enhancement technique based infarct size (%) measured by magnetic resonance imaging (MRI) after 6 weeks. Left ventricular function was assessed by left ventricular ejection fraction (LVEF) by MRI after 6 weeks. MRI measures were available in 16 patients.

2.4. Statistical Analyses. As the majority of variables were skewly distributed, nonparametric statistics were used throughout. Group differences were assessed by Mann-Whitney *U* Test or Fisher's Exact Test as appropriate, while overall change within a group and change between two time points within a group were assessed by Friedman Test and Wilcoxon Signed Rank Test, respectively. Correlation analyses were performed by Spearman rho. Due to the hypothesis generating nature of the study, Bonferroni corrections for multiple comparisons were not performed. The level of significance was set to $p < 0.05$. All statistical analyses were performed by SPSS software package, version 23.0.

3. Results

Patient characteristics at baseline are shown in Table 1. Beyond more established CAD in the stable AP group and thus more frequent use of secondary prophylactic cardiovascular drugs, the groups were comparable.

3.1. The Time Profile of Circulating Markers of NETs

3.1.1. Double-Stranded Deoxyribonucleic Acid (dsDNA). Levels of dsDNA were significantly higher in the STEMI group than in the AP group at all time points until day 3 ($p < 0.03$ for all) (Figure 1(a)).

Within both groups, a significant overall change in dsDNA levels was observed ($p \leq 0.02$), with values declining from 3 hours in the STEMI group and from baseline in the stable AP group, respectively, to day 5 and all later time points ($p < 0.04$ for all) (Figure 1(a)).

3.1.2. Nucleosomes (DNA-Histone Complexes). Levels of nucleosomes were significantly higher in the STEMI group compared to the stable AP group at 3 and 12 hours ($p < 0.03$ for both) (Figure 1(b)).

No significant overall change or change from 3 hours to later time points was observed in the STEMI group. In the stable AP group, levels declined from baseline until day 3 ($p < 0.05$ for all), although no significant overall change was observed (Figure 1(b)).

3.2. The Time Profile of MPO. No difference between the groups was observed for levels of MPO at any time point (Figure 1(c)).

In both groups, a significant overall change was observed ($p < 0.01$) with declining levels from 3 hours to all later time points ($p < 0.01$ for all). Within the stable AP group,

Table 1: Clinical characteristics of the study population.

	Acute MI group $n = 20$	Stable AP group $n = 10$	p
Age (yrs)	60 (54–68)	64 (54–71)	ns
Female gender	5	1	ns
Established CAD	0	7	<0.01
Hypertension	7	4	ns
Diabetes mellitus (type 1 or 2)	2	2	ns
Previous or current smoking	10	3	ns
Medication at study inclusion			
Acetylsalicylic acid	1	7	<0.01
Clopidogrel	0	0	ns
ACE/AT II antagonists	5	2	ns
Beta-blocker	2	4	0.02
Aldosterone antagonist	0	0	ns
Insulin	0	0	ns
Diuretics	0	0	ns
Statins	1	7	<0.01
Medication at hospital discharge			
Acetylsalicylic acid	20	10	ns
Clopidogrel	20	9	ns
ACE/AT II antagonists	11	2	ns
Beta-blocker	18	6	ns
Aldosterone antagonist	0	0	ns
Insulin	0	0	ns
Diuretics	1	0	ns
Statins	20	10	ns
Indices of infarct size and left ventricular function			
Peak troponin T (μg/L)	3.8 (2.1–6.1)		
Peak CKMB (μg/L)	158 (93–268)		
Infarct size (MRI, %)[a]	6.6 (3.2–10.7)		
LVEF (MRI, %)[a]	58 (53–66)		

Values are given as numbers or medians (25–75 percentiles) unless otherwise stated. CAD: coronary artery disease, defined as previous angina, Q- or non-Q infarction, percutaneous intervention (PCI), or coronary artery bypass grafting (CABG). ACE/AT II antagonists: angiotensin converting enzyme/angiotensin II antagonists. CKMB: creatine kinase MB. LVEF: left ventricular ejection fraction. [a]Measured 6 weeks after study inclusion.

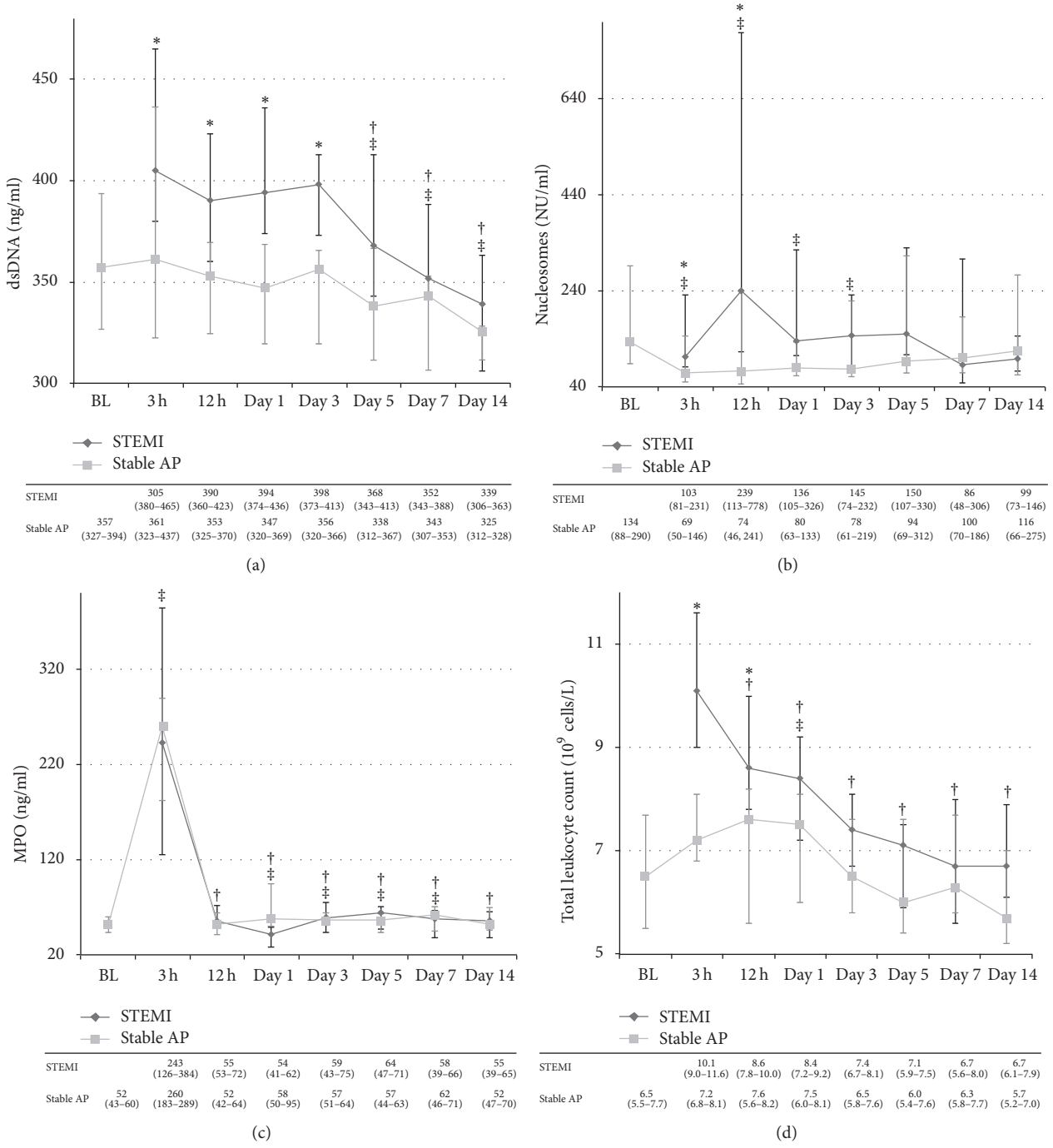
a significant increase from baseline to 3 hours was observed, sustained elevated at days 1, 3, 5, and 7 ($p \leq 0.03$ for all) (Figure 1(c)).

3.3. Correlations between Markers of NETs and Neutrophil Proteins. In the total cohort, dsDNA and nucleosome levels intercorrelated significantly at the majority of time points (Table 2). Levels of MPO did not correlate with either dsDNA or nucleosome levels beyond a negative correlation to nucleosomes at 3 hours ($r = -0.43$, $p = 0.02$). Previously, we have investigated the time profile of pentraxin 3 (PTX3), another neutrophil granule protein and thus potential NETs component in the same cohort and observed that PTX3 levels were elevated shortly after PCI [23]. PTX3 levels did not

Table 2: Correlation between levels of dsDNA and nucleosomes at corresponding time points in the total study population ($n = 30$).

	R	p
BL	0.17	ns
3 hours	0.51	<0.01
12 hours	0.63	<0.01
Day 1	0.40	0.03
Day 3	0.28	ns
Day 5	0.53	<0.01
Day 7	0.21	ns
Day 14	0.50	<0.01

R: Spearman rho. BL: baseline.



	BL	3 h	12 h	Day 1	Day 3	Day 5	Day 7	Day 14
STEMI		305 (380–465)	390 (360–423)	394 (374–436)	398 (373–413)	368 (343–413)	352 (343–388)	339 (306–363)
Stable AP	357 (327–394)	361 (323–437)	353 (325–370)	347 (320–369)	356 (320–366)	338 (312–367)	343 (307–353)	325 (312–328)

(a)

	BL	3 h	12 h	Day 1	Day 3	Day 5	Day 7	Day 14
STEMI		103 (81–231)	239 (113–778)	136 (105–326)	145 (74–232)	150 (107–330)	86 (48–306)	99 (73–146)
Stable AP	134 (88–290)	69 (50–146)	74 (46, 241)	80 (63–133)	78 (61–219)	94 (69–312)	100 (70–186)	116 (66–275)

(b)

	BL	3 h	12 h	Day 1	Day 3	Day 5	Day 7	Day 14
STEMI		243 (126–384)	55 (53–72)	54 (41–62)	59 (43–75)	64 (47–71)	58 (39–66)	55 (39–65)
Stable AP	52 (43–60)	260 (183–289)	52 (42–64)	58 (50–95)	57 (51–64)	57 (44–63)	62 (46–71)	52 (47–70)

(c)

	BL	3 h	12 h	Day 1	Day 3	Day 5	Day 7	Day 14
STEMI		10.1 (9.0–11.6)	8.6 (7.8–10.0)	8.4 (7.2–9.2)	7.4 (6.7–8.1)	7.1 (5.9–7.5)	6.7 (5.6–8.0)	6.7 (6.1–7.9)
Stable AP	6.5 (5.5–7.7)	7.2 (6.8–8.1)	7.6 (5.6–8.2)	7.5 (6.0–8.1)	6.5 (5.8–7.6)	6.0 (5.4–7.6)	6.3 (5.8–7.7)	5.7 (5.2–7.0)

(d)

Figure 1: Time profiles of the NETs markers, myeloperoxidase, and leukocyte count. (a) Double-stranded deoxyribonucleic acid (dsDNA), (b) nucleosomes (DNA-histone complexes), (c) myeloperoxidase (MPO), and (d) total leukocyte count. The various time points (x-axis) and levels (y-axis) in the ST-elevation myocardial infarction (STEMI) and stable angina pectoris (AP) groups, as well as between- and within-group comparisons. Values are given as median (25–75 percentiles). BL: baseline. $^{*}p < 0.05$ for between-group difference at the various time points. $^{\dagger}p < 0.05$ for within-group difference from 3 hours in the STEMI group. $^{\ddagger}p < 0.05$ for within-group difference from baseline in the stable AP group.

correlate significantly with either dsDNA or nucleosomes at any time point (data not shown).

3.4. Correlations with Total Leukocyte Count. In the total cohort, levels of dsDNA correlated positively with total leukocyte count at the majority of time points while the nucleosomes correlated to a lesser degree. Levels of MPO correlated with total leukocyte count only at day 14 (Table 3). The correlations were less obvious when analyzing the groups separately (data not shown), although the time profile of

Table 3: Correlation between markers of NETs, MPO, and total leukocyte count at corresponding time points in the total study population ($n = 30$).

	dsDNA		Nucleosomes		MPO	
	R	p	R	p	R	p
Baseline ($n = 10$)	0.61	ns	0.13	ns	0.06	ns
3 hours	0.42	0.03	0.37	0.05	−0.04	ns
12 hours	0.37	0.05	0.30	ns	0.36	ns
Day 1	0.22	ns	0.45	0.01	0.07	ns
Day 3	0.57	<0.01	0.51	<0.01	0.17	ns
Day 5	0.54	<0.01	0.59	<0.01	0.36	ns
Day 7	0.49	<0.01	0.18	ns	0.09	ns
Day 14	0.39	0.04	0.21	ns	0.47	0.01

R: Spearman rho. BL: baseline.

total leukocyte count in both groups moderately imitated the pattern of dsDNA (Figure 1(d)).

3.5. Correlations with Myocardial Injury. Levels of dsDNA correlated positively with peak levels of troponin T and CKMB at day 5 ($r = 0.48$, $p = 0.03$ for both), while day 5 and 7 levels correlated with infarct size assessed by MRI ($r = 0.61$, $p = 0.01$ and $r = 0.52$, $p \leq 0.04$, resp.) (Figures 2(a)–2(d)). Also, nucleosome levels at day 5 correlated with infarct size by MRI ($r = 0.58$, $p = 0.02$) (Figure 2(e)), while levels of MPO were not correlated with any indices of myocardial injury. Either dsDNA, nucleosome, or MPO levels correlated with LVEF at any time point (data not shown).

4. Discussion

The main findings of this explorative, hypothesis generating study were as follows: (1) the two circulating surrogate markers of NETs, dsDNA and nucleosomes, were higher in patients with STEMI compared to patients with stable AP shortly after PCI, and levels were to a certain degree associated with indices of myocardial injury; (2) dsDNA and nucleosome levels decreased after PCI also in patients with stable AP, indicating that transient coronary ischemia might be associated with production of these NETs markers; (3) circulating levels of MPO did not reflect levels of dsDNA or nucleosomes and were not affected by the STEMI, but apparently by the PCI procedure itself.

Although circulating DNA has been reported to be higher in AMI than in both stable AP and healthy controls [11–15], no studies have to the best of our knowledge reported on the detailed time profile of NETs surrogate markers during STEMI and stable AP. The observed elevated levels of dsDNA and nucleosomes in patients with STEMI shortly after PCI are noticeable and may reflect enhanced neutrophil cell activation, myocardial cell necrosis with subsequent release of nuclear content, or a combination of more events. As strong correlations between dsDNA and nucleosome levels obtained in the subacute phase following PCI and infarct size measured by MRI after 6 weeks were observed, these potential markers of NETs could reflect myocardial cell necrosis directly. The possibility that they also represent neutrophil cell activation is nevertheless present, as strong correlations between circulating total leukocyte count and both dsDNA and nucleosome levels were observed at most time points and because the time profile of total leukocyte count imitated the time profile of dsDNA. Neutrophils have further previously been suggested to be the major source of circulating levels of both dsDNA and nucleosomes [10, 24]. As NETs probably hold prothrombotic properties like platelet activation [18], coagulation activation by factor XII and tissue factor, tissue factor pathway inhibitor (TFPI) suppression [19, 20] and inhibition of fibrinolysis [21], a role in coronary thrombosis would be plausible. As the present sample size was limited and the infarcts were relatively small, possible linkages between the assessed markers of NETs and coronary thrombosis need further exploration.

Elevated levels of dsDNA and nucleosomes shortly after PCI were not limited to the STEMI group, as decreasing levels of both markers also were observed in the stable AP group throughout the study period. In line with our observations, levels of both dsDNA and nucleosomes have previously been reported to associate with the severity of coronary atherosclerosis [10, 12], indicative of a link between AP with coronary ischemia and stimulated production of NETs (so-called NETosis). The pathophysiological properties of NETs in atherosclerosis and thus potentially also in AP have not been explored in detail, but have in murine models been suggested to include stimulation and migration of various innate and adaptive immune cells into the atherosclerotic plaque, partly through macrophage-mediated cytokine release [16, 25] and endothelial dysfunction mediated through metalloproteinases [26]. Given the prothrombotic properties of NETs, it would also be intriguing to speculate whether NETs participate in microthrombosis following intraplaque hemorrhage, a well-established cause of atherosclerotic plaque progression [27].

Levels of MPO, a well-known neutrophil granule protein and a proposed component of NETs [7, 28], were not reflected in neither dsDNA nor nucleosome levels. If dsDNA and nucleosomes indeed are part of NETs, this observation suggests that circulating MPO were mainly not NETs-derived. The lack of association between MPO and

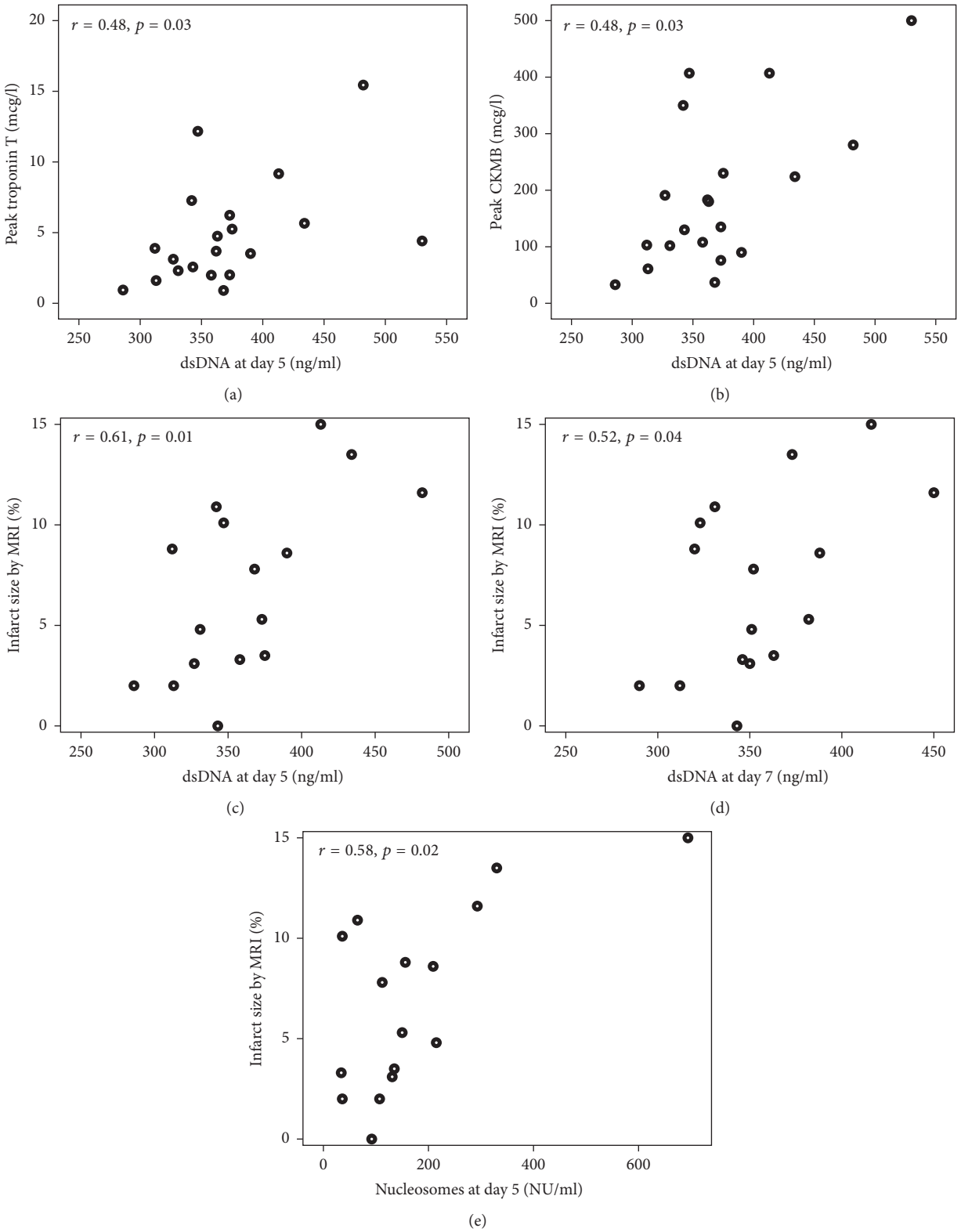


Figure 2: Correlations between NETs markers and indices of infarct size. dsDNA: double-stranded deoxyribonucleic acid. CKMB: creatine kinase MB. MRI: magnetic resonance imaging.

dsDNA/nucleosomes is consistent with that of another neutrophil granule protein, PTX3 [23], suggesting that despite release from neutrophil granules upon neutrophil cell activation, these two proteins did not enter circulating blood simultaneously with dsDNA and nucleosomes and did thus presumably not reflect NETosis in this cohort. Further, while MPO levels were not influenced by the STEMI, increased levels shortly after PCI in the stable AP group may be indicative of a direct effect of the PCI procedure per se. The mechanistic explanation for this apparent PCI-effect is unclear. Lastly, MPO was not correlated with any indices of myocardial injury or left ventricular function in contrast to the previously reported adverse properties of MPO in left ventricular remodeling after AMI [29, 30]. Again, limited sample size and small infarct sizes may have influenced these results.

The discovery of markers of NETs in atherothrombosis is of particular interest as NETs could be subject to therapeutic manipulations, that is, through administration of enzymes able to dissolve the NET structure like deoxyribonucleases (DNAses) or through inhibition of peptidylarginine deiminase 4 (PAD4), an essential enzyme in NETosis. Although in vivo experiments in humans have not been performed so far, adding DNAse to standard thrombolysis (with t-PA) has been shown to accelerate thrombus lysis in human ex vivo thrombi [8] and to reduce myocardial no-flow area, infarct size, and ischemia-reperfusion-induced left ventricular remodeling in rats [31]. Likewise, PAD4 inhibition has been shown to reduce NETosis in both murine and human neutrophils and further to directly interfere with atherosclerotic burden in mice [17, 32, 33].

5. Limitations

Beyond limited sample size and small infarctions as previously described, the hypothesis generating nature of this study carries methodological limitations, as the high CV for the nucleosome analysis. To what extent circulating levels of dsDNA and nucleosomes are specific for NETs is further not clear. Moreover, aspirin, heparin, and statins have all been reported to suppress or destabilise NETs in murine and human models [18, 34–37], which could explain falling levels of dsDNA and nucleosomes in both groups, as well as lower levels generally in the AP group. Finally, as differential counts for neutrophils, the major source of dsDNA [24], were not available, our analyses are based on total leukocyte counts. These are though to a high degree representative for circulating neutrophils.

6. Conclusions

High levels of the NETs markers dsDNA and nucleosomes were observed in patients with STEMI shortly after revascularisation and did partly reflect infarct size. The decline over time, also observed in AP patients, may indicate that transient coronary ischemia also stimulates release of NETs markers. Together, these observations may imply roles for NETs in atherothrombosis.

Disclosure

Study identification number (Regional Committee for Medical Research Ethics) is 126-03032.

Competing Interests

The authors declared no competing interests.

Authors' Contributions

Ragnhild Helseth, Svein Solheim, Harald Arnesen, Ingebjørg Seljeflot, and Trine Baur Opstad all contributed significantly to the conceptualization, methodology, visualization, and writing. Trine Baur Opstad also performed the laboratory analyses, while Ragnhild Helseth performed the formal analyses.

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Research Article

Dyslipidemia and Diabetes Increase the OPG/TRAIL Ratio in the Cardiovascular System

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Background. Dyslipidemia and diabetes are two of the most well established risk factors for the development of cardiovascular disease (CVD). Both of them usually activate a complex range of pathogenic pathways leading to organ damage. Here we hypothesized that dyslipidemia and diabetes could affect osteoprotegerin (OPG) and TNF-related apoptosis-inducing ligand (TRAIL) expression in the vessels and the heart. *Materials and Methods*. Gene and protein expression of OPG, TRAIL, and OPG/TRAIL ratio were quantified in the aorta and the hearts of control mice, dyslipidemic mice, and diabetic mice. *Results*. Diabetes significantly increased OPG and the OPG/TRAIL ratio expression in the aorta, while dyslipidemia was the major determinant of the changes observed in the heart, where it significantly increased OPG and reduced TRAIL expression, thus increasing cardiac OPG/TRAIL ratio. *Conclusions*. This work shows that both dyslipidemia and diabetes affect OPG/TRAIL ratio in the cardiovascular system. This could contribute to the changes in circulating OPG/TRAIL which are observed in patients with diabetes and CVD. Most importantly, these changes could mediate/contribute to atherosclerosis development and cardiac remodeling.

1. Introduction

In industrialized countries, cardiovascular disease (CVD) remains the predominant cause of morbidity and mortality. It is current scientific opinion that CVD occurs as a part of a series of events that form a continuum [1]. The first stage of this continuum corresponds to the presence of risks factors that predispose to tissue injury, such as hypertension, dyslipidemia, and diabetes [2]. These risk factors usually activate a complex range of pathogenic pathways leading to the development of atherosclerosis and ischemic heart disease. Failure to effectively manage any stage of this continuum results in ventricular hypertrophy, heart failure, end-stage heart disease, and cardiovascular death.

One of the molecular pathways that could be activated in response to these risk factors and be involved in CVD development is that of osteoprotegerin (OPG) and TNF-related apoptosis-inducing ligand (TRAIL), as reviewed in [3]. OPG and TRAIL are both members of the TNF superfamily, and they are widely expressed in different tissues, including the heart and the vessels, in health and disease states [4, 5]. Although initially OPG actions seemed to be limited to bone metabolism [6] and those of TRAIL to host defense against tumors [7], recent studies have suggested that both molecules might actually be involved in CVD development and progression [3].

Epidemiological studies have shown that there is an association between circulating OPG, TRAIL, and CVD. OPG was found to be directly correlated [5, 8, 9], while TRAIL was inversely correlated, with cardiovascular morbidity and mortality [10, 11]. Likewise, OPG was significantly associated with the level of C-reactive protein [12], which is an independent predictor of acute vascular events and adverse outcomes, while TRAIL was negatively correlated with it [10]. Recently, Secchiero and colleagues have shown that patients with coronary artery disease have an increased OPG/TRAIL ratio, which further increases in patients developing heart failure [13]. Consistent with this epidemiological data, experimental studies suggest that OPG and TRAIL have opposite actions, as OPG has proinflammatory [12] and proatherogenic effects [14, 15], while TRAIL seems to be anti-inflammatory [16, 17] and antiatherogenic [18–20].

Based on these premises, we hypothesized that dyslipidemia and diabetes, which are two well known risk factors for CVD, could modify the vascular and cardiac expression of OPG and TRAIL, leading to an increased OPG/TRAIL ratio at a tissue level. The aim of this study was to explore the vascular and cardiac changes of OPG and TRAIL in experimental models of dyslipidemia and diabetes.

2. Materials and Methods

2.1. Animal Model and Experimental Protocol. A total of 12 male C57BL/6J (CNT) mice and 24 male apolipoprotein E null ($ApoE^{-/-}$) mice on a C57BL/6J background, aged 6 weeks, were studied for 14 weeks. At 6 weeks of age, $ApoE^{-/-}$ mice were further randomized to saline (ApoE) or streptozotocin (ApoE + DM), which were delivered intraperitoneally in five consecutive daily doses. The daily dose of streptozotocin (STZ) (Sigma-Aldrich) was 55 mg/kg of body weight. After one week, blood glucose was checked and only the mice with blood glucose levels greater than 15 mM were included in the ApoE + DM group. During the study period, all the mice were fed with a standard diet. The animals were kept in a temperature-controlled room (22 ± 1°C) on a 12 h light/12 h dark cycle with free access to food and water and they were fed ad libitum for the length of the study. After 14 weeks, the animals were anesthetized with an intraperitoneal injection of thiobutabarbital sodium (Inactin, Sigma-Aldrich) (60 mg/kg body weight) and sacrificed by exsanguination via cardiac puncture. Bloods and tissues (aortas and hearts) were collected for further analysis.

This study was carried out at the Animal House of the University of Trieste. All the animal experiments were approved by the animal ethics committee of the University of Trieste (ID 28.0.2008) and the Italian Ministry of Health, conforming to the Guide for the Care and Use of Laboratory Animals.

2.2. General Parameters. At the end of the study, we measured body weight and systolic blood pressure (SBP). In particular, SBP was assessed by a computerized noninvasive tail cuff system in conscious mice. Glucose, total cholesterol, HDL cholesterol, and triglycerides were measured in the sera by autoanalyzer technique (Hitachi 917, Tokyo, Japan). Circulating levels of OPG and TRAIL were measured in the sera by ELISA (R&D Systems DuoSet #DY459 and #DY1121).

2.3. Aortic and Cardiac Structural Changes. In half of the animals in each group, aortae were collected and placed in 10% neutral-buffered formalin for atherosclerosis quantification, before being processed for immunohistochemical analyses. In the other half, aortae were snap-frozen in liquid nitrogen and stored at −80°C for RNA extraction and gene expression analyses. Plaque area was quantified as described previously [15]. Briefly, aortae were cleaned of excess fat and stained with Sudan IV-Herxheimer's solution. Then, they were dissected longitudinally and pinned flat onto wax. Images were acquired with a dissecting microscope (Olympus BX-50) equipped with a high-resolution camera (Sony XC-77CE). Digitized images were then evaluated with an image analysis system (Image-Pro Plus 6.3 software, Media Cybernetics). Total plaque area was quantified as the percentage area of aorta stained red.

As for the hearts, they were divided and half was put in formalin and half was put in liquid nitrogen. The part that was fixed in formalin was embedded in paraffin, cut in 4 μm thick sections, and stained with hematoxylin and eosin (H&E) in order to evaluate cardiomyocyte hypertrophy. Cardiomyocyte hypertrophy was assessed by measuring the shortest diameter of 200 cells, which were selected when they showed the spindle-shaped transverse section including the elliptical nucleus. The shortest transverse diameter was measured 3 times per cell, and the values were averaged, as reported by [21]. The other half that was snap-frozen in liquid nitrogen was used either for gene expression analyses or for immunohistochemical stainings.

2.4. Quantitative Real-Time RT-PCR. Gene expression of OPG and TRAIL was determined by real-time quantitative RT-PCR (reverse transcription-polymerase chain reaction) in aortic and cardiac tissue. In order to isolate mRNA from the aorta and the heart, tissue was homogenized and processed as previously reported [22]. Then, mRNA was treated with the DNase inactivation reagent (Ambion DNA-free product #AM-1906), and 3 μg of treated mRNA was subsequently used to synthesize cDNA with Superscript First Strand synthesis system for RT-PCR (Gibco BRL). The gene expression of OPG and TRAIL was analyzed by real-time quantitative RT-PCR using the TaqMan system (Life Technologies) for OPG and the SYBR Green system (Life Technologies) for TRAIL. Fluorescence for each cycle was quantitatively analyzed by an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Gene expression of OPG was normalized to 18S mRNA, while that of TRAIL was normalized to Rps9. Results were reported as ratio compared with the level of expression in untreated controls, which were given an arbitrary value of 1.

2.5. Immunohistochemistry. The presence of OPG and TRAIL in the aortae was evaluated by immunostainings on 4 μm thick paraffin sections. In particular, after antigen retrieval in citrate buffer (pH6) endogenous peroxidase was quenched for 10 minutes using 3% H_2O_2 in PBS. To localize OPG, we used a biotinylated goat anti-OPG antibody (R&D Systems, 1 : 10 dilution) followed by a catalyzed signal amplification system (Dako). To localize TRAIL, we used a monoclonal mouse anti-TRAIL antibody (R&D Systems, 1 : 50 dilution), applied overnight at 4°C. Biotinylated goat anti-mouse immunoglobulins (Vector Laboratories, dilution 1 : 200) were then used as secondary antibody, followed by streptavidin-HRP (Dako).

The presence of OPG and TRAIL in the hearts was evaluated by immunostaining on 5 μm thick frozen sections. After neutralization of endogenous peroxidase, sections were incubated overnight with the goat anti-OPG antibody (R&D Systems, 1 : 10 dilution) and the monoclonal mouse anti-TRAIL antibody (R&D Systems, 1 : 50 dilution). Biotinylated

Table 1: General parameters.

Parameter	CNT	ApoE	ApoE + DM
Body weight (g)	30.89 ± 0.74	33.46 ± 1.40	24.86 ± 0.61*#
LV/BW (mg/g)	3.42 ± 0.09	3.57 ± 0.06	3.29 ± 0.07#
SBP (mmHg)	81.57 ± 8.31	86.11 ± 5.12	87.33 ± 3.78
Fasting glucose (mmol/L)	9.48 ± 1.17	11.94 ± 0.91	54.77 ± 11.73*#
Total cholesterol (mmol/L)	2.60 ± 0.35	11.97 ± 0.92*	21.10 ± 3.94*#
Triglycerides (mmol/L)	0.84 ± 0.19	1.28 ± 0.14*	1.70 ± 0.37*
Cholesterol HDL (mmol/L)	1.91 ± 0.24	1.86 ± 0.15	2.60 ± 0.51

* $p < 0.05$ versus CNT; # $p < 0.05$ versus ApoE; LV, left ventricle; BW, body weight.

immunoglobulins, diluted 1 : 200, were then applied as secondary antibodies, followed by streptavidin-HRP (Dako). In both tissues, the staining was visualized by reaction with 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich). After counterstaining with hematoxylin, all the sections were examined by light microscopy (Olympus BX50WI) and digitized using a high-resolution camera (Q-Imaging Fast 1394). The proportional area of brown staining was measured by an image analysis system (Image-Pro Plus 6.3 software, Media Cybernetics) in order to quantify OPG and TRAIL expression in aortae and hearts.

2.6. Statistical Analysis. Results are expressed as means ± standard error of the mean. Differences in the mean among groups were analyzed using two-way ANOVA. Pairwise multiple comparisons were made using Bonferroni posttest analysis. $p < 0.05$ was considered statistically significant.

3. Results

3.1. General Parameters. The general parameters of the mice are reported in Table 1. ApoE$^{-/-}$ mice had higher levels of total cholesterol and triglycerides as compared to the CNT mice. In line with the study protocol, the mice in the ApoE + DM group were hyperglycemic. Moreover, diabetes was associated with a significant reduction of body weight and a significant increase of total cholesterol as compared to nondiabetic ApoE$^{-/-}$ mice.

3.2. Structural Changes in the Aorta and the Heart. ApoE$^{-/-}$ mice had a significant increase in total plaque area, which increased further after the induction of diabetes (Figures 1(a) and 1(b)). As for cardiac changes, dyslipidemia was associated with a significant increase in cardiomyocyte size (Figures 1(c) and 1(d)), indicating cardiomyocyte hypertrophy, with no further increase after the induction of diabetes.

3.3. OPG and TRAIL Gene Expression in the Aorta and the Heart. In the aorta, the induction of diabetes was associated with a significant increase in OPG gene expression and in the OPG/TRAIL ratio, while dyslipidemia had no effect on OPG/TRAIL ratio as compared to controls (Figure 2(a)). By contrast, in the heart, dyslipidemia was the major determinant of OPG/TRAIL tissue changes, not only by increasing OPG but also by reducing TRAIL gene expression. So dyslipidemia increased significantly cardiac OPG/TRAIL ratio, with no further differences after the induction of diabetes (Figure 2(b)).

3.4. OPG and TRAIL Protein Expression in the Aorta and the Heart. Immunostainings showed that diabetes significantly increased aortic OPG expression as compared to the other groups, while TRAIL was unchanged (Figure 3). On the other hand, in the heart, it was dyslipidemia that significantly increased OPG protein expression, with no further changes after the induction of diabetes. As for cardiac TRAIL, TRAIL protein expression decreased in the ApoE and ApoE + DM groups (data not shown).

3.5. Circulating OPG and TRAIL. The levels of circulating OPG mirrored its vascular changes. Circulating OPG was 1.91 ± 0.21 ng/mL in the control group, 2.05 ± 0.1 ng/mL in the ApoE group, and 3.1 ± 0.19 in the ApoE + DM group, such that OPG increased significantly in diabetic mice as compared to the other groups ($p < 0.05$, ApoE + DM versus CNT and versus ApoE group). Dyslipidemia alone had no effect on OPG circulating levels. As for TRAIL, we were unable to measure its circulating levels, as they were below the detection levels of the ELISA we used. It has already been argued that OPG, which circulates at much higher levels than its ligands (RANKL and TRAIL), may be a more stable overall measure of OPG/TRAIL activity than TRAIL [23].

4. Discussion

Epidemiological studies report associations between OPG, TRAIL, and CVD. Patients with coronary artery disease have an increased OPG/TRAIL ratio, which further increases in the group of patients who develop heart failure [13]. Dyslipidemia and diabetes are two of the most firmly established risk factors for CVD [2]. Here, we evaluated the effects of

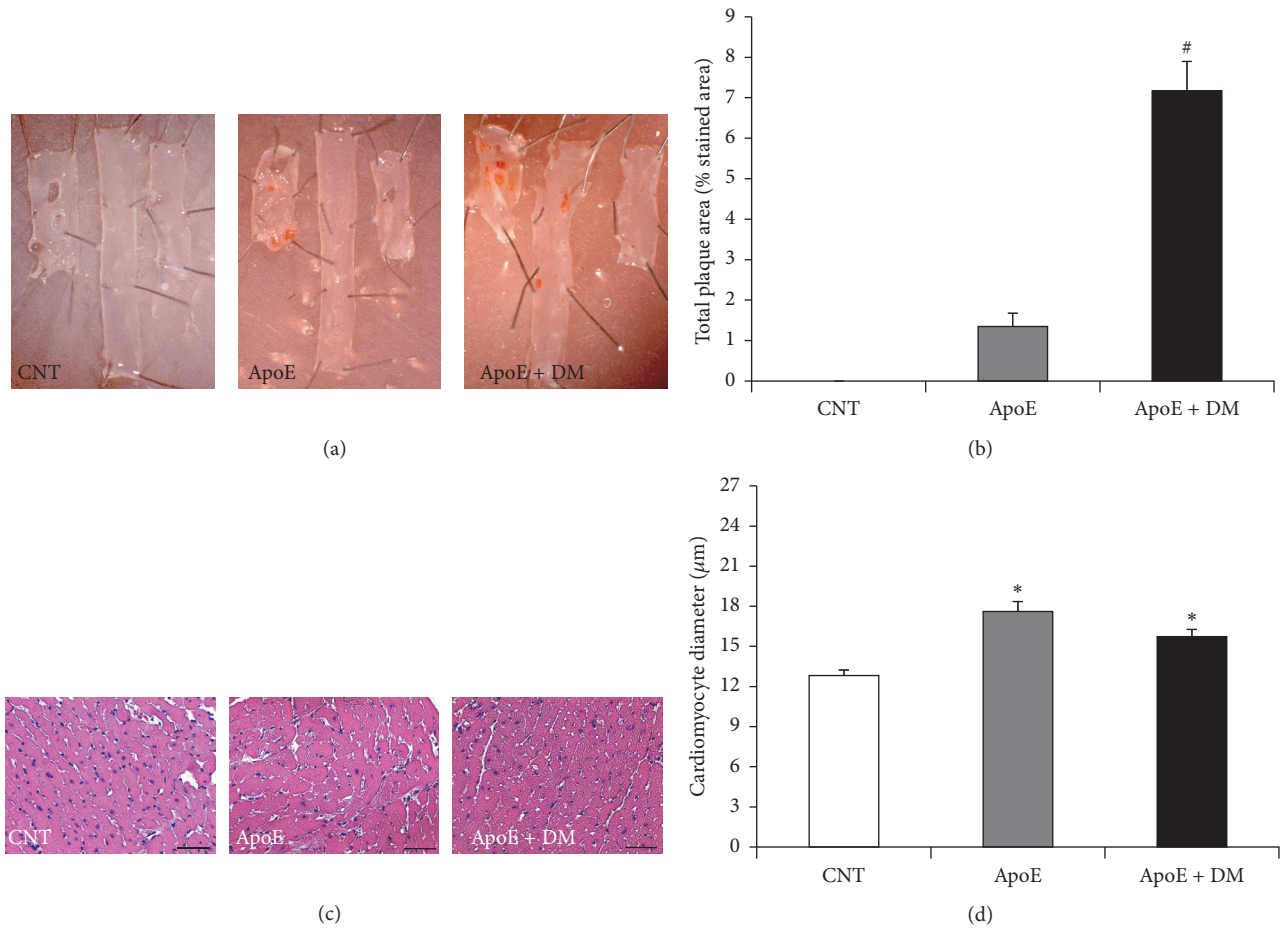

Figure 1: Effect of dyslipidemia and diabetes on atherosclerosis and cardiomyocyte hypertrophy. (a) Representative en face aortic sections showing atherosclerosis. (b) Percentage of the aorta stained red with Sudan IV in each group. Result from CNT is not shown, as there was no plaque present. (c) Representative H&E-stained hearts (original magnification 25x) and (d) related cardiomyocyte size quantification (in μm). Scale bar = 50 μm. Data show mean ± SEM. $^{*}p < 0.05$ versus CNT; $^{\#}p < 0.05$ versus ApoE. CNT is for C57BL/6J mice; ApoE is for ApoE$^{-/-}$ mice; ApoE + DM is for ApoE$^{-/-}$ mice + diabetes mellitus.

dyslipidemia, alone and in combination with diabetes, on aortic and cardiac OPG and TRAIL expression and found that while diabetes is the major determinant of OPG/TRAIL tissue changes in the vessels, dyslipidemia is it in the heart.

This data is in line with previous experimental works showing that diabetes increased OPG expression and the OPG/TRAIL ratio in the aorta [24] and that the onset of diabetes was associated with an increase of circulating OPG [25]. Consistent with these observations, in our study, we found that aortic and circulating OPG increased significantly only in the group of mice with diabetes. Taken together, these findings seem to support the hypothesis that the vessels might be the source of increased circulating levels of OPG in patients with diabetes and/or CVD.

It has to be noted that the increase of circulating and tissue OPG could be not only a risk marker but also a risk factor for atherosclerosis and CVD development. Firstly, animal models point to a role for OPG in glucose metabolism regulation, as OPG injections increased significantly glucose levels [12, 26], which represent one of the risk factors for CVD. This effect could be mediated by TRAIL blockade, as TRAIL lowers glucose levels [16]. By offsetting TRAIL effects, OPG could also promote body weight gain and dyslipidemia [20], as well as atherosclerotic plaque development [18, 20]. Secondly, several studies have shown that OPG has proinflammatory and profibrotic effects on the vasculature. Consistent with these actions, our group has shown that OPG increases leukocyte adhesion to endothelial cells [14], that it increases TGF-beta-mediated fibrogenesis and proliferation in vascular smooth muscle cells [27], and that it increases atherosclerosis extension in diabetic ApoE$^{-/-}$ mice [15].

By contrast, dyslipidemia alone did not affect OPG/TRAIL ratio in the vasculature. This data is consistent with a few in vitro studies showing that oxidized LDL did not change OPG expression in human coronary artery smooth muscle cells [28] and lymphocytes [29]. Nevertheless, in our study, we found that dyslipidemia significantly increased OPG/TRAIL ratio in the heart, while diabetes had no additional effect on it.

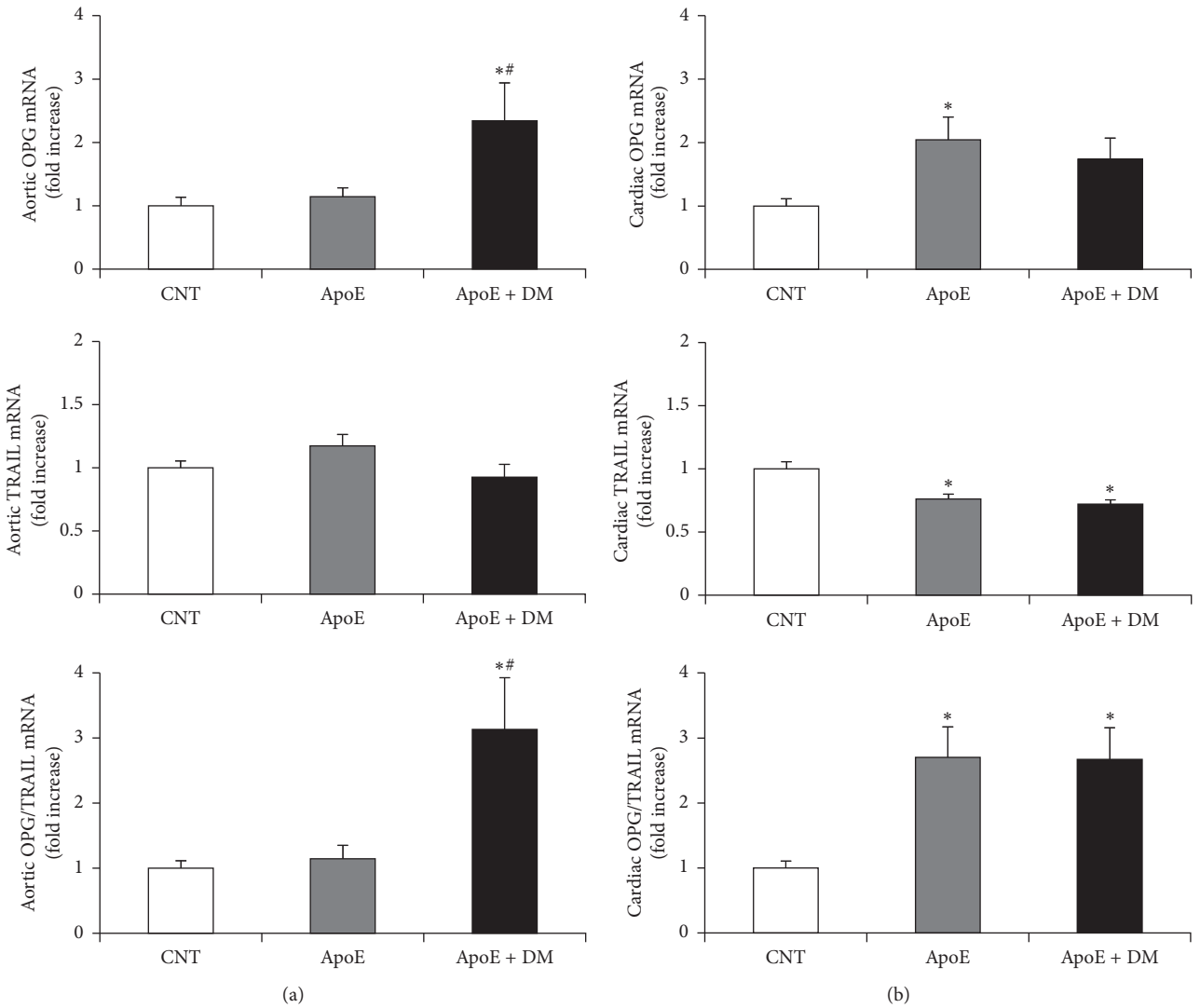

Figure 2: Effect of dyslipidemia and diabetes on OPG, TRAIL gene expression, and OPG/TRAIL ratio in the aorta and the heart. (a) Aortic messenger RNA expression of OPG, TRAIL, and OPG/TRAIL ratio, reported as relative gene units. (b) Cardiac messenger RNA expression of OPG, TRAIL, and OPG/TRAIL ratio, reported as relative gene units. Data are expressed as mean ± SEM. $^{*}p < 0.05$ versus CNT; $^{\#}p < 0.05$ versus ApoE. CNT is for C57BL/6J mice; ApoE is for ApoE$^{-/-}$ mice; ApoE + DM is for ApoE$^{-/-}$ mice + diabetes mellitus.

It has been shown that dyslipidemia is independently associated with left ventricle (LV) hypertrophy [5, 30, 31]. This was confirmed by experimental studies on ApoE$^{-/-}$ mice, where dyslipidemia was associated with increased LV mass in the absence of hemodynamic stress [32]. Consistent with previous reports, here we found that dyslipidemia was associated with cardiomyocyte hypertrophy. This data suggests that hyperlipidemia might be an early initiator of cardiac remodeling. It has been demonstrated that triacylglycerol overload and the increased availability of other lipid intermediates, such as ceramides, diacylglycerol, and oxidized phospholipids, can all activate inflammatory pathways and oxidative stress, leading to cellular apoptosis and myocardial scarring [33, 34].

It is possible that the increase in cardiac OPG/TRAIL ratio that was associated with dyslipidemia represents one of the mediators of lipid-induced cardiac remodeling. As for OPG, a recent study has demonstrated that OPG delivery induced LV hypertrophy, while, in vitro, cardiomyocytes transfected with AAV-OPG increased dramatically the expression of hypertrophy-related proteins, such as ANP, α-MHC, and troponin I [35]. In the cardiac fibroblast, OPG overexpression led to a significant increase in the fibrosis-related proteins [35]. By contrast, we showed that TRAIL administration to mice with diabetic cardiomyopathy had cardioprotective effects, as it reduced cardiac fibrosis and apoptosis, which are generally contributing to cardiac remodeling [36].

In conclusion, here we found that both dyslipidemia and diabetes affect OPG/TRAIL ratio in the cardiovascular system. This could contribute to the changes in circulating OPG/TRAIL which are observed in patients with diabetes

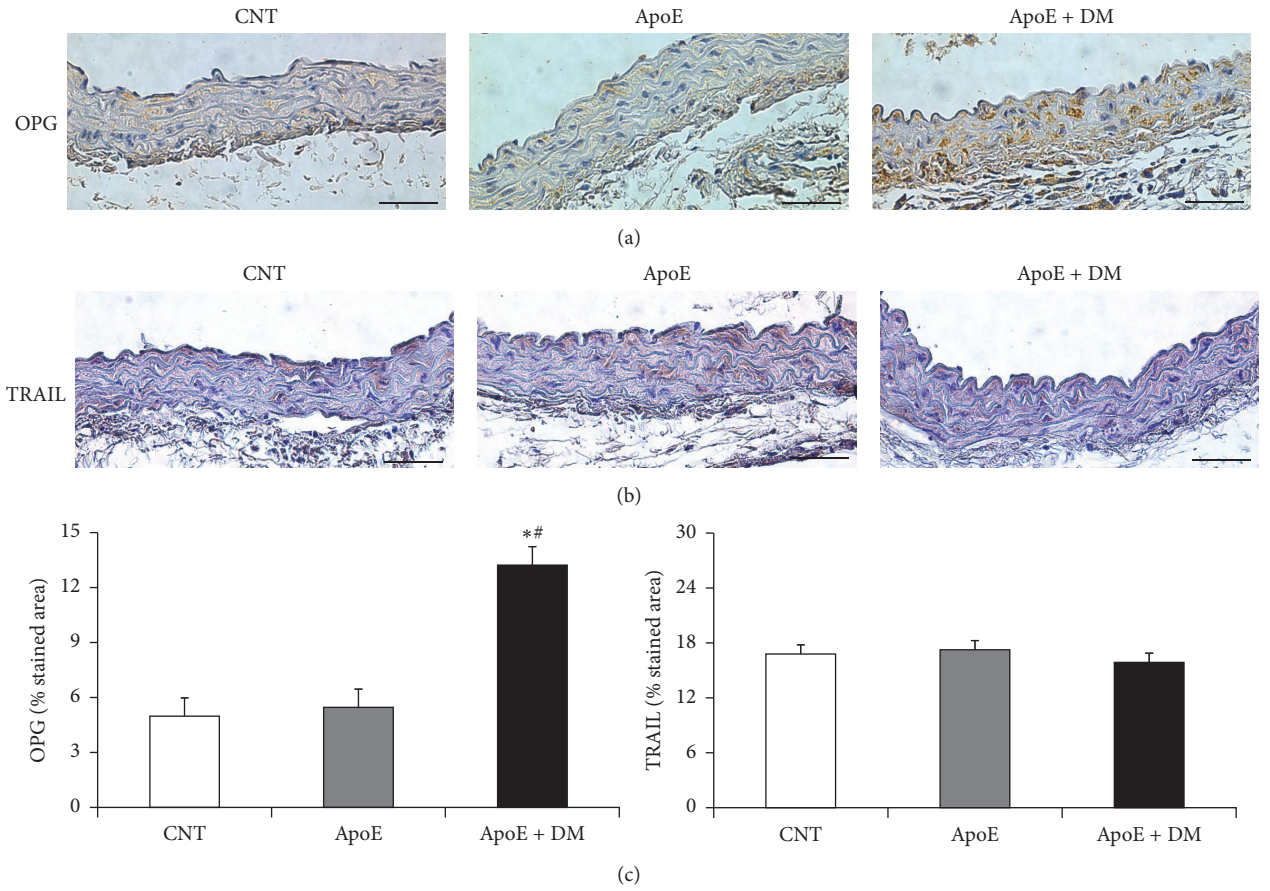

Figure 3: Effect of dyslipidemia and diabetes on aortic OPG and TRAIL protein expression. (a) Representative sections of aorta immunostained for OPG and (b) TRAIL (original magnification 25x). (c) Aortic OPG and TRAIL protein expression reported as percentage stained area. Scale bar = 50 μm. Data show mean ± SEM. $^{*}p < 0.05$ versus CNT; $^{\#}p < 0.05$ versus ApoE. CNT is for C57BL/6J mice; ApoE is for ApoE$^{-/-}$ mice; ApoE + DM is for ApoE$^{-/-}$ mice + diabetes mellitus.

and CVD. Most importantly, these changes could mediate/contribute to atherosclerosis development and cardiac remodeling.

Competing Interests

The authors declare that they have no competing interests.

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