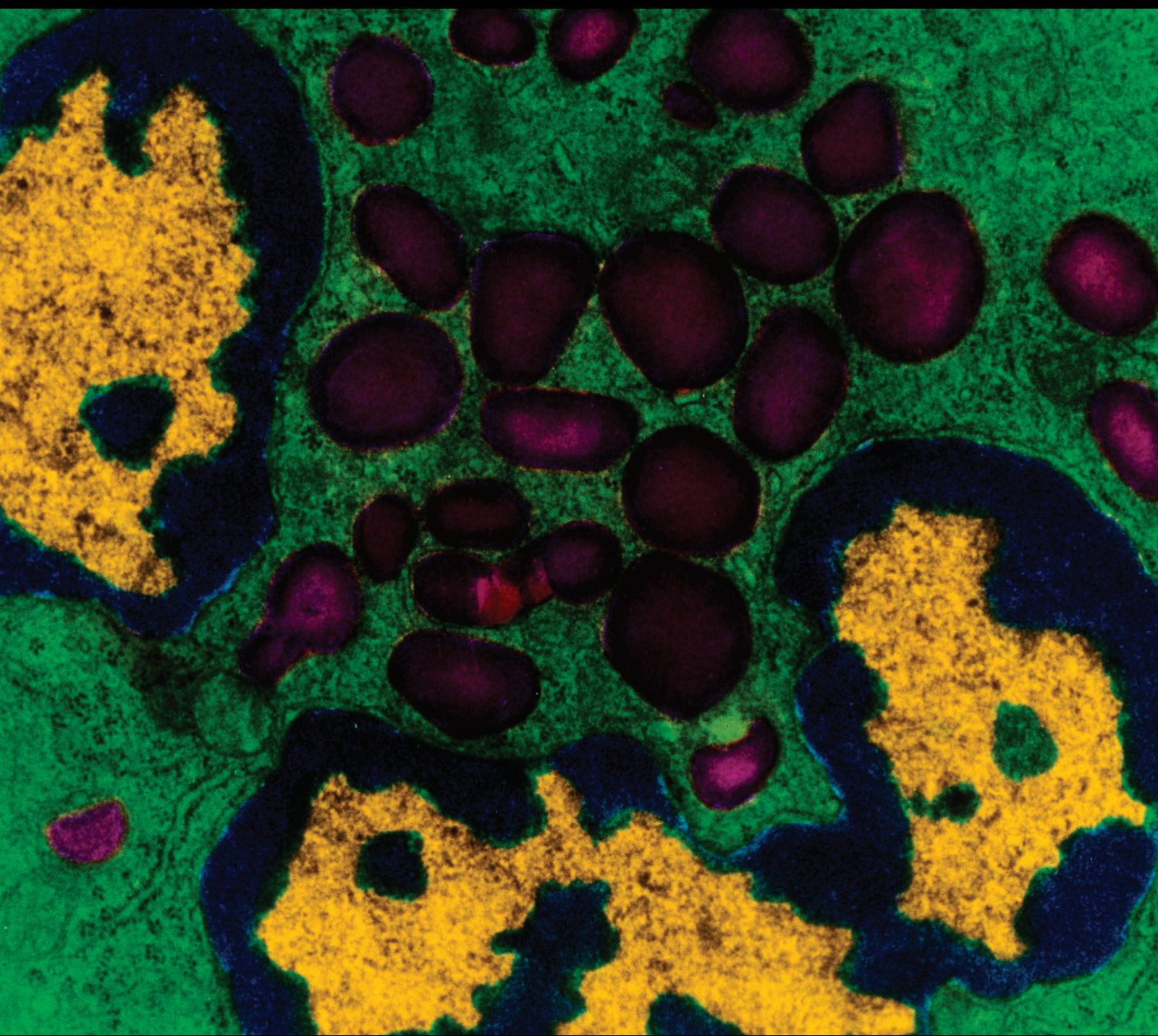
Mediators of Inflammation

Atherosclerosis and Autoimmunity

Lead Guest Editor: Francesca R. Spinelli
Guest Editors: Francesca Barone, Fabio Cacciapaglia, Arbi Pecani, and Matteo Piga



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Editorial Board

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Hindawi
Mediators of Inflammation
Volume 2018, Article ID 6730421, 2 pages
https://doi.org/10.1155/2018/6730421

Editorial

Atherosclerosis and Autoimmunity

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Received 31 December 2017; Accepted 1 January 2018; Published 29 March 2018

This special issue brings together original research and review articles focusing on the contribution of innate and adaptive immune response in the endothelial dysfunction and in the progression of atherosclerosis in autoimmune rheumatic diseases (AIRDs). By pointing out the relationship between immune response and atherosclerosis, it opens a window of opportunity for therapeutic intervention in the management of cardiovascular comorbidity.

AIRDs have been linked to a high risk of cardiovascular (CV) morbidity and mortality, mainly due to premature atherosclerosis (ATS). Recently, more attention has been attributed to ATS as an inflammatory, immune-mediated disease leading to premature vascular damage; mounting evidence supports an independent role for both humoral and cellular immune response, together with the involvement of innate immunity, in the development of the atherosclerotic plaque [1]. The immune system contributes mainly to endothelial dysfunction (ED), the earliest and reversible stage of ATS [2]. Recent evidence supports the presence of premature, accelerated ATS in AIRD patients that cannot be fully explained by the traditional cardiovascular risk factors. In this special issue, Z. Wang et al. reviewed the emerging role of the interplay between cigarette smoking and adipose tissue in the progression of atherosclerotic plaque, describing how the exposure to chemicals affects the status and functions of adipocytes.

Rheumatoid arthritis (RA) and ATS share many common mechanisms responsible for local and systemic inflammation [3]. In their original paper, G. L. Erre et al. evaluated the microvascular endothelial response in a large cohort of RA patients without any previous CV event and detected peripheral microvascular ED in one-third of the patients, which was only partially related to traditional cardiovascular risk factors. In their review of the literature, M. Di Franco et al. addressed the methylarginine metabolism in RA patients; in particular, the authors focused on asymmetric dimethyl-arginine (ADMA) and its implication in the accelerated atherosclerosis both as a surrogate biomarker of ED and as a possible target of treatment.

Immune-mediated mechanisms seem to interplay and converge into a proinflammatory and proatherogenic phenotype. In this regard, Y. Jeng et al. explored how the abundance of CD16+ monocyte subset predicts mortality: in a longitudinal cohort study, the authors showed that higher number of CD16+ monocytes correlates with increased mortality (overall mortality and CV deaths) in hemodialysis patients, shedding light on early recognition of immune dysfunction in this context.

The metabolic effects linking atherosclerosis, autoimmunity, and chronic inflammation are intriguing and still not well explored [4]. S. Gao et al. created a transgenic rabbit model that successfully expressed liver-specific human

cholesteryl ester transfer protein (hCETP) with the ability to enhance macrophage-derived foam cell formation and increasing HDL cholesterol and triglyceride plasmatic levels; the authors reported an atherogenic effect of increased CETP activity during cholesterol-fed diet, supporting a role for CETP inhibition as a cardioprotective intervention. In the light of these results, the immune system interference with CETP activity should be evaluated in human clinical trials and considered for therapeutic strategies.

Through this special issue, we also provide new scientific evidence on the potential effects that medications used to treat AIRDs may have on the endothelial function and progression of atherosclerosis. *In vitro* experiments from K. Lakota et al. showed that both methotrexate and fluvastatin are highly effective in lowering proatherogenic cytokines. Such an evidence may partly explain the protective effect of methotrexate and hydroxychloroquine against accelerated ATS in patients with RA and systemic lupus erythematosus as reviewed by A. A. Mangoni et al. and by A. Floris et al., respectively. Moreover, the effects of methotrexate and TNF-inhibitors on ADMA serum levels are reviewed in the paper by M. Di Franco and colleagues.

In this special issue, we emphasized some important topics concerning the contribution of the immune system in the development of ATS in AIRDs, thus shedding light not only on the understanding of these mechanisms but also on their practical implications.

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Francesca Barone
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Hindawi
Mediators of Inflammation
Volume 2018, Article ID 3424136, 11 pages
https://doi.org/10.1155/2018/3424136

Review Article

Protective Effects of Hydroxychloroquine against Accelerated Atherosclerosis in Systemic Lupus Erythematosus

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Received 28 July 2017; Accepted 10 December 2017; Published 18 February 2018

Academic Editor: Yona Keisari

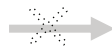
Cardiovascular (CV) morbidity and mortality are a challenge in management of patients with systemic lupus erythematosus (SLE). Higher risk of CV disease in SLE patients is mostly related to accelerated atherosclerosis. Nevertheless, high prevalence of traditional cardiovascular risk factors in SLE patients does not fully explain the increased CV risk. Despite the pathological bases of accelerated atherosclerosis are not fully understood, it is thought that this process is driven by the complex interplay between SLE and atherosclerosis pathogenesis. Hydroxychloroquine (HCQ) is a cornerstone in treatment of SLE patients and has been thought to exert a broad spectrum of beneficial effects on disease activity, prevention of damage accrual, and mortality. Furthermore, HCQ is thought to protect against accelerated atherosclerosis targeting toll-like receptor signaling, cytokine production, T-cell and monocyte activation, oxidative stress, and endothelial dysfunction. HCQ was also described to have beneficial effects on traditional CV risk factors, such as dyslipidemia and diabetes. In conclusion, despite lacking randomized controlled trials unambiguously proving the protection of HCQ against accelerated atherosclerosis and incidence of CV events in SLE patients, evidence analyzed in this review is in favor of its beneficial effect.

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune inflammatory disease characterized by a broad range of clinic manifestations and serologic findings [1, 2]. The prevalence of SLE ranges between 28.3 and 149.5 cases per 100,000 people and is higher in females of childbearing age [3]. Patients with SLE have a 2 to 3 times increased risk of premature death. Cardiovascular disease (CVD) is the leading cause of mortality regardless of time after diagnosis [4, 5]. The overall risk of myocardial infarction (MI) in SLE patients is 10-fold higher than that in the general population; however, it is much greater in young SLE women aged 35–44 years old, who are over 50 times more likely to have a MI, than in age-matched women without SLE [6, 7]. Noteworthy, the increased awareness of the burden of CVD in patients with SLE has not yet translated into decreased rates of hospitalization for acute MI or stroke [8, 9].

The higher risk of CVD in SLE patients is mostly related to accelerated atherosclerosis, which leads to clinical symptoms and manifestations at an earlier age compared to the general population [10]. Despite the pathobiological bases of accelerated atherosclerosis are not fully understood, it is thought that this process is driven by the complex interplay between autoimmunity, inflammation, vascular repair,

Table 1: Possible protective effects of HCQ on the interplay between atherosclerosis and SLE pathogenesis.

Features of SLE pathogenesis	HCQ	Features of atherosclerosis pathogenesis
Imbalance between endothelial damage and repair mechanisms		Endothelial dysfunction
Increased oxidative stress		Endothelial damage and impaired vasodilatation
Increased macrophage activation		Monocyte recruitment and activation in atherosclerotic plaques
Hyperactive T-cell with increased survival		T-cell recruitment and activation in atherosclerotic plaques
Dysregulation of TLR2 and TLR4 activation; activation of TLR7 and TLR9 by anti-DNA		Overexpression and activation of TLRs (especially TLR2/TLR4)
Increased levels of IFNα		Increased activation of macrophages and foam cells in the atherosclerotic plaques
Increased levels of TNF-α, IL-17, IL-6		Increased macrophage activation, adhesion molecule expression, chemotaxis, and inhibition of SMC proliferation
Increased levels of IFN-γ		Increased expression of adhesion molecule expression and inhibition of SMC proliferation and collagen production
Increased prevalence of anti-ApoA-1 antibodies and proinflammatory HDL		Decreased antiatherosclerosis HDL function

The arrows represent the interplay between SLE and atherogenesis. The crosses represent the proved (black) or potential (blank) action of HCQ in inhibiting the proatherogenic effect of SLE.

traditional risk factors, and therapeutic agents [10, 11]. As a result, not surprisingly, the traditional Framingham cardiac risk factors do not fully explain the increased prevalence of CVD observed in SLE [6, 12–14]. Moreover, multiple SLE-related features of autoimmunity have been associated with accelerated atherosclerosis [10, 11, 15, 16].

Hydroxychloroquine (HCQ) has been used for more than 50 years in the treatment of SLE patients. Over the last decades, an increasing number of *in vitro* and *in vivo* studies have highlighted the potential protective effect of HCQ against CVD through multiple mechanisms of action. This review discusses the role of SLE-related and SLE-unrelated factors in the pathophysiology of accelerated atherosclerosis, the pharmacology of HCQ, and the available evidence regarding the effects of this agent in reducing CV risk in SLE patients.

2. SLE and Accelerated Atherosclerosis

Roman et al. reported an increased prevalence of atherosclerosis, as determined by ultrasound assessment of carotid plaques, in patients with SLE (RR 2.4; 95% confidence interval (CI), 1.7–3.6; $P < 0.001$), particularly in those younger than 40 years which prevalence was 5.6 times higher than healthy controls [17]. Similarly, Asanuma et al. found a significantly higher prevalence of coronary calcification (OR 9.8, 95%CI 2.5–39.0, $P = 0.001$) and greater coronary artery calcium scores ($P < 0.001$) in SLE patients than in healthy controls [18].

Longer disease duration (OR 2.14, 95%CI 1.28–3.57; $P = 0.004$) and higher disease-related Systemic Lupus International Collaborating Clinics (SLICC)/damage index (SDI) (OR 1.26 per SDI point score, 95%CI 1.03–1.55, $P = 0.03$) were identified as independent predictors of carotid plaque in SLE [17]. In some studies, lupus disease activity was significantly associated with subclinical measures of atherosclerosis in univariate analysis, but its independent effect was not confirmed in multivariate analysis [19–21].

3. Interplay between SLE and Atherogenesis

The increasing evidence that both adaptive and innate immunity take part in the initiation and progression of atherosclerosis suggests that the dysregulation of the immune system of SLE could play an independent role in atherogenesis (Table 1) [22].

3.1. Endothelial Dysfunction. Endothelial dysfunction is one of the earliest signs of atherosclerosis [16, 23], resulting in increased expression of adhesion molecules and impaired vasodilation [24]. A recent meta-analysis, of 25 case-control studies involving 1313 SLE patients and 1012 healthy controls, confirmed that patients with SLE who are naïve of cardiovascular disease have impaired endothelial function as determined by brachial artery flow-mediated dilation [25].

An imbalance between circulating apoptotic endothelial cells (ECs), indicative of vascular damage, endothelial progenitor cells (EPCs), and circulating myelomonocytic angiogenic cells (CACs), expression of vascular repair mechanisms, was described in SLE patients [26, 27]. Such findings correlate with the presence of endothelial dysfunction (beta = −4.5, $P < .001$) assessed by brachial artery flow-mediated dilation [26].

Both endothelial damage and the initiation of the atherogenic process are influenced by the redox environment.

Patients with SLE have increased concentrations of reactive oxygen species (ROS) and decreased antioxidant defense mechanisms which provide a favorable environment for oxidation of lipoproteins and atherosclerosis development [28, 29]. Moreover, a positive correlation between SLE disease activity and oxidative stress was observed in some studies [28, 30, 31], but not in others [32, 33].

Further potential mechanisms involved in endothelial dysfunction in SLE include alterations in lipid profile with increased oxidized LDL (ox-LDL) and proinflammatory high-density lipoproteins (HDL) [11], high frequency of low-density granulocytes (LDG) with direct toxic effect on the endothelium [34], renal involvement, and antiphospholipid antibodies [35, 36].

3.2. Monocytes and T-Cell Recruitment and Activation. Due to the overexpression of adhesion molecules and the increased chemokine releasing by activated ECs, monocytes can migrate into the intima and differentiate into macrophages. The uptake of ox-LDL by scavenger receptors leads to a further transformation into foam cells that secrete proinflammatory cytokines under the toll-like receptor (TLR) stimuli [22]. Macrophage activation, as assessed by serum neopterin measurement, was demonstrated to be increased in SLE patients (median (IQR) serum neopterin nmol/L: 8.0 (6.5–9.8) versus 5.7 (4.8–7.1) in SLE and healthy controls, resp.) [37] and to correlate with SLE disease activity [38, 39]. However, a significant association with coronary calcium in SLE patients was not observed [37].

T-cells, consisting predominately of CD4+ T helper 1, are recruited to nascent atherosclerotic plaques similarly to monocytes and represent approximately 7–17% of the cells in the lesion [40]. T-cells have been shown to be hyperactive in lupus patients, with reduced apoptosis rate and increased survival [41–43]. In support of the role of CD4+ T-cells in the link between SLE and atherosclerosis, Stanic et al. demonstrated an increased infiltration of CD4+ T-cells into the atherosclerotic lesions of $LDLr^{-/-}$ mice following transfer of bone marrow from lupus-susceptible mice [44].

3.3. Toll-Like Receptors. The toll-like receptors (TLRs), a class of pattern recognition receptors expressed on multiple cells involved in innate immunity, were demonstrated to be involved in atherogenesis [45, 46]. Edfeldt et al. found that the expression of TLR1, TLR2, and TLR4 was markedly enhanced in human atherosclerotic plaques [47]. Miller et al., in their *in vitro* experiments, reported that the binding of TLR4 and CD14 to ox-LDL on macrophages inhibits the phagocytosis of apoptotic cells, upregulates the expression of the scavenger receptor, and increases the uptake of ox-LDL [48].

Recent studies described a dysregulated activation of TLR2 and TLR4 in SLE patients, resulting in upregulated production of autoantibodies and cytokines [49]. Moreover, the endogenous anti-DNA antibody immune complexes typical of SLE can bind TLR7 and TLR9 on active plasmacytoid dendritic cells (DCs) and promote the release of IFNα. This leads to the recruitment of activated inflammatory cells, self-perpetuating the process of inflammation and plaque formation [46].

3.4. Cytokines. Many cytokines are involved both in atherosclerosis and SLE pathogenesis. IFNα is a multifunctional cytokine which plays a pivotal role in SLE pathogenesis. IFNα concentrations are increased in SLE patients, associate with disease activity [50], and seem to be involved in endothelial dysfunction. Denny et al. demonstrated that IFNα induces EPC and CAC apoptosis and skews myeloid cells toward nonangiogenic phenotypes, whilst neutralization of IFN pathways led to a normalization of the EPC/CAC phenotype [27, 43]. Recently, IFNα has been claimed to serve as a proatherogenic mediator through repression of endothelial NO synthase-dependent pathways promoting the development of endothelial dysfunction and cardiovascular disease in SLE [51].

IFNγ, a key regulator of immune function, was demonstrated to be highly expressed and to play a crucial role both in SLE and in atherosclerosis [52, 53]. IFNγ participates in atherogenesis by stimulating ECs and macrophage activation, proinflammatory mediator production, and adhesion-molecule expression and by inhibiting smooth muscle cell proliferation and collagen production [22, 54].

Other cytokines overexpressed in SLE, such as TNF-α, IL-17, and IL-6, participate in the initiation and perpetuation of the atherosclerotic process by stimulating the activation of macrophages, inducing the secretion of matrix metalloproteinases, upregulating the expression of adhesion molecules on the ECs, increasing the concentration of chemotactic messengers, and affecting the proliferation of smooth muscle cells [15, 55–59]. In SLE, serum TNF-α concentrations have been reported to be elevated and to correlate with CVD and altered lipid profiles [60, 61].

3.5. Reduced Protective Effect of High-Density Lipoproteins. HDL have atheroprotective effects through the inhibition of oxidative modification of LDL, stimulation of reverse cholesterol transport, and attenuation of endothelial dysfunction. During the acute phase of inflammation, HDL can be converted from anti-inflammatory to proinflammatory molecules that promote LDL oxidation [62, 63]. McMahon et al. found that a higher proportion of SLE patients had proinflammatory HDL (44.7% of SLE patients versus 4.1% of controls, $P < 0.006$ between all groups), which correlated with ox-LDL concentrations ($r = 0.37$, $P < 0.001$) and coronary artery disease ($P < 0.001$) [64].

The prevalence of antibodies against apolipoprotein A1 (anti-ApoA-1), the main component of HDL, is significantly higher in patients with acute coronary syndrome (21%) and in patients with SLE and/or antiphospholipid syndrome (13–32%), than in healthy subjects (1%) [65, 66]. Although the direct demonstration of a cause-effect relationship is needed, the high prevalence of anti-ApoA-1 autoantibodies in SLE patients is supposed to play a role in accelerated atherosclerosis.

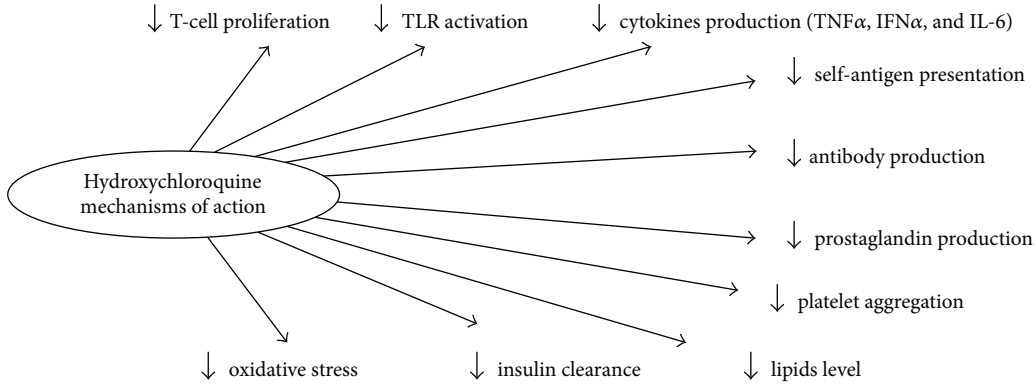


Figure 1: HCQ mechanisms of action.

4. Increased Prevalence of Traditional Cardiovascular Risk Factors in SLE

Some of the traditional risk factors for atherosclerosis, such as dyslipidemia, diabetes, and hypertension, have an increased prevalence in SLE patients [67].

4.1. Dyslipidemia. SLE patients exhibit an increased incidence of proatherogenic lipid profile, consisting in low concentrations of HDL and high concentrations of triglycerides, total cholesterol, and LDL [43]. The increased prevalence of dyslipidemia in SLE may be due to both steroid therapy and disease-related pathogenetic mechanisms, including increased C-reactive protein levels, cytokine release (e.g., TNF-alpha and IL-6), and antibodies against lipoprotein lipase (LPL) affecting the balance between pro- and antiatherogenic lipoproteins [68]. In 918 SLE patients of the Systemic Lupus International Collaborating Clinics' cohort, the prevalence of hypercholesterolemia was 36% at diagnosis and 60% 3 years later [69]. Moreover, in the same cohort, hypercholesterolemia was significantly associated with CV events (OR = 4.4, 95%CI 1.51–13.99) [70].

4.2. Hypertension. Hypertension is an independent risk factor CV in SLE (OR 5.0; 95%CI 1.3–18.2) [70]. In a case-control study, Bruce et al. reported a 2.59 RR (95%CI 1.79–3.75) of hypertension in women with SLE [12]. In a multivariate analysis, Doria et al. found that hypertension was associated with atherosclerosis by means of higher carotid intima-media thickness in SLE patients [21].

4.3. Diabetes and Insulin Resistance. An increased prevalence of insulin resistance and diabetes was reported in several studies [70–72], but not in all [73]. Bruce et al. reported a 6.6 RR (95%CI 1.36–26.53) of diabetes, which is an established risk factor for CVD, in SLE women [12].

An unbalance in adipokine production, consisting of lower concentrations of adiponectin and higher concentrations of leptin, was proposed as a potential cause of the increased prevalence of insulin resistance in SLE, as well as corticosteroid use [74]. However, neither insulin resistance nor diabetes has been shown to independently predict CV events in SLE cohorts [70, 72].

Dyslipidemia, hypertension, and insulin resistance can be part of metabolic syndrome that was observed to be more frequent in SLE patients compared with controls (32.4% versus 10.9%; $P < 0.001$) and associated to an increased risk of atherosclerosis by means of aortic pulse wave velocity [75, 76].

5. Hydroxychloroquine Pharmacology

HCQ is an antimalarial agent that has been used for many years in treating inflammatory rheumatic diseases, especially SLE and rheumatoid arthritis. HCQ is administered orally as the sulphate salt and, being a weakly basic drug, is rapidly absorbed in the upper gastrointestinal tract with a large volume of distribution. HCQ is then dealkylated by cytochrome P450 enzymes into its active metabolite desethyl-HCQ [77]. The systemic clearance is by renal excretion with a long tissue half-life of 40–50 days. HCQ may take up to 4–6 weeks for the onset of therapeutic action and 3–6 months to achieve the maximal clinical efficacy. The recommended dose of HCQ is 200–400 mg daily or about 5 mg/kg/day in a weight-based regimen [77]. According to Durcan et al. [78], HCQ dosing based on actual body weight, instead of ideal weight, is appropriate for patients with SLE. Blood HCQ concentrations can be measured with available commercial kits, which may help in adherence monitoring and the identification of individualized therapeutic regimens [79].

HCQ has numerous and complex mechanisms of action (Figure 1). The increasing pH in the intracellular compartments ("lysosomotropic action") favors HCQ-mediated interference with phagocytosis, receptor recycling, antibody production, and selective presentation of self-antigens [67]. Moreover, HCQ blocks T-cell and monocyte proliferation, inhibits TLR signaling, and downregulates cytokine production including TNF-alpha, IL-17, IL-6, IFNα, and IFNγ [77].

6. Hydroxychloroquine Clinical Benefits in SLE

6.1. Disease Activity. The first study on HCQ clinical efficacy in SLE randomized 25 patients to continue HCQ on stable dose therapy and 22 patients to switch to placebo for 24 weeks. A lower rate of flare (36% versus 73%, $P = 0.02$;

RR 2.5 95%CI 1.1–5.6) was observed in the HCQ group [80]. More recently, Ruiz-Irastorza et al. systematically reviewed the effect of HCQ on lupus activity and identified 8 studies, of which 3 were randomized controlled trials [81]. All studies were of high quality and consistently found lupus disease activity and flares to be significantly reduced in patients treated with HCQ [81, 82].

6.2. Atherosclerosis. Some studies did not find any effect of current [20, 83] or past [84–87] treatment with HCQ on the presence of atherosclerosis. On the other hand, Roman et al., in multivariate analysis, found a borderline-independent effect of current or former treatment with HCQ (adjusted OR 0.49; 95%CI 0.21–1.12; $P = 0.09$) in reducing plaque burden, on carotid ultrasound, of SLE patients [17]. Moreover, the current use of HCQ was associated with significantly lower (partial R2 0.025; $P = 0.032$) aortic stiffness, measured by pulse wave velocity, in premenopausal SLE women [88]. Noteworthy, the only study specifically designed to analyze the effect of treatment with HCQ on atherosclerosis, albeit conducted in a relatively small population ($n = 41$), found increased large artery elasticity (13.7 versus $8.3\,\text{mmHg} \times \text{ml} \times 10$; $P = 0.006$) and reduced systemic vascular resistance (14.4 versus 18.4 $\text{dyne} \times \text{sec} \times 10^{-3}$; $P = 0.05$) among patients treated with HCQ compared with those receiving corticosteroids only [89]. Overall, the available evidence is inconclusive, mainly as a result of poor study quality and design [81].

6.3. Irreversible Target Organ Damage and Survival. The beneficial effects of HCQ on target organ damage and survival in SLE patients have been demonstrated by several high-quality evidence studies [81, 90–93]. For example, HCQ was protective (HR 0.73; 95%CI 0.52 to 1.00) against damage accrual, calculated using the SLICC damage index, in the prospective LUMINA (Lupus in Minorities: nature versus nurture) study cohort, particularly in those patients without damage at baseline (HR 0.55, 95%CI 0.34 to 0.87) (94). In the same cohort, 17% of patients not taking HCQ died during the follow-up versus 5% of those treated with HCQ ($P < 0.001$), accounting for a 0.28 unadjusted OR (95%CI 0.05 to 0.30) and 0.32 adjusted OR (95%CI 0.12 to 0.86) [94]. Moreover, HCQ use was associated with less cerebrovascular damage on brain MRI of SLE patients (OR 0.08; 95%CI 0.01–0.73) [95], less thrombosis (OR 0.31, 95%CI 0.13–0.71) [96], less CV events (HR 0.04, 95%CI 0.004–0.48) [97], and less, albeit not statistically significant, cardiovascular mortality (0% versus 36.8%) [98].

In a multinational Latin American inception cohort, a lower mortality rate was observed in antimalarial users compared with nonusers (4.4% versus 11.5%; $P < 0.001$), and, after adjustment for potential confounders in a Cox regression model, antimalarial use was associated with a 38% reduction in the mortality rate (hazard ratio 0.62, 95%CI 0.39–0.99) [99].

It remains to be established whether HCQ exerts its protective effects on damage accrual and survival in SLE patients through lowering disease activity, preventing atherosclerosis, or both.

7. Hydroxychloroquine and SLE-Related Risk Factors for Atherosclerosis

7.1. Endothelial Dysfunction. Endothelial dysfunction (ED) is a potentially reversible alteration thus representing an attractive target for CVD prevention and treatment. Gómez-Guzmán et al. [100] found that short-term treatment with HCQ in advanced disease stages is able to reverse large artery ED in a murine model of SLE. This effect was mediated by a reduction of nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase activity, which is a major ROS source. Recently, Virdis et al. confirmed that early treatment with HCQ exerts protective effect by decreasing vascular oxidative stress and improving endothelium-dependent relaxation, essentially by preserving the NO-mediated component [101].

7.2. Toll-Like Receptor Signaling and Cytokine Production. Evidence that HCQ acts by blocking the nucleic acid-sensing TLRs (TLR3, TLR7, TLR8, and TLR9) is the most important advance in our understanding of its mechanism of action. Nucleic-sensing TLRs, located in intracellular compartments, are activated when interacting with foreign nuclear material presented by specialized molecules such as FC-gamma receptor on DCs or B-cell receptor on the surface of B-cells. HCQ interferes with the TLR7 and TLR9 signaling pathways, reducing the production of IFNα, IL-6, and TNF-α [102]. It has been postulated that, by altering the lysosomal pH, HCQ prevents TLR functional transformation and activation [103]. However, it is also possible that, by binding nucleic acids, HCQ masks their TLR-binding epitope preventing TLR activation [104].

Beyond the inhibition of TLR signaling, experimental evidence showed that HCQ reduces the concentration of proatherogenic cytokines, such as IFNα, IL6, TNF-α, IL17, and IL22, in SLE patients through different mechanisms [105, 106]. The observation that HCQ reduces the expression of miR155 in NZB/NZW mice, a SLE animal model, suggests additional therapeutic effects through an epigenetic control of cytokine gene expression [107].

7.3. Actions on Immune System Cells and Autoantibody Production. T-cell and B-cell activities may be directly or indirectly affected by HCQ [103]. The HCQ "lysosomotropic action" is responsible for altering the process of self-antigen presentation, whilst preserving that of exogenous antigens, and may also inhibit the intracellular calcium signals after T-cell-receptor stimulation, preventing T-cell activation and proliferation [103, 108]. Furthermore, the inhibition of IFNα, IL6, IL17, and TNF-α production affects B-cell activation and autoantibody production and favors the differentiation of endothelial cells [103].

The reported HCQ-mediated effects may theoretically reduce the initiation and progression of atherosclerosis by inhibiting the monocyte adhesion to endothelial cells, reducing smooth cell proliferation and favoring vascular repair. However, to date, no study has investigated whether the described effects of HCQ may have a direct benefit in

preventing atherosclerosis in SLE patients. More research is warranted to confirm, or refute, this hypothesis.

8. Hydroxychloroquine and Traditional Atherosclerosis Risk Factor

8.1. Effects on Lipid Profile. The beneficial effect of HCQ on dyslipidemia in patients with SLE has been known for some time. Potential mechanism underlying the beneficial effect of antimalarials on dyslipidemia may be represented by upregulation of LDL receptors with an enhancement of the plasma removal of this lipoprotein [109]. This potential effect of antimalarials would minimize the increased lipoprotein hepatic synthesis induced by steroids [110]. Petri et al. [111] found that HCQ treatment was independently associated with lower serum cholesterol concentrations in multivariate analysis (effect on mg% −8.94; $P = 0.009$). In a cohort of 815 patients, Rahman et al. [13] showed that the lipid lowering effect of antimalarials (mainly HCQ) was higher in patients on a stable dose of steroids and consisted of a reduction in total cholesterol concentrations of 11.3% at 3 months ($P = 0.0002$) and 9.4% at 6 months ($P = 0.004$). Contrasting results have been reported on the different lipoprotein profiles [112–114]. However, two recent prospective studies specifically designed to analyze the effect of HCQ on lipoprotein concentrations, after correction for the confounding effect of other variables, found lower LDL ($P = 0.036$) [113], VLDL ($P = 0.002$), and triglyceride concentrations ($P = 0.043$) and higher HDL concentrations ($P = 0.03$) [114] in patients treated with HCQ.

8.2. Effects on Glucose Level. Hypoglycemia has been reported in patients treated with antimalarials. *In vitro* and animal studies, antimalarials affected insulin metabolism, increasing insulin binding to its receptor, altering hepatic insulin metabolism, potentiating insulin action, and reducing the insulin clearance [115–117]. A small randomized study in decompensated diabetic patients showed that HCQ significantly lowered glycated hemoglobin A1c (3.3%; 95%CI, −3.9 to −2.7, $P = 0.001$) when added to insulin therapy, possibly by improving insulin secretion and peripheral sensitivity [118].

Recently, the use of HCQ has been associated with lower concentrations of serum glucose (85.9 versus 89.3 mg/dl, $P = 0.04$) [119] and a lower incidence of diabetes mellitus in SLE patients, in a dose-dependent manner (HR 0.26; 95%CI 0.18–0.37; $P < 0.001$) [120].

8.3. Effects on Thrombosis. HCQ has a protective effect against thrombosis both in SLE patients with and without antiphospholipid antibodies [86]. Such an effect seems mediated by reduced platelet aggregation and protection of the annexin A5 anticoagulant shield from disruption by aPL antibodies [121].

9. Discussion

There is good evidence from prospective studies of an increased CV risk in SLE patients [4–7]. Accelerated atherosclerosis, in the presence of traditional risk factors, may explain at least in part this enhanced risk. However, SLE-related factors, as endothelial dysfunction and inflammation, autoantibodies, damage accrual, and disease activity are equally or even more important [10–14]. Such a complex interplay of pathogenetic mechanisms presents clinical challenges, particularly because of the lack of data on the effects of the modification of traditional and SLE-specific CVD risk factors. Presently, in order to lower the CV risk in SLE, the main objectives should be treating the disease targeting remission or low disease activity [122] and sparing corticosteroids when possible, whilst monitoring traditional CVD risk factors at least once a year [123].

HCQ should be an essential part of SLE treatment strategy and should be started as soon as the diagnosis has been made and maintained for an indefinite period if toxicity does not occur [81]. Although for a long time it has been considered a minor component in the management of SLE, in fact, increasing evidence demonstrates that HCQ has a broad spectrum of beneficial effects on disease activity, prevention of damage accrual, and mortality [124]. Furthermore, HCQ is thought to protect against accelerated atherosclerosis by means of several mechanisms of action targeting both SLE-related and traditional CV risk factors.

One of the main limitations to be considered, when interpreting the available data, is the lack of a direct demonstration of the cause-effect relationship between HCQ treatment and atheroprotection from randomized controlled trials. On the other hand, given the many evidences of beneficial effects on HCQ in SLE patients, a placebo-controlled trial would be probably not ethically sustainable. Studies addressing the potential effect of HCQ on CV risk in patients with no existing rheumatic disease with a very high risk of a recurrent CV event, such as the OXI trial (NCT02648464), may shed some light on mechanistic insights regarding the cardioprotective effect of HCQ [125].

In conclusion, despite the lack of randomized controlled trials, the available evidence strongly suggests that HCQ exerts beneficial effects against atherosclerosis and CVD in SLE patients.

Conflicts of Interest

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

Authors' Contributions

Alberto Floris and Matteo Piga contributed equally to this work.

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Hindawi
Mediators of Inflammation
Volume 2018, Article ID 8237209, 11 pages
https://doi.org/10.1155/2018/8237209

Research Article

Analysis of Drug Effects on Primary Human Coronary Artery Endothelial Cells Activated by Serum Amyloid A

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Received 27 July 2017; Revised 3 November 2017; Accepted 14 November 2017; Published 13 February 2018

Academic Editor: Matteo Piga

Background. RA patients have a higher incidence of cardiovascular diseases compared to the general population. Serum amyloid A (SAA) is an acute-phase protein, upregulated in sera of RA patients. *Aim*. To determine the effects of medications on SAA-stimulated human coronary artery endothelial cells (HCAEC). *Methods*. HCAEC were preincubated for 2 h with medications from sterile ampules (dexamethasone, methotrexate, certolizumab pegol, and etanercept), dissolved in medium (captopril) or DMSO (etoricoxib, rosiglitazone, meloxicam, fluvastatin, and diclofenac). Human recombinant apo-SAA was used to stimulate HCAEC at a final 1000 nM concentration for 24 hours. IL-6, IL-8, sVCAM-1, and PAI-1 were measured by ELISA. The number of viable cells was determined colorimetrically. *Results*. SAA-stimulated levels of released IL-6, IL-8, and sVCAM-1 from HCAEC were significantly attenuated by methotrexate, fluvastatin, and etoricoxib. Both certolizumab pegol and etanercept significantly decreased PAI-1 by an average of 43%. Rosiglitazone significantly inhibited sVCAM-1 by 58%. *Conclusion*. We observed marked influence of fluvastatin on lowering cytokine production in SAA-activated HCAEC. Methotrexate showed strong beneficial effects for lowering released Il-6, IL-8, and sVCAM-1. Interesting duality was observed for NSAIDs, with meloxicam exhibiting opposite-trend effects from diclofenac and etoricoxib. This represents unique insight into specific responsiveness of inflammatory-driven HCAEC relevant to atherosclerosis.

1. Background

A healthy endothelium provides for an antiadhesive/antithrombogenic surface, which can prevent the development of atherosclerosis and thrombosis. Systemic autoimmune diseases, such as rheumatoid arthritis (RA), exhibit accelerated atherosclerosis (AS) [1–4] as a consequence of endothelial dysfunction, leading to higher incidence of cardiovascular (CV) disease (at least 2-fold enhanced CV risk) and premature and higher mortality [5, 6]. The pivotal role of inflammation in the development of AS and amplification of CV risk in RA has been extensively and well documented [7–10].

Inflammation mediates all stages of atherosclerotic CV events, from preclinical initiation to thrombotic complications of AS [11]. Serum amyloid A (SAA), a major acute-phase protein and inflammatory marker, has long been implicated as a predictor of clinical progression and outcome in RA [12] and a predictor of coronary artery disease, CV outcome [13], and early mortality in acute coronary syndromes [14]. SAA was shown to exhibit causal properties in AS, as a consequence of endothelial dysfunction (elevating tissue factor, as well as a variety of cytokines/chemokines) and early lesions (biglycan synthesis) [15] to plaque destabilization by inducing matrix metalloproteinases [16]. The first report in 2007, on SAA-stimulated human coronary artery

endothelial cells (HCAEC), exhibited a substantial and significantly higher induction of released IL-6 protein and mRNA levels as compared to HUVEC [17], as well as increased responsiveness to IL-1β [18]. SAA dose-dependently increased IL-6 protein levels in HCAEC, to a much larger extent than in HUVEC (4-fold higher at a concentration of 1000 nM SAA). These changes were not only confirmed by IL-6 mRNA expression levels but also showed larger changes (>20-fold), judging by densitometry [17]. It is unclear, however, how drugs used routinely in rheumatology for treating RA and other chronic diseases can affect HCAEC, in the presence of SAA.

A wide variety of drugs from different groups of functionality was tested in our cellular model, namely, (a) a glucocorticoid (GC), for example, dexamethasone; (b) disease-modifying antirheumatic drugs (DMARDs), for example, methotrexate; (c) biologicals and anti-TNFα inhibitors, for example, etanercept and certolizumab pegol; (d) an angiotensin-converting enzyme (ACE) inhibitor, for example, captopril; (e) an antilipemic agent, for example, fluvastatin; (f) an antidiabetic thiazolidinedione (TZD), for example, rosiglitazone; and (g) three nonsteroidal anti-inflammatory drugs (NSAIDs), for example, diclofenac, meloxicam, and etoricoxib.

Dexamethasone is a synthetic GC that binds to cytosolic glucocorticoid receptors, translocates to the nucleus, and physically interacts with NF-κB and AP-1 thereby affecting expression of IL-1, IL-6, TNFα, and VCAM, among others, and attenuating the inflammatory response [19, 20].

Methotrexate (MTX) is an antimetabolite used in low doses for treatment of autoimmune diseases. It is the most widely used classic DMARD, inhibiting dihydrofolate reductase and purine synthesis, acting as anti-inflammatory by causing adenosine release and signaling through adenosine G-protein-coupled receptors [21]. MTX reduced levels of proinflammatory cytokines in patients on one hand and increased anti-inflammatory cytokines on the other [22].

TNFα is a cytokine, central for the development of the inflammatory response in RA [23], present in soluble (17 kDa) and precursor membrane-bound form (26 kDa) found also on the endothelium [24, 25]. Clinical trials using anti-TNFα biologicals, such as etanercept and certolizumab pegol, to treat rheumatic diseases started in the mid-1990s [26] and today represent an important part of RA patient therapy, especially for those who fail to respond to traditional nonbiological DMARDs.

Captopril was the first marketed ACE inhibitor. ACE is mainly expressed on the endothelium surface [27, 28] with oxLDL shown to induce ACE in HCAEC [29]. This class of drugs affects the renin-angiotensin-aldosterone system by cleaving angiotensin I in angiotensin II, increasing water retention and vasoconstriction, making captopril an antihypertensive agent. ACE also degraded bradykinin, a potent vasodilator [30, 31], exhibited anti-inflammatory actions, affected scavenging reactive oxygen species, and influenced prostaglandin production, as well as levels of certain inflammatory cytokines [32, 33].

Statins were first marketed in 1987 [34], with the main indication for hypercholesterolemia and ischemic heart disease prevention. Their mechanism was shown to go through inhibition of liver HMG-CoA reductase, influencing cholesterol synthesis by producing mevalonate and lowering low-density lipoprotein (LDL). Studies also reported beneficial effects on C-reactive protein (CRP) lowering (as reviewed by Liao [35]), and specifically, the JUPITER study pointed out that subjects with increased CRP without hypercholesterolemia could benefit from statin therapy, regardless of LDL levels [36]. Fluvastatin is a synthetic statin, shown to reduce coronary events when started after percutaneous coronary intervention [37].

Thiazolidinediones, such as rosiglitazone, are exogenous agonists of peroxisome proliferator-activated receptor γ (PPARγ), a nuclear receptor acting as a transcription factor also found present in atherosclerotic plaques. Rosiglitazone improved endothelial dysfunction; decreased CRP, SAA, and E-selectin [38]; and was shown to promote generation of the anti-inflammatory lipid mediator 15-epi lipoxin A_4 [39].

NSAIDs are widely used for their anti-inflammatory and analgesic properties in rheumatic diseases, promoting inhibition of COX-2 activity and prostaglandin synthesis as the main mechanisms of action. In addition, they were reported to inhibit NF-κB [40] and activate PPARs [41]. However, different NSAIDs showed differential modes of activity; for example, diclofenac, a derivative of acetic acid, acted similarly to COX-2 selective inhibitors in increasing risk of myocardial infarction (MI) [42], as was the case for all NSAIDs depending on dose administered, as they all inhibit COX-2 enzyme activity [6, 43]. Because it is unclear how NSAIDs affect the coronary artery endothelium, we set out to compare three different NSAIDs, specifically potent diclofenac, highly selective COX-2 inhibitor etoricoxib, and enolic acid-derived meloxicam on stimulated HCAEC.

Besides traditional risk factors, therapy might influence both the development and even more importantly, the regression of AS [5, 6]. Thus, the main aim of our study was to determine the impact of the aforementioned drugs used for therapy of systemic autoimmune diseases, such as RA, on inflammatory responses of SAA-activated HCAEC, suggesting their effects on the coronary artery endothelium.

2. Materials and Methods

2.1. Cell Culture. Human coronary artery endothelial cells (HCAEC) were purchased from Cambrex BioScience (Walkersville, Maryland, USA). Cells were plated into 6-well plates (TPP, Trasadigen, Switzerland) at 37°C in a humidified atmosphere at 5% CO_2 and grown in EGM-2M medium containing 5% fetal bovine serum, following the manufacturer's instructions (Cambrex BioScience, Walkersville, MD, USA).

2.2. Materials. Lyophilized human recombinant SAA1/2 (hrSAA1/2) (Peprotech EC Ltd., London, UK) was spun down and reconstituted according to the manufacturer's instructions in cell culture-grade sterile water to a stock concentration of 1 μg/μl and stored until used at −20°C or −80°C.

Scheme 1: Timeline protocol.

The following medications were tested: (a) dexamethasone (Krka, Slovenia; stock 4 mg/ml), final concentration 5 μM; (b) methotrexate (Medac, Germany; stock 10 mg/ml), final concentration 1 μM; (c) certolizumab pegol (UCB Pharma, Belgium; stock 200 mg/ml), final concentration 100 μg/ml; (d) etanercept (Pfizer, UK; stock 50 mg/ml), final concentration 100 μg/ml; (e) captopril (Krka, Slovenia; stock 25 mg), final concentration 10 μM, dissolved in medium; (f) fluvastatin sodium (Novartis, Germany; 40 mg), final concentration 10 μM, dissolved in DMSO; (g) rosiglitazone (Cayman Chemical, USA; stock 10 mg/ml), final concentration 30 μM, dissolved in DMSO; (h) diclofenac sodium (Krka, Slovenia; 75 mg), final concentration 10 μM, dissolved in DMSO; (i) meloxicam (Boehringer Ingelheim, Germany; 15 mg), final concentration 100 μM, dissolved in DMSO; and (j) etoricoxib (MSD, Netherlands; 90 mg) final concentration 100 μM, dissolved in DMSO.

2.3. HCAEC Treatments. HCAEC at passage 5, grown to confluency in 6-well plates, were incubated in serum-free media for 2 hours prior to experiments. Preincubation was performed for 2 hours with the specific medications from sterile ampules or resuspended, at above indicated final concentrations, followed by the addition of SAA1/2 to stimulate HCAEC at a final 1000 nM concentration for 24 h (Scheme 1), and supernatants were collected, aliquoted, and stored at −20°C until tested.

2.4. Enzyme-Linked Immunosorbent Assay. Protein levels of IL-6, IL-8, PAI-I, and sVCAM-1 (all Invitrogen, Frederick, MD, USA) were measured in cell culture supernatants using ELISA.

The assays were performed in duplicates according to the manufacturer's instructions. Briefly, samples were diluted with standard diluent buffer 1 : 50 for IL-6, 1 : 2 for sVCAM-1, 1 : 50 for IL-8, and 1 : 80 for PAI-1 ELISA. In all ELISAs, biotin-labeled conjugates were incubated with samples for 2 hours and, after washing, incubated with streptavidin-horseradish peroxidase enzyme. Tetramethylbenzidine was used as a substrate, and after the reaction was stopped, absorbance was measured at 450 nm with a Sunrise Tecan microplate absorbance reader (Tecan, Groening, Austria). The concentrations of analytes were calculated from standard curves and multiplied by the dilution factor.

In order to compare the results of many cell culture experiments, we had to normalize the data—so a response in a well with the SAA treatment was taken as 1 in each experiment and responses in all other wells were calculated accordingly.

2.5. Viability. The number of viable cells was determined colorimetrically (CellTiter MTS assay, Promega). Cell toxicity and cell viability were assessed by cell morphology and with CellTiter 96 Aqueous One Solution Reagent (Promega, Madison, WI, USA), respectively. The viability assay was modified for use with adherent cells. After completion of treatments in 6-well plates, cells were washed with PBS and 200 μl of fresh serum-free medium was added together with 20 μl of reagent. Following 20 minutes, 100 μl of medium was transferred to a 96-well plate and absorbance read at 490 nm.

2.6. Statistical Analysis. All experiments were repeated at least in biological triplicate. Data are presented as mean ± standard deviation (SD). Means were compared among the various treated and control groups using Student's t-test. p values of <0.05 were accepted as statistically significant, unless otherwise stated.

3. Results

In order to determine the inflammatory response of HCAEC, stimulated for 24 h with pathological concentrations of SAA (1000 nM), in the presence and absence of drugs, released IL-6 and IL-8 protein levels were measured (Figures 1 and 2). Of all tested drugs, only captopril treatment significantly increased IL-6 in SAA-stimulated HCAEC (by 19%), while methotrexate and etoricoxib reduced IL-6 levels to 67%, with fluvastatin exhibiting the largest inhibition, down to 58% of initial SAA stimulatory levels. The three NSAIDs showed different modes of activity, with meloxicam increasing IL-6 (by 14%), diclofenac not affecting IL-6 levels, and etoricoxib significantly decreasing IL-6 levels (to 67%) (Figure 1).

Similarly, IL-8 protein production exhibited a marked, significant inhibition in the presence of fluvastatin (down to 24% of SAA-treated HCAEC) followed by methotrexate (to 77%) and etoricoxib (to 52%). On the other hand, meloxicam increased IL-8 (by 46%), similar to IL-6 (Figure 2).

Since elevated plasminogen activator inhibitor-1 (PAI-1), a serine protease inhibitor, represents a risk factor for thrombosis and atherosclerosis [44], we set out to investigate its concentrations in SAA-stimulated HCAEC in the presence/absence of drugs. PAI-1 secretion, as measured by ELISA in cell culture supernatants, was significantly increased in SAA-treated HCAEC in the presence of diclofenac (by 52%), while meloxicam, fluvastatin, etanercept, and certolizumab pegol all significantly decreased its levels (to 71, 73, 57, and 58%, resp.) (Figure 3).

In order to examine the effects of drugs on the SAA-stimulated adhesion molecule, sVCAM-1 in HCAEC, ELISA

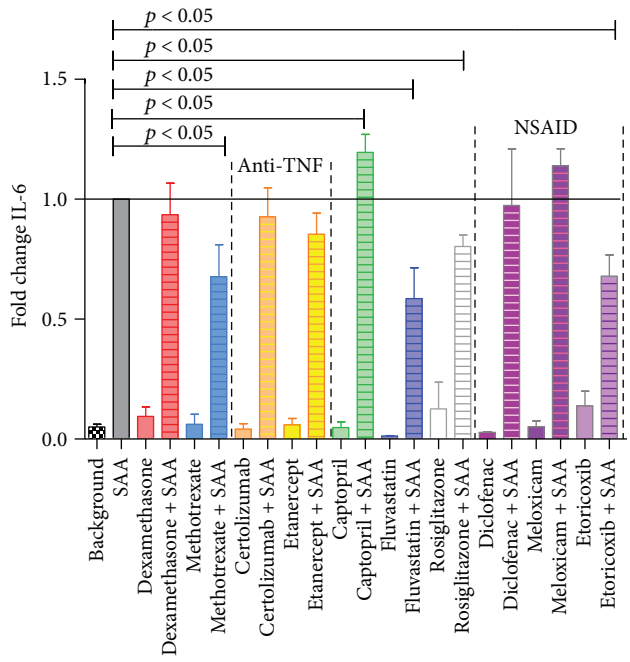

Figure 1

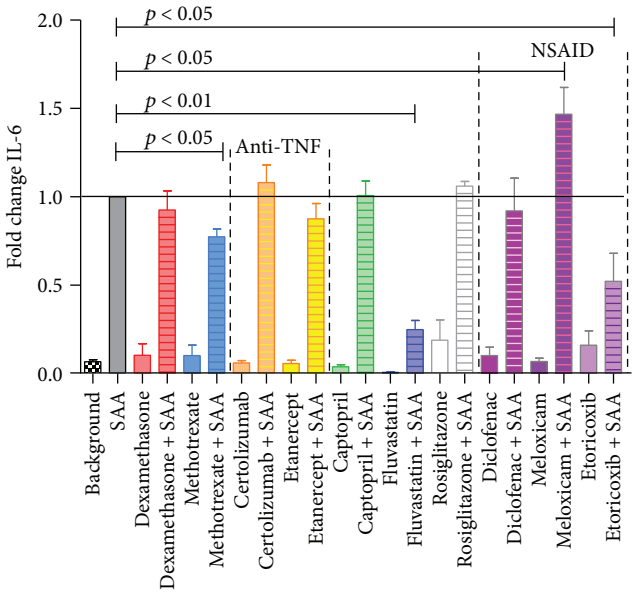

Figure 2

was performed. Soluble VCAM-1 levels in HCAEC supernatants were significantly inhibited by methotrexate (to 69%), by fluvastatin and rosiglitazone (both to 42%), by diclofenac (to 46%), by meloxicam (to 67%), and most potently by etoricoxib (to 29%), while neither of the TNF inhibitors significantly changed sVCAM-1 levels (Figure 4).

To determine the effects of SAA treatment in the presence/absence of drugs on HCAEC viability, proliferation was assessed based on tetrazolium reduction. No significant changes in absorbance were observed after treatment of HCAEC with drugs alone or in combination with SAA, with respect to the untreated cells (Figure 5).

4. Discussion

HCAEC have previously been shown to exhibit increased responsiveness to inflammation and coagulation compared to HUVEC or human microvascular endothelial cells (HMVEC), which could account for greater susceptibility of coronary arteries to inflammation and atherogenesis leading to CV pathology [18]. SAA has previously been reported to play a causal role in atherogenesis in animal and human studies [45]; however, the role of drugs in SAA-stimulated HCAEC has not been investigated till now. Thus, HCAEC represent an optimal cellular model system for evaluating drug effectiveness in an elevated SAA milieu, mimicking *in vivo* activated endothelium.

No drugs applied alone to HCAEC, in our study, exhibited significantly changed levels of tested parameters, including viability, with respect to the untreated cells.

Interestingly, the most effective drug in the presence of SAA was fluvastatin, with the greatest inhibition of all parameters tested, specifically IL-8, VCAM-1, IL-6, and PAI-1 (Figures 1–4). Fluvastatin was reported to induce eNOS, as well as NO and prostaglandin I_2 production in HUVEC and in human aortic endothelial cells within the first 24 h. In the next 24 h, statins also induced COX-1 and prostacyclin synthase expression [46]. The biphasic effect in vasodilatation is presumably potentiated, as researchers found that eNOS activation leads to iNOS and nitrosylation of COX-2 [39, 47]. Nitrosylated COX-2 produces epi-lipoxin A_4 (epi-LXA_4), a potent anti-inflammatory mediator and competitor ligand of SAA for their common LXA_4 receptor, ALX/FPR2 [48, 49]. Numerous studies on fluvastatin showed, in addition to LDL modification and endothelial function, also effects on smooth muscle cell proliferation, immunomodulation, plaque stabilization, and antithrombotic activity [50]. In HUVEC, multiple studies showed that fluvastatin inhibited CRP-induced TNFα expression and NF-κB activation [51], as well as attenuated PAI, tPA [52], and endothelin, while increasing prostacyclin [53]. Inoue et al. [54] reported on fluvastatin reducing IL-6, IL-1β, COX-1, and COX-2 and increasing PPARα and PPARγ, in response to different stimuli (specifically lipopolysaccharides, phorbol 12-myristate 13-acetate, and TNFα). However, the current study is the first to our knowledge, showing marked decrease of IL-6, IL-8, VCAM-1, and PAI-1 following fluvastatin application to HCAEC, in combination with SAA. In a rabbit model, fluvastatin was reported to reduce TF expression and content of macrophages at atherosclerotic lesions [55]. Fluvastatin has pleiotropic, anti-inflammatory, and antiatherogenic effects including suppression of leukocyte cytokine release, reduction in ROS, amelioration of platelet hyperreactivity, and smooth muscle cell proliferation [6, 56]. Statins prevent oxidative stress and increase vascular nitric oxide (NO) production, so even acute use with intravenous application has been suggested [57]. One fact leading to suppressive effects in inflammatory processes is that by inhibiting mevalonate synthesis, isoprenylation of small GTP-binding proteins is also inhibited, which is required for maintaining NADPH oxidase activity [58] and Ras-like proteins (Rho, Rac). Important for improving endothelial function is

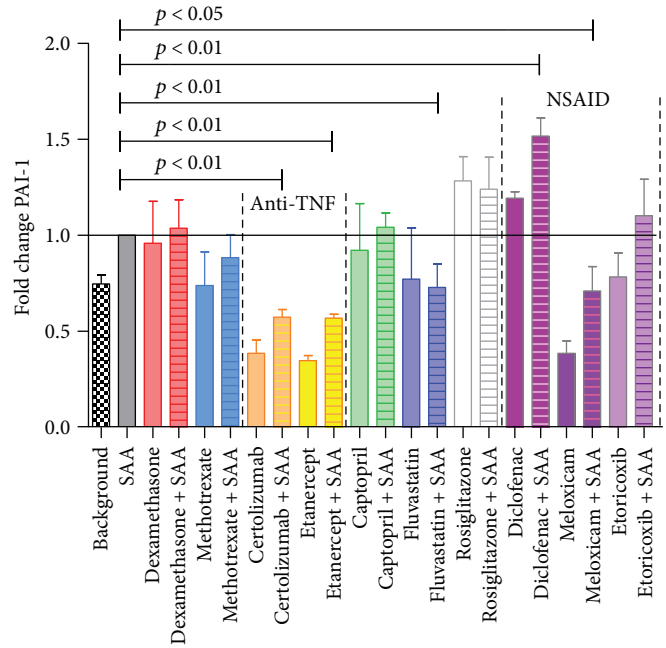

Figure 3

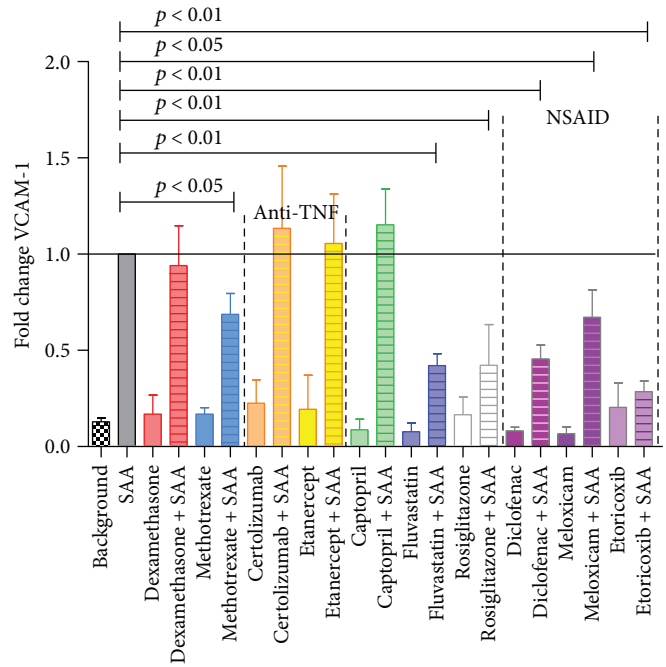

Figure 4

that statins induce eNOS through various mechanisms and eNOS-deficient mice are resistant to statin-mediated cardioprotection, mainly due to limiting adherence and leukocyte accumulation [35, 56].

Methotrexate also lowered the effects of SAA on inflammatory cytokines IL-6, IL-8, and sVCAM-1 in HCAEC. We used a final concentration of 1 μM, as doses 0.1–1 μM represent levels achieved in vivo with a low-dose regimen [59]. MTX is known to significantly reduce risk of CV disease in RA and, in contrast to COX-2 inhibitors, demonstrate also atheroprotective properties [60–62]. Besides the reported improvement of systemic autoimmune patient lipid profile [6], Yamasaki et al. [63] found decreased ICAM and VCAM expression with MTX treatment in HUVEC, which was confirmed by Johnston et al. who showed that MTX anti-inflammatory action is predominantly due to suppression of adhesion molecules (e.g., ICAM and cutaneous lymphocyte antigen) through

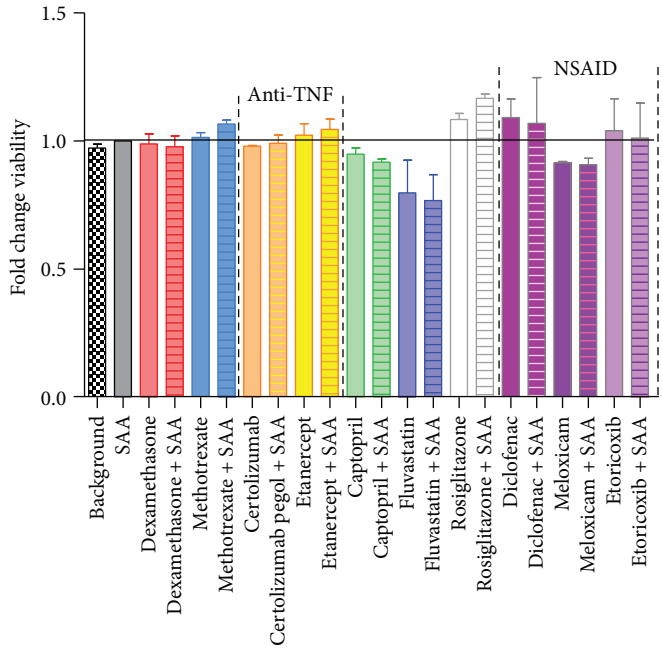

Figure 5

adenosine-mediated or polyglutamate MTX [64]. MTX also decreased AS lesion size, inhibited macrophage migration, and lowered TNFα-stimulated HUVEC expression of proinflammatory cytokines (e.g., TNFα, vascular adhesion protein 1, IL-1β, CXCL2, and TLR2) [65].

While dexamethasone (5 μM) did not affect the levels of proinflammatory cytokines or adhesion molecules in our HCAEC model system, EULAR recommendations promote dexamethasone in early arthritis, at doses 7–10 mg/day for less than 6 months [6], while long-term standard therapy is suggested for other rheumatic diseases, such as giant cell arteritis. Dexamethasone reduced IL-6, IL-8, and PGE_2 induced by IL-1β in osteoarthritic and RA fibroblasts [66]; reduced IL-6 and only minor IL-8 in HUVEC in response to TNFα [67]; and decreased constitutive MCP-1, but not induced MCP-1 by TNFα [68]. Surprisingly, researchers found connections with thromboembolic events and acceleration of inflammation during inflammatory disease states in long-term GC use with increased acute myocardial infarction and CV events [6, 69–71]. This could be due, in part, to nonresponse of IκB transcription to dexamethasone in endothelial cells, contrary to HeLa and THP-1, where IκB is increased under GC thereby suppressing NF-κB [72]. High-dose (1 mM) dexamethasone primed HUVEC for higher expression of adhesion molecules (VCAM, ICAM, and E-selectin) enhancing neutrophil migration, as well as coagulation/fibrinolysis with increased expression of vWf, PAI-1, and tissue factor [73].

In our assays, we used two different anti-TNFα biological drugs, specifically etanercept (soluble TNFR2 fusion protein with Fc fragment of human IgG) and certolizumab (human Fab fragment binding TNFα with attached pegol to improve pharmacokinetics). Both showed significant decreases in only PAI-1 in our SAA-treated HCAEC (Figure 3), while not exerting major effects on proinflammatory IL-6 or IL-8. Data suggest that neutralizing soluble TNFα is not sufficient to attenuate gastrointestinal Crohn's disease [74, 75]. The influence of TNFα inhibitors on CV events in RA patients is still elusive, since many studies on larger sample sizes report different results, but an overall trend to reduce CV disease is indicated [76, 77].

We have previously tested for detection of released levels of TNFα from SAA-stimulated HCAEC and found them to be very low [78]. That is why TNFα has not been included in the compilation of tested molecules, for example, IL-6, IL-8, PAI-1, and VCAM-1, in this study. TNFα itself had been previously tested as a single inducer of HCAEC and was shown to upregulate GROα, IL-6, IL-8, and MCP-1 [79]. Consequently, it would be of further interest to determine the effects of drugs, such as anti-TNFα inhibitors, methotrexate, and steroids on TNFα-activated HCAEC. In such a model, one might speculate that besides etanercept acting to block circulating TNFα levels, another hypothetical mode of action could, in part, also come from etanercept binding to the transmembrane form of TNFα [75], which could be tested for.

Captopril did not act inhibitory for any of the tested molecules in HCAEC, with only an increased effect on IL-6 observed. Protection of bovine endothelial cells against oxidative stress-induced apoptosis was shown with captopril [80], while reduced ROS, glutathione (GSH) consumption, and inhibition of NF-κB activation were observed with the ACE inhibitor zofenoprilat in HUVEC [81]. There was a short-term antioxidant suppressive effect on redox-sensitive NF-κB activation with captopril reported in sarcoma cells [82], while a long-term role in activating NF-κB and transcription of only certain, protective proteins was suggested, such as manganese superoxide dismutase

[83]. Captopril was shown to increase prostacyclin and reduce PAI-1 in porcine aortic endothelial cells and smooth muscle cells [84, 85].

Many beneficial effects were suggested for PPARγ activity, starting with influencing endothelial dysfunction [86, 87]. PPARγ is constitutively active in endothelial cells, suppressing adhesive molecules [88] and cytokine/chemokine expression caused by NF-κB and AP-1 activation. TZDs have been shown to reduce superoxide generation and inhibit expression of VCAM-1, ICAM-1, and lectin-like oxidized LDL receptor and hence inhibit inflammation of endothelial cells [89–92], suggesting an important role of endothelial PPARγ in the development of AS. Our results confirm the data by Xin et al. [93] who reported that a PPARγ agonist (in our case, rosiglitazone in the presence/absence of SAA) increased PAI-1 above background, and further reports indicate decreased levels of VCAM-1 in HUVEC [86, 92], whereby we also show attenuated levels of IL-6 in HCAEC.

All NSAIDs, diclofenac, meloxicam, and etoricoxib, significantly lowered the adhesion molecule VCAM-1 in SAA-treated HCAEC as compared to untreated (Figure 4). Besides this beneficial effect, we observe that diclofenac increased PAI-1, while meloxicam elevated IL-8. Etoricoxib was the only NSAID used in our study to lower both proinflammatory IL-6 and IL-8, while meloxicam was the only NSAID significantly lowering PAI-1 in SAA-stimulated HCAEC (Figures 1–3).

Few studies have been published on the effects of NSAIDs at the cellular levels, making direct comparisons difficult. When etoricoxib was administered preoperatively to patients requiring hip replacement surgery, there was a significant reduction in IL-6 levels in patient plasma observed, with better pain relief, after the surgery [94], which together with our study indicates that etoricoxib could be the NSAID of choice, for lowering proinflammatory cytokines, such as IL-6 and IL-8. Rainsford et al. [95] reported on the effects of meloxicam on human and porcine cartilage explants, as well as human synovial tissue explants. They observed that meloxicam did not affect synovial production of the proinflammatory IL-1 or IL-8 but significantly increased IL-6. This is closer to our study, which otherwise shows an elevation in IL-8 but unchanged IL-6 in HCAEC. Chu et al. [96] reported that meloxicam suppressed PAI-1 secretion from *ex vivo* cultured human osteoarthritic cartilage, meniscus, and synovium at 48 h, similarly, as we currently report for HCAEC at 24 h. However, as the 2010 review on diclofenac showed [42] the modalities of action of NSAIDs could extend well beyond COX inhibition, to further modulate substrate P, peroxisome proliferator activated receptor γ, acid sensing ion channels, and nitric oxide-cGMP antinociceptive pathway, among others.

The reason for the specific responsiveness of HCAEC to different drugs, contrary to other types of cells, could be that the endothelium of arteries (versus veins) exhibits (a) specific and intrinsic expression patterns and unique response profiles leading to inflammation and atherosclerosis and (b) greater susceptibility of HCAEC to inflammatory stimuli, specifically pathological concentrations of SAA and IL-1β, as opposed to HUVEC and HMVEC [17, 18].

However, our model has some more or less obvious limitations. One limitation related to this cellular HCAEC experimental model is, at the same time, its benefit, namely that HCAEC are primary endothelial cells of the coronary artery, taken from the human body and expanded *ex vivo* and cultured *in vitro*. Thus, they represent a nonsynchronous population of cells and a more optimal model closely mimicking the situation in coronary arteries, as opposed to cell lines, which would otherwise give more homogeneous results, but would be further from the *in vivo* situation. Furthermore, our HCAEC model portrays the limitation of looking at a single inducer (e.g., acute phase SAA), which never occurs *in vivo*; however, a chronically elevated acute phase response, even one conveying low-grade inflammation, is a threat to the coronary arteries and early development of cardiovascular diseases. Clear limitations of the current experimental HCAEC model are that tissue remodelling or vascular aging important in the development of atherosclerosis cannot be addressed, nor the effects of lifestyle changes, such as diet and/or exercise. On the other hand, a cellular model enables rapid screening for drug candidates, restricts the necessary number of animal experiments, and allows for an unlimited access to cells. Taken together, the marked and differential influence of the tested medications on SAA-activated HCAEC could be important for controlling atherogenesis in RA patients. In addition to the well-known protective effects of methotrexate, confirmed by the current study (e.g., lowering of IL-6, IL-8, and VCAM-1), there was a lack of response observed with anti-TNFα inhibitors, presumably due to the fact that SAA itself does not induce TNFα in HCAEC [78]. In regard to the lack of response of SAA-treated HCAEC to dexamethasone, there could be several considerations: (a) hydrocortisone is present in the endothelial cell medium, which could already mask some of the effects; (b) dexamethasone actually enhances inflammatory responses in ATP-induced endothelial cells [97], and high-dose dexamethasone sensitizes HUVEC to the effect of inflammatory mediators and induces a proadhesive environment [73]; (c) dexamethasone exerted limited effects on TNFα- or IL-1β-treated HUVEC at 24 h on the gene expression of IL-6, IL-8, and VCAM-1 [98], similar to our model; and (d) long-term use of glucocorticoids increased the rate of acute myocardial infarction and cardiovascular events [6]. One explanation is that dexamethasone does not increase IκBα in endothelial cells, as it does in other cell types, such as monocytes and lymphocytes [72], providing a mechanism of why dexamethasone does not inhibit inflammatory responses in HCAEC.

Finally, we emphasize the beneficial role of fluvastatin in our model of primary human coronary artery endothelial cells. It is interesting to speculate whether the beneficial effects in HCAEC of fluvastatin could be the consequence of epi-lipoxin A_4, a potent anti-inflammatory mediator, produced from the nitrosylated COX-2 (in absence of acetylation) via iNOS and eNOS [39].

In the future, more data on patients already taking fluvastatin could be beneficial, in order to determine possible effects in preventing premature atherosclerosis and CV disease in RA.

Disclosure

K. Lakota and D. Hrušovar shared the first coauthorship.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

K. Lakota and D. Hrušovar contributed equally to the work.

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Hindawi
Mediators of Inflammation
Volume 2018, Article ID 6548715, 8 pages
https://doi.org/10.1155/2018/6548715

Research Article

Prevalence and Determinants of Peripheral Microvascular Endothelial Dysfunction in Rheumatoid Arthritis Patients: A Multicenter Cross-Sectional Study

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Received 26 July 2017; Revised 27 October 2017; Accepted 12 November 2017; Published 1 February 2018

Academic Editor: Shin-ichi Yokota

Objectives. To define the prevalence and determinants of peripheral microvascular endothelial dysfunction (ED) in a large series of rheumatoid arthritis (RA) patients free of previous cardiovascular events. *Materials and Methods*. Data from 874 RA patients enrolled in the EDRA study (Endothelial Dysfunction Evaluation for Coronary Heart Disease Risk Estimation in Rheumatoid Arthritis—ClinicalTrials.gov: NCT02341066) were analyzed. Log-transformed reactive hyperemia index (Ln-RHI) was evaluated by peripheral arterial tonometry (PAT) using the EndoPAT2000 device: values of Ln-RHI < 0.51 were considered indicative of peripheral ED. *Results*. Peripheral microvascular ED was documented in one-third of RA patients (33.5%); in multiple logistic regression analysis, ACPA negativity and higher triglycerides concentrations were independently associated with the presence of peripheral ED [OR (95% CI) = 1.708 (1.218–2.396), $p < 0.01$ and OR (95% CI) = 1.005 (1.002–1.009), $p < 0.01$, respectively]. Multiple regression analysis showed a positive correlation between Ln-RHI values and systolic blood pressure and HDL cholesterol levels; furthermore, higher values of Ln-RHI were associated with ACPA positivity, while smoking habit was associated with lower Ln-RHI values. *Conclusions*. This study demonstrates for the first time a high prevalence of peripheral microvascular ED in patients with RA free of previous cardiovascular events that appear to be only partially driven by traditional cardiovascular risk factors. The association between ACPA negativity and ED warrants further exploration.

1. Introduction

Rheumatoid arthritis (RA) is a chronic progressive disease associated with systemic inflammation that mainly affects synovial joints leading to tissues destruction, disability, and excess of mortality.

RA patients suffer a significantly reduced life expectancy (by 3 to 18 years) with respect to the general population with

a standardized mortality ratio ranging from 1.2 to 2.7 [1]. This excess of mortality in RA patients has not changed over the past 20 years [2].

About one-third of premature deaths in RA are due to cardiovascular disease (CVD) [3], primarily coronary heart disease (CHD). Mortality risk for CHD in RA patients has been estimated to be >50% higher than the general population [4]. Moreover, unlike the general population, global CV mortality in RA has not appeared to have fallen over time [5] despite relevant improvements in early diagnosis and treatment.

This excess of CHD is not fully explained by the higher prevalence of traditional CV risk factors (smoking, dyslipidemia, hypertension, and diabetes) in RA patients with respect to the general population [6, 7]. Thus, it is conceivable that other nonconventional risk factors, likely related to systemic inflammatory RA burden, may be involved in chronic vascular atherosclerotic damage ultimately resulting in CHD and global cardiovascular disease. Therefore, there is an urgent need to develop novel CV risk scores encompassing novel risk factors to provide a more reliable estimate of CV risk in RA.

A significant impairment of both the compliance of the central arterial system, termed arterial stiffness, and the endothelial function was frequently reported in the RA population [8, 9].

Endothelial dysfunction (ED), the earliest pathological alteration of the arterial wall in atherosclerosis, is a measure of impaired nitric oxide (NO) synthesis and availability, hence a reduced vasodilatory and atheroprotective function. ED is associated with virtually all known CV risk factors [10] and independently predicts the risk of future CV events in the general population [11]. Therefore, measuring ED should be seen as a valuable tool for CV risk stratification, over and above established scoring systems such as the Framingham Risk Score (FRS).

However, a poor correlation between peripheral microvascular and macrovascular endothelial function has been reported in RA patients [12, 13]. Furthermore, until now, very little attention has been paid to the assessment of microvascular ED and its associations with a comprehensive panel of clinical and demographic factors in this population.

Microvascular ED can be evaluated noninvasively by laser Doppler imaging (combined with iontophoresis of acetylcholine and sodium nitroprusside) to the forearm and by pulse amplitude tonometry (PAT) of the small digital artery.

PAT has recently gained attention as a useful tool to measure peripheral microvascular ED in an outpatient setting because it is a simple, rapid, noninvasive, and operator-independent technique. Briefly, PAT measures reactive hyperemia of the small digital artery (sa-RH) after an ischemic stimulus in the forearm. PAT shows high grade of correlation with gold standard measures of coronary ED [14]. Moreover, ED determination by PAT is related to CV risk factors in the Framingham cohort [15] and has proven to predict CHD [16] and future CV events [17, 18].

To the best of our knowledge, the available evidence on peripheral microvascular ED evaluated by PAT in RA is limited to a single study enrolling 55 patients [19]. In this study, ED did not show any significant associations with conventional cardiovascular risk factors and its prevalence was not assessed.

Therefore, we sought to determine prevalence and determinants of peripheral microvascular ED by PAT in a large RA population free of previous overt CVD.

2. Patients and Methods

2.1. Patient Selection. We reported data on the peripheral microvascular endothelial function of 874 RA patients aged 45–85 years without evidence of clinically overt cardiovascular disease. Patients were prospectively enrolled in the multicenter 3-year prospective cohort EDRA study (Endothelial Dysfunction Evaluation for Coronary Heart Disease Risk Estimation in Rheumatoid Arthritis study (EDRA) – ClinicalTrials.gov: NCT02341066) between October 2015 and July 2017. The EDRA study was approved by the Azienda ASL 1 of Sassari (Italy) Institutional Review Board (2126/CE-2015) and was conducted in accordance with the guidelines of our institutional ethics committees and the Declaration of Helsinki. Written informed consent was obtained from each patient before participation.

The EDRA study aimed to evaluate the incremental value of ED, assessed by Endo-PAT, when added to the FRS, in predicting CHD events in a cohort of 3000 RA patients. Inclusion criteria were (a) men and women aged > 45 and <84 years, and (b) RA as defined by the ACR/EULAR 2010 RA classification criteria [20]. Exclusion criteria were (a) previous CV or cerebrovascular events (acute coronary syndrome, stable angina, stroke, interventional procedures, carotid endarterectomy, and symptomatic peripheral artery ischemia), (b) abnormal ECG at rest, (c) sign or symptoms of autonomic nervous system dysfunction, (d) serious infections in the previous 6 months, (e) concomitant severe illness (overt hepatic insufficiency and renal disease, GFR < 30 ml/min, Cockroft-Gault formula), (f) recent diagnosis of cancer, and (g) pregnancy.

The EDRA study is ongoing at three sites, two regional hospitals (site 1, Sassari; site 2, Cagliari) and one central (site 3, Roma) hospital. A total of 270 patients (100 at site 1, 150 at site 2, and 20 at site 3) denied informed consent; 5 Endo-PAT tests were not reliable to low signal; 3 Endo-PAT tests were not correctly performed due to severe digital deformities related to RA. A total of 874 Endo-PAT tests where then available for the subsequent analysis.

2.2. Baseline Characteristics. The following baseline characteristics were registered on the same day of PAT assessment: hypertension (blood pressure ≥ 140/90 mmHg or treatment with antihypertensive medications), diabetes mellitus (patient history and/or treatment with insulin or oral hypoglycaemic agents), family history of CHD in first-degree relatives, and smoking habit. We also performed a 12-lead conventional ECG. Lipid profile data (total HDL and LDL cholesterol and triglycerides concentrations, all expressed in mg/dL) collected within 3 months prior to the study as routine clinical practice were registered. To assess potential correlations between the RA phenotype and

Table 1: Demographics and cardiovascular risk factors of RA population.

	Overall $n = 874$	ED $n = 293$	No ED $n = 581$	p value
Age (yrs)	60.9 ± 9	61.3 ± 9	60.7 ± 9	ns
Smoking habit (%)	20.8	25.2	18.6	0.02
Hypertension (%)	44	48	43	ns
Systolic blood pressure (mmHg)	127.8 ± 16	125.4 ± 15	129 ± 17	0.002
Diastolic blood pressure (mmHg)	76.9 ± 9	75.8 ± 8	77.4 ± 10	0.02
BMI (kg/m^2)	28.3 ± 33	29.4 ± 32	27.8 ± 34	ns
Dyslipidemia (%)	32.5	28.3	34.7	ns
Diabetes (%)	7.4	8.3	6.9	ns
Total cholesterol (mg/dL)	205.7 ± 36	207.8 ± 36	204.7 ± 36	ns
HDL cholesterol (mg/dL)	61.3 ± 15	60.2 ± 16	61.8 ± 15	ns
LDL cholesterol (mg/dL)	124.5 ± 31	126.2 ± 31	123.7 ± 32	ns
Triglycerides (mg/dL)	98 ± 44	103.7 ± 48	95.3 ± 42	0.01

ns: not significant.

peripheral ED, the following disease specific scores, disease descriptors, and treatment data were recorded and collected: steroid treatment; cumulative steroid dose in the last month; treatment with synthetic or biological disease-modifying antirheumatic drugs (DMARDs); number of swollen joints; number of tender joints; C-reactive protein (CRP) concentrations, mg/dL; erythrocyte sedimentation rate (ESR), mm/h; Disease Activity Score-28 (DAS-28); Health Assessment Questionnaire (HAQ); positivity for IgM-rheumatoid factor (IgM-RF); and anticitrullinated cyclic peptide antibodies (ACPA).

2.3. Endo-PAT. Patients were studied in a fasting state. Antihypertensive drugs were withheld on the study day. Finger probes consisting of thimble a-shaped sensor cap which register pulsatile volume changes were placed on the middle finger of each subject's hand. Changes in digital pulse amplitude were sensed by pressure transducers, filtered, amplified, and then recorded for further analysis by the EndoPAT 2000 device (Itamar Medical Inc., Caesarea, Israel).

After a 5 min baseline measurement, arterial flow in the brachial artery was interrupted by a cuff placed on a proximal forearm and inflated to 200 mmHg or 60 mmHg above baseline systolic blood pressure for 5 min. Then, the cuff was deflated and the digital pulse amplitude was recorded for a further 6 min. The ratio of the postischemic pulse amplitude signal compared with baseline was calculated, normalized for the baseline signal, and indexed to the contralateral one. The log-transformed ratio, expressed as Ln-RHI, reflects the small artery reactive hyperemia. Bonetti et al. reported that a RHI value of < 1.67 (corresponding to a Ln-RHI < 0.51) had a sensitivity of 82%, a specificity of 77%, and an AUC of 0.82 for diagnosing coronary ED [14]. Therefore, we used a Ln-RHI cutoff value <0.51 to define the presence of a significant ED.

2.4. Statistical Analysis. Continuous variables are presented as mean ± SD whereas categorical variables are presented as frequencies (n) or percentages (%). Variables with a nonnormal distribution were log-transformed for further analysis. Univariate association was tested by Pearson correlation analysis or by Mann–Whitney U test analysis. Multiple linear regression analysis was performed to analyze linear correlation between predictors and Ln-RHI. The variables related to ED with a $p < 0.05$ at the univariate logistic regression analysis entered into a multivariate logistic regression model in which the "presence of ED" was the variable to be explained. Results are expressed as the odds ratio (OR) and 95% confidence interval (95% CI). Analyses were performed using SPSS (Version 20, SPSS Inc., Chicago, IL, USA). A $p < 0.05$ was considered statistically significant.

3. Results

The demographic, cardiovascular, and biochemical characteristics of RA patients are shown in Tables 1 and 2.

The median Ln-RHI value for the overall RA population was 0.67 ± 0.3. Men had a trend towards lower median Ln-RHI values than women (0.63 ± 0.3 versus 0.68 ± 0.3, $p = 0.055$). One-third (33.5%) of RA patients exhibited peripheral ED (Ln-RHI < 0.51) (Table 3).

In a bivariate correlation analysis, Ln-RHI was positively, albeit weakly, correlated with systolic blood pressure, ACPA positivity, and HDL cholesterol concentrations. RHI was also inversely correlated with smoking habit (Table 4). In multiple regression analyses, systolic blood pressure, HDL cholesterol concentrations, and ACPA positivity remained independently associated with higher Ln-RHI whereas smoking was independently associated with lower Ln-RHI (Table 4).

After stratification for the ACPA status, none of the previously identified factors was independently associated with Ln-RHI in ACPA-negative patients. On the other hand, the effect of systolic blood pressure (B coefficient 0.002; 95% CI 0.001–0.004) and smoking (B coefficient −0.088; 95% CI from −0.162 to −0.015) was confirmed in ACPA-positive patients whereas disease duration (B coefficient per year −0.000; 95% CI from −0.001 to −0.000) was

Table 2: RA descriptors.

	Overall $n = 874$	ED $n = 293$	No ED $n = 581$	p value
Disease duration (months)	131.8 ± 116	141.1 ± 116	127 ± 115	ns
ACPA positivity (%)	62.8	55.9	66.2	0.009
IgM-RF positivity (%)	67	62.8	69	ns
ESR (mm/h)	26.4 ± 20	25.9 ± 21	26.7 ± 20	ns
CRP (mg/dL)	0.59 ± 0.9	0.56 ± 0.7	0.61 ± 0.9	ns
DAS-28	3.53 ± 1.3	3.51 ± 1.2	3.52 ± 1.3	ns
HAQ	0.75 ± 0.6	0.79 ± 0.7	0.73 ± 0.6	ns
Steroid use (%)	37.6	38.4	37.2	ns
Steroid dose (mg/day)	2.9 ± 3.9	3 ± 4.6	2.8 ± 3.6	ns
Cumulative steroid dose (mg/month)	87 ± 119	91.2 ± 139	84.9 ± 108	ns
NSAID use (%)	23.4	25.1	22.6	ns
DMARD use (%)	78.6	78.8	78.5	ns
TNFi use (%)	28.7	29.1	28.5	ns
Tocilizumab use (%)	7.2	7.2	7.1	ns
Abatacept use (%)	5.1	5.1	5.1	ns
Rituximab use (%)	2.4	2.4	2.4	ns

ns: not significant.

Table 3: Peripheral endothelial dysfunction by EndoPAT2000 in RA population.

	Overall $n = 874$	Males $n = 213$	Females $n = 661$	p value
Ln-RHI	0.67 ± 0.32	0.63 ± 0.31	0.68 ± 0.33	0.055
Ln-RHI ≤ 0.51	293 (33.5)	35.2	33	ns
Ln-RHI ≤ 0.44 (1Q)	223 (25.5)	25.6	25.4	ns

Values are expressed as median ± 1SD; Ln-RHI: logarithmic reactive hyperemia index; 1Q: first quartile.

independently associated with lower Ln-RHI in this subgroup (Supplementary file (available here)). However, these findings cannot be considered reliable as the study sample was not designed for this kind of subanalysis.

RA patients with pathological Ln-RHI values had lower systolic blood pressure and diastolic blood pressure, higher levels of triglycerides, a higher BMI, and a longer disease duration (Table 5). RA patients with ED were in higher percentage smokers compared to patients with normal Ln-RHI values. A higher frequency of ACPA negativity was found among patients with ED, compared to patients without ED (Table 5).

In logistic regression analysis, ACPA negativity and higher serum triglyceride concentrations were independently associated with the presence of peripheral ED, whereas higher systolic blood pressure values were modestly associated with a reduced risk of ED (Table 5). In multiple logistic regression analysis, ACPA negativity was the factor that was most strongly associated with the presence of peripheral ED [OR (95% IC) = 1.708 (1.218–2.396); $p < 0.01$] even after adjustment for smoking habit (Table 5).

Other than expected, we found no significant relationship between measures of inflammatory burden (ESR and CRP), disease severity (DAS28 and HAQ), RA treatment patterns (use and dosage of steroids, use of synthetic DMARDs, and use of biological DMARDs), and microvascular reactivity (data not shown).

4. Discussion

Despite an increasing number of studies assessing ED in RA, mostly based on the measurement of flow-mediated dilatation (FMD) of the brachial artery [12], the prevalence and the factors associated with its presence remain largely unknown.

To our knowledge, this is the first study assessing microvascular endothelial function by PAT in a large series of prospectively enrolled RA patients without previous cardiovascular events. Our results are of interest in basic research and, possibly, in clinical practice.

We used PAT technology, instead of brachial FMD, due to its independence of operator, easy of use, and simplicity in implementation in our outpatient clinics. Furthermore, although largely based on the same physiological mechanism (endothelium-dependent vasodilation), peripheral microvascular and macrovascular endothelial function are shown to be largely independent from each other in RA [13]. No significant correlation between FMD and PAT, after adjustment for confounders, was reported in two large community studies [21, 22]. Similarly, a small study found no association between FMD and PAT in SLE, an autoimmune rheumatic disease, sharing with RA some common pathogenetic mechanisms and systemic features [23].

This lack of concordance between PAT and FMD may suggest distinct pathophysiologies in conductance vessels and digital microvascular bed.

The primary novel finding of this study is that up to a third of RA patients free of previous cardiovascular

Table 4: Independent determinants of Ln-RHI.

Independent variable	Bivariate correlation Spearman rho	Univariate linear regression B coefficient (95% IC)	Multiple linear regression B coefficient (95% IC)
Systolic blood pressure (mmHg)	0.10*	0.002 (0.001–0.003)*	0.003 (0.001–0.004)*
ACPA positivity	0.10*	0.054 (0.006–0.102)^	0.089 (0.035–0.144)*
Smoke habit	−0.10*	−0.078 (from −0.132 to −0.025)*	−0.085 (−0.153–0.017)^
Dyslipidemia	0.08^	0.060 (0.008–0.111)^	
BMI	−0.086^		
HDL cholesterol (mg/dL)	0.073*	0.001 (0.000–0.003)^	0.002 (0.000–0.004)^
Triglycerides (mg/dL)	−0.07^	−0.001 (from −0.001 to −0.000)^	
Disease duration	−0.06^		

A linear regression for multiple variables (stepwise method) was performed including into the model variables showing significant association ($p < 0.05$) with the dependent variable Ln-RHI at the univariate regression analysis. Age and gender were forced in the model. ^$p < 0.05$, *$p < 0.01$.

Table 5: Independent determinants of peripheral ED.

	ED $n = 293$	No ED $n = 581$	Binary logistic analysis OR (95% IC)	Multivariate logistic analysis OR (95% IC)	Cox and Snell R^2
Systolic blood pressure (mmHg)	125.4 ± 15.5	129 ± 17.1	0.98 (0.97–0.99)[a*]	0.98 (0.97–0.99)[a*]	
Triglycerides (mg/dL)	103.7 ± 48.2	95.4 ± 42.7	1.004 (1.001–1.007)[b^]	1.005 (1.002–1.009)[b*]	0.04
ACPA negativity [n (%)]	44.1	33.8	1.546 (1.126–2.122)*	1.708 (1.218–2.396)*	
Smoking habit [n (%)]	25.2	18.6	1.468 (1.047–2.059)^	—	
Diastolic blood pressure (mmHg)	75.9 ± 8.9	77.4 ± 10.2	0.98 (0.97–0.99)^	—	
BMI (kg/m^2)	29.5 ± 32.5	27.9 ± 34.4	—	—	
Disease duration (months)	141.2 ± 116.8	127.1 ± 115.8	—	—	

Odds ratio (OR) is based on the risk of the dependent variable (low Ln-RHI) given the presence of the independent variable. 95% CI: 95% confidence interval. Multivariate logistic analysis with backward logistic regression method has been performed including in the model variables showing significant ($p < 0.05$) association with the dependent variable (low Ln-RHI) at the binary logistic analysis. *$p < 0.01$; ^$p < 0.05$; [a]per mmHg; [b]per mg/dL.

events exhibit significant peripheral microvascular ED, as demonstrated by an impairment of microvascular hyperemic response.

Of note, lower peripheral microvascular vasodilatory function has proven to significantly predict the risk of future coronary events [16, 24] in the general population: a relative risk of 0.76 (95% CI 0.65–0.88) per each 0.1 increase of Ln-RHI was reported from a meta-analysis of 6 studies reporting prospectively collected data on cardiovascular outcomes [18]. Therefore, it is conceivable that a high prevalence of peripheral microvascular ED might translate into accelerated atherosclerosis and increased risk of future cardiovascular events also in RA population.

In the present investigation and consistent with previous reports, only a weak correlation was observed between microvascular reactive hyperemia and major conventional cardiovascular risk factors, such as advancing age and gender [14, 15].

Unexpectedly, Ln-RHI correlated positively with systolic blood pressure; the same result was obtained from 3 large community-based studies involving in total over 7500 subjects [15, 22, 25]. The mechanism behind this association is not well understood, but the possibility that factors related to blood pressure-dependent brachial artery blood flow may significantly impact on RHI cannot be ruled out. Indeed, Lee et al. demonstrated a close relationship between basal blood flow in the brachial artery and reactive hyperemia-induced changes in the digital artery diameter and flow velocity [26]. It could be hypothesized that a higher basal pulse amplitude results into a higher microvascular reactivity. However, further research in experimental models and humans is warranted to address this issue.

Total cholesterol and LDL cholesterol concentrations were not associated with impaired microvascular reactivity in our series of RA patients. However, it should be taken into account that RA patients exhibit significantly lower concentrations of these lipid fractions with respect to the general population [27]. Accordingly, the increased CVD risk related to RA has shown to be paradoxically associated to relatively low cholesterol concentrations, a phenomenon known as the "lipid paradox" that has been related to systemic inflammation [28]. On the contrary, HDL cholesterol concentrations were positively associated with higher microvascular reactivity in this study. Of note, HDL cholesterol has shown to be protective on the endothelium, increasing the endothelium nitric oxide synthase- (eNOS-) mediated production of the vasodilator NO [29].

Similar to our findings, Ferré et al. in a series of 816 subjects at intermediate to high cardiovascular risk reported that

HDL cholesterol was the main determinant of microvascular reactivity [30].

Similarly to previous reports [31], hypertriglyceridemia and smoking were inversely associated with RHI in our population. Smoking habit negatively impacts on macro and microvascular bed vasodilatory capacity trough impaired eNOS-related NO availability. Therefore, pharmacological and nonpharmacological measures aimed at smoking cessation, increasing HDL cholesterol, and reducing triglycerides concentrations may have a significant positive impact on peripheral ED in RA.

Failure of other traditional cardiovascular factors in showing significant associations with Ln-RHI suggests that further "unexplained" factors, such as systemic inflammation, may drive microvascular reactivity in RA.

However, in this study, other than expected, we were not able to find significant correlations between measure of systemic inflammation (CRP, ESR, and DAS-28) and digital hyperemic response.

Similarly, in age- and sex-adjusted analyses, no significant relationship was demonstrated between CRP and PAT ratio in the large cross-sectional study by Hamburg et al. from the Framingham cohort [15].

Therefore, interactions between inflammation, conventional cardiovascular risk factors, and vascular function, more than inflammation alone, may explain impairment of microvascular reactivity in RA patients.

ACPA and RF are the most characteristic RA-specific autoantibodies. ACPA-positive RA patients differ from seronegative ones in genetic and environmental risk factors and response to treatment. The presence of a significant relationship between autoantibody positivity, including ACPA, and ED accelerated atherosclerosis, CVD incidence, and mortality in RA is a matter of debate.

A significant process of protein citrullination has been demonstrated into atheroma suggesting that ACPA positivity mirrors ongoing accelerated atherosclerosis. However, ACPA positivity was not related to carotid intima-media thickness in a cross-sectional controlled study in RA patients [32]. Similarly, in a prospective study looking at identifying parameters associated with the development of subclinical atherosclerosis in a very early arthritis cohort, ACPA positivity was associated with thinner carotid intima-media thickness after 7 years of follow-up [33]. By contrast, available data on ACPA status and microvascular dysfunction in a previous study [19] reported significantly lower mean RHI values in 33 ACPA-positive patients when compared to 22 ACPA-negative RA patients (RHI = 1.78 versus 2.19, respectively, $p = 0.008$) [17].

A large study including 937 RA patients reported an association between ACPA positivity and risk of CHD (OR 2.58, 1.17–5.65) [34] while other studies did not observe a significant relationship between ACPA status and CVD incidence in RA population [35, 36]. In the Women Health Initiative study following postmenopausal women with self-reported RA, ACPA positivity was not significantly associated with higher rates of incident CVD morbidity or mortality. Accordingly, RF, but not ACPA, was related to CVD mortality in a longitudinal observational study of US veterans with RA [37]. Furthermore, in a recent Canadian prospective multicentre inception cohort study of 2626 RA patients, cardiovascular event rates in seropositive versus seronegative subjects were not significantly different. Although seropositivity was not associated with incident cardiovascular events in multivariable Cox regression models, the calculated relative risk was 0.81, suggesting a potential protective effect of ACPA positivity towards cardiovascular disease [38].

Collectively taken, these data do not clearly support a significant correlation between ACPA positivity and atherosclerotic CVD burden in the RA population. Even considering this scenario, our data showing a significant negative association between ACPA status and microvascular peripheral ED are unexpected and a firm biologically plausible explanation is lacking. Therefore, further studies are needed to explore whether ACPA may act (in)directly to prevent ED or merely associate to specific factors involved in endothelial function in RA patients.

The main limitation of our work was that RA patients were under treatment for the control of cardiovascular risk factors at the moment of PAT evaluation. Nevertheless, the treatment regimen had no significant effect on PAT measures in multiple logistic regression analysis (data not shown).

Moreover, the observational design of this study did not enable us to make conclusive considerations about cause-and-effect relationship and direction of association between microvascular function and covariates.

Finally, we did not use a comparator, for example, healthy subjects. However, although the comparison between patients and the general population could be of general interest for the understanding of accelerated atherosclerosis in RA, the purpose of the EDRA study was to investigate ED as a risk factor for new CV events in RA, and therefore it did not include a control population.

5. Conclusions

This is the first study to show that small artery reactive hyperaemia as measured by PAT is reduced in up to a third of RA patients free of previous cardiovascular events. HDL cholesterol and systolic blood pressure were positively associated with RHI, whereas smoking, ACPA negativity, and triglycerides concentrations were inversely associated with RHI. Furthermore, systemic inflammation per se does not appear to influence peripheral ED in the RA population. The negative association between ACPA and peripheral ED warrants further exploration in prospective studies.

We expect that the prospective data from the EDRA study will offer more conclusive data on the clinical relevance of microvascular ED evaluation by PAT in improving the prediction of CVD in the RA population.

Conflicts of Interest

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

Acknowledgments

The EDRA study is a project funded by the Italian Ministry of Health and by Regione Sardegna (RAS): GR-2011-02352816, Ricerca Finalizzata 2011.

Supplementary Materials

Table S1: Independent determinants of Ln-RHI according to ACPA status. Table S2: Independent determinants of peripheral ED according to ACPA status. *(Supplementary Materials)*

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Hindawi
Mediators of Inflammation
Volume 2018, Article ID 3897295, 13 pages
https://doi.org/10.1155/2018/3897295

Review Article

Asymmetric Dimethyl Arginine as a Biomarker of Atherosclerosis in Rheumatoid Arthritis

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Received 13 September 2017; Accepted 27 November 2017; Published 18 January 2018

Academic Editor: Mirella Giovarelli

Cardiovascular disease is the main cause of morbidity and mortality in rheumatoid arthritis (RA). Despite the advent on new drugs targeting the articular manifestations, the burden of cardiovascular disease is still an unmet need in the management of RA. The pathophysiology of accelerated atherosclerosis associated to RA is not yet fully understood, and reliable and specific markers of early cardiovascular involvement are still lacking. Asymmetric dimethylarginine is gaining attention for its implication in the pathogenesis of endothelial dysfunction and as biomarkers of subclinical atherosclerosis. Moreover, the metabolic pathway of methylarginines offers possible targets for therapeutic interventions to decrease the cardiovascular risk. The purpose of this review is to describe the main causes of increased methylarginine levels in RA, their implication in accelerated atherosclerosis, the possible role as biomarkers of cardiovascular risk, and finally the available data on current pharmacological treatment.

1. Introduction

Patients with rheumatoid arthritis (RA) have a significantly higher risk of cardiovascular diseases (CVD) compared to general population, comparable to patients with diabetes mellitus or non-RA subjects 10 years older [1]. In RA patients, cardiovascular events account for over 50% of the excess premature mortality [2]. Accelerated atherosclerosis plays a pivotal role in the pathogenesis of RA-related CVD: indeed, in RA patients, the atherosclerotic process starts in the early phases of the disease and it is determined by both an increased prevalence of traditional risk factors and the inflammatory nature of RA itself [3, 4]. The systemic inflammation has a major role in the pathogenesis of accelerated atherosclerosis. Proinflammatory cytokines involved in the pathogenesis of RA, such as TNF, IL-1, and IL-6, are also involved in the development and in the progression of atherosclerotic plaque. The first step in plaque development is the activation of endothelial cells and the induction of endothelial dysfunction (ED) by proinflammatory cytokines. The proatherogenic and prothrombotic endothelium is characterized by upregulation of adhesion molecules, raised vascular permeability, cytokine and chemokine expression, and reduced production of vasodilatory molecules, such as nitric oxide [5]. ED is the earliest, reversible, preclinical phase of plaque development, leading to the accumulation of lipoproteins and inflammatory cells in the subendothelial layer and to subsequent plaque formation [5]. Other than activating endothelial cells, TNF and IL-6 activate monocytes and immune cells contributing to the progression of the atherosclerotic disease, until rupture and thrombotic complication of the plaque [6]. There is a growing interest around the prevention of CVD in RA patients, although there is no clear evidence that any intervention can actually reduce that risk [7]. Early identification of ED may allow clinicians to characterize patients with subclinical atherosclerosis, establishing early risk factor modification or pharmacological intervention [5]. The imbalanced production of endothelial vasoactive mediators is a key step in the development of ED. Nitric oxide (NO) is the main endothelial-derived vasodilatory and antiproliferative molecule, inhibiting activation and vessel wall adhesion of

leukocytes and platelets [8]. The impaired ability of endothelial cells to produce NO is a main driver of ED. Dysregulation of other vasoactive mediators of NO metabolism predispose to subsequent pathological abnormalities such as platelet activation, abnormal fibrinolytic activity, lipoprotein deposition, and oxidative stress: all these modifications contribute to impaired vascular integrity [5, 9]. The role of endogenous inhibitors of NO synthase (NOS) activity in the induction of ED has gained the attention of rheumatologists. Asymmetric dimethylarginine (ADMA) is an analogue of L-arginine—the precursor of NO—naturally released in biological fluids following proteolysis; it inhibits NO synthesis by competing with L-arginine at the active site of NOS [10]. ADMA emerged as novel markers of ED and cardiovascular risk in RA [11]. The aim of this review is to summarize the available data on the role of ADMA in the pathogenesis of ED in RA patients, its role as potential biomarkers of CVD risk, and the possible therapeutic interventions.

2. Methylarginine Metabolism

Dimethylarginines are naturally occurring endogenous products of the degradation of methylated proteins. Methylation of arginine residues is a posttranslational modification catalyzed by a family of enzymes called protein arginine methyltransferases (PRMTs) which use S-adenosylmethionine as source of methyl groups; methylation of arginine is a two-step process of monomethylation [12, 13]. The first methylation leads to the formation of monomethylarginine (MMA), while the second one can produce either symmetric dimethylarginine (SDMA) or ADMA, according to the PRMT isoform involved in the methylation reaction [14]. After their proteolysis, MMA, SDMA, and ADMA are released in the cytosol, where the asymmetric methylarginines (MMA and ADMA) inhibit NOS activity by competing with L-arginine for the active site of the enzyme [15]. Cationic amino acid transporters (CATs) are the transmembrane enzymes which carry out methylarginines and arginine from the cellular cytosol to extracellular fluids and then in the bloodstream [16]. In physiological conditions, intracellular levels of arginine are much higher than those required for NOS activity; however, intravenous supplementation of arginine can increase endothelial-dependent vasodilatation [17]. This apparently incongruous phenomenon is called "arginine paradox": several hypotheses have been proposed to explain this effect. The activity of the enzyme arginase, which converts arginine in ornithine and urea, may reduce the availability of arginine, decreasing NOS activity. However, arginine is converted by NOS in an intermediate state, the hydroxy-L-arginine, which inhibits arginase, increasing substrate bioavailability for NOS. Another possible explanation is the competitive occupation of CATs by arginine excess for intracellular space transportation instead of other cationic amino acids [17]. CATs and NOS are located in the plasmatic membrane caveolae, ensuring a stable supply of the substrate (i.e., arginine) from the plasmatic compartment [18]. A relative abundance of plasmatic arginine may overtake NOS inhibition by raising intracellular arginine/ADMA ratio in the strict proximity of NOS [19]. However, using the same transporter, plasmatic ADMA may also gain a selective access to NOS, thus reducing NO bioavailability and explaining the association with the ED and, subsequently, with the increase in cardiovascular risk [16]. Once in the circulation, methylarginine can be eliminated through renal excretion or tissue catabolic pathways [13]. About 20% of ADMA is removed from plasma by the kidney while SDMA is mostly excreted unmodified through the urine [20]. The main pathway for asymmetric methylarginine catabolism is the hydrolytic reaction mediated by dimethylarginine dimethylaminohydrolase (DDAH) enzymes which catalyze the degradation of MMA and ADMA to citrulline and monomethylamine or dimethylamine, respectively [21]. Different tissues and cells express DDAH including heart, endothelium, kidney, lung, pancreas, liver, brain, and placenta as well as macrophages and neutrophils; however, ADMA is mostly catalyzed by the kidney and liver [22, 23]. A further catabolic pathway for both symmetric and asymmetric methylarginines is the transamination mediated by alanine-glyoxylate aminotransferase; however, the contribution of transamination to ADMA metabolism has not been fully investigated [24]. The methylarginine metabolism is depicted in Figure 1.

3. Physiopathology of ADMA and Endothelial Dysfunction in Rheumatoid Arthritis

3.1. Factors Affecting ADMA Levels in RA Patients. Different mechanisms can account for the increase in ADMA levels detected in RA patients. The inducible NOS (iNOS) is an isoform that can be induced in various cellular types under inflammatory stimuli; iNOS has a crucial role in the intracellular clearance of pathogens and in the vasodilatation of inflamed tissues [25]. However, the increased production of NO by iNOS, primed by inflammatory cytokines, leads to an S-nitrosylation of reactive cysteine in DDAH, inhibiting ADMA catabolism, thus increasing its levels and lastly inhibiting all three isoforms of NOS [26]. *In vitro* studies on endothelial cells demonstrated that TNF, a cytokine playing a key role in RA pathogenesis, exerts an inhibitory effect on DDAH leading to the impairment in ADMA degradation [27]. In RA patients, free radicals and nitrotyrosine produced by rheumatoid synovia as well as by the reduced expression of DDAH enzyme in the hypoxic environment of inflamed synovia may further contribute to DDAH inhibition and rise in plasmatic ADMA levels [28–30]. Another explanation for the high ADMA levels is an increase in its production by PRMT activity: Böger et al. described an enhanced production of ADMA in endothelial cells exposed to native and oxidized LDL (oxLDL), partially due to enhanced PRMT gene expression [31]. oxLDL levels are higher in RA patients than in healthy subjects because of the oxidative stress coexisting with the inflammatory state [32, 33]. Moreover, other posttranslational modifications of LDL may also account for NO uncoupling [34]. In the rheumatoid synovia, endothelial cells undergo a phenotypic change characterized by an increase in activation, angiogenesis, and apoptosis [35]. The increased turnover of endothelial cells as well as the increased number of proliferating cells associated with angiogenetic microenvironment of the

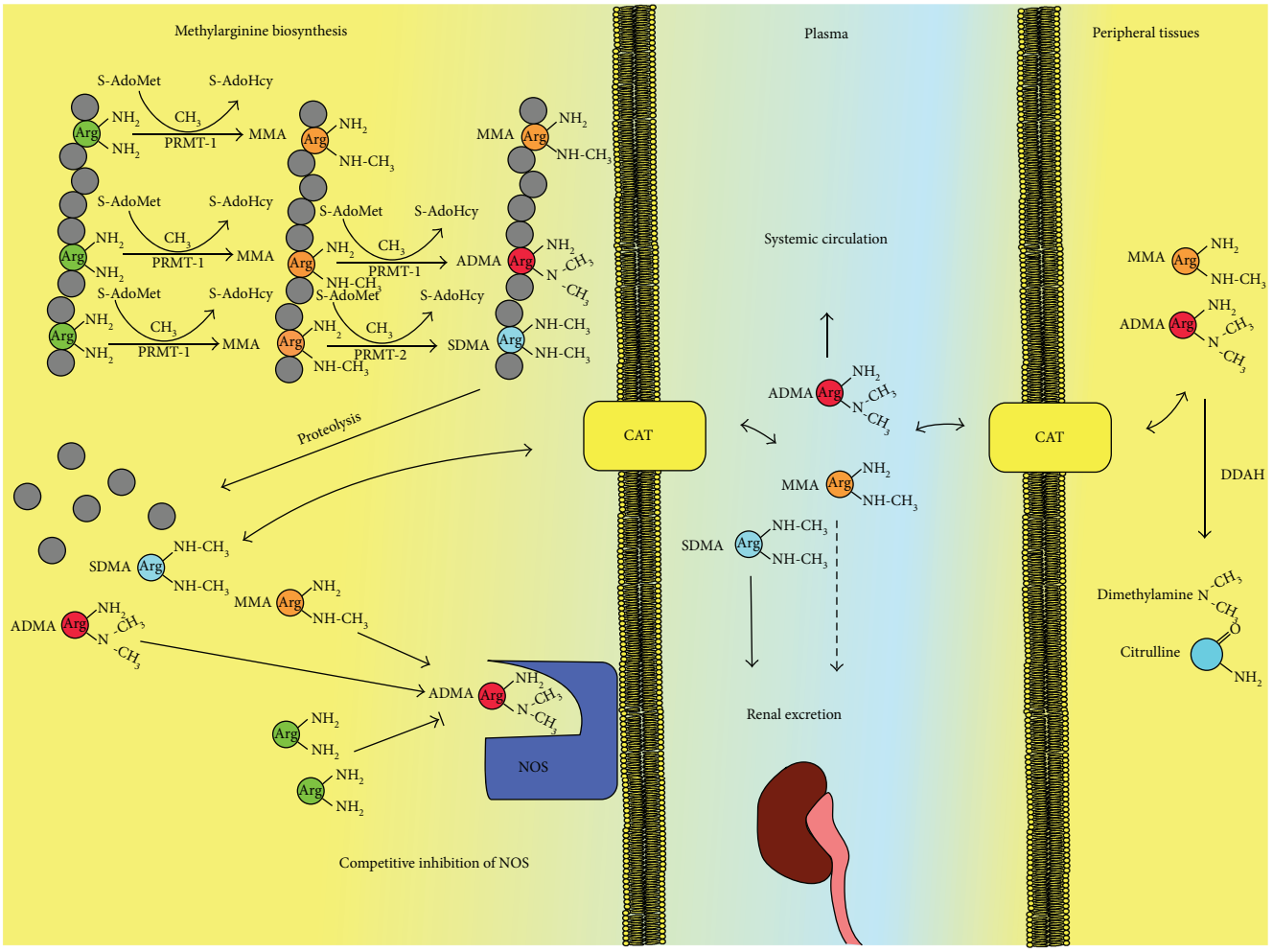

Figure 1: Metabolic pathways of methylarginines. S-AdoMet: S-adenosyl-l-methionine; S-AdoHcy: S-adenosylhomocysteine; PRMT: protein arginine methyltransferases; MMA: monomethyl arginine; ADMA: asymmetric dimethyl arginine; SMDA: symmetric dimethyl arginine; CAT: cationic amino acid transporter; DDAH: dimethylarginine dimethylaminohydrolase; NOS: nitric oxide synthase.

inflamed joint may be a source of methylarginines. ADMA production is enhanced in apoptotic and senescent endothelial cells, as a result of methylated protein turnover [36].

Patients with RA have a high basal level of insulin and a tendency toward insulin resistance which is associated with the inflammatory status and seems to be reverted by TNF inhibitors [37, 38]. Proinflammatory cytokines such as TNF and IL-6 prevent muscular glucose uptake and induce lipolysis in adipocytes, leading to an impaired plasmatic glucose regulation; moreover, free fatty acids released by stimulated adipocytes determine a positive feedback loop both by inducing an insulin-resistant phenotype of skeletal muscle and liver and by stimulating TNF and IL-6 production by macrophages [39, 40]. In diabetic patients, both increased and decreased levels of ADMA were reported [41, 42]. Chronic hyperglycemia increases ADMA levels by inhibiting DDAH activity [43]. On the contrary, insulin upregulates CAT expression in various cell types [44]. In healthy subjects and in type 1 diabetic patients, acute hyperinsulinemia reduces ADMA levels, probably increasing the cellular uptake related to CAT regulation [45, 46]. Raising the production of ADMA (via DDAH inhibition) and increasing cellular uptake (via CAT upregulation), insulin resistance may contribute to ADMA-mediated NOS inhibition [16].

Homocysteine (Hcy) is a sulfhydryl-containing amino acid mainly produced from the essential amino acid methionine. Several factors affect Hcy levels, including age, sex, lifestyle factors (coffee consumption, smoking habit, physical activity, and alcohol), genotype of the enzymes involved in Hcy catabolism, drugs and diseases interfering with its metabolism, and most importantly group B vitamins (folic acid, pyridoxine, and cobalamin) [47]. Hyperhomocysteinemia (HHcy) is a well-known risk factor for CVD in general population and in patients with RA [48, 49]. Some authors suggested a link between HHcy and increased ADMA levels; indeed, Hcy inhibits DDAH activity and the endoplasmic reticulum stress response in the dysfunctional endothelium seems to increase proteolysis, and thus ADMA levels [50, 51]. In RA patients, several factors contribute to the increase in Hcy serum levels. Chronic inflammation enhances immune cell turnover increasing the folate requirement, and the use of methotrexate contributes to folate deficiency

by inhibiting the enzyme dihydrofolate reductase [52, 53]. The reduced bioavailability of the methylenetetrahydrofolate, the key substrate of methylenetetrahydrofolate reductase, limits the conversion of Hcy to methionine, causing HHcy [53]. The link between NO metabolism and HHcy is not completely clear since Hcy-lowering agents seem not to significantly affect ADMA levels [54].

3.2. Linking ADMA to Endothelial Dysfunction in RA. Normal endothelium is responsible for many physiological functions needed to maintain vascular integrity, such as regulation of vascular tone and anticoagulating and anti-inflammatory functions [55]. NO is a key mediator of many functions of a healthy and functional endothelium, and consequently, the impaired ability to produce NO is a main feature of ED [56]. A dysfunctional endothelium is characterized by cytokine and chemokine production, adhesion molecule expression, platelet activation, abnormal fibrinolytic activity, lipoprotein deposition, and immune cell migration in the subendothelial layer leading to the early and subclinical phases of the atherosclerosis and driving all the steps of CVD until acute complications [5, 8, 9, 55].

Methylarginines affect endothelial function in different ways. Asymmetric methylarginines inhibit the three isoforms of NOS, reducing the NO production [15]. Furthermore, ADMA and MMA can compete with arginine for transmembrane transport through CAT, reducing the availability of the substrate for NO synthesis [57, 58]. Besides the interference with arginine-dependent NO production, ADMA determine "NOS uncoupling," a shift in NOS enzymatic activity from reductase to oxidase [59]. In the absence of its substrate, NOS transfers electrons to molecular oxygen, instead of arginine, leading to the formation of superoxide, instead of NO [59]. Superoxide is a free radical which rapidly combines with NO producing peroxynitrite, a highly reacting intermediate and powerful source of oxidative stress that entails DNA and protein oxidation and at high concentration, cytotoxicity [60]. Therefore, superoxide and peroxynitrite produced by ADMA-related NOS uncoupling contribute to oxidative stress and endothelial cell dysfunction [61].

Endothelial progenitor cells (EPCs) are bone marrow derived, circulating endothelial precursors able to differentiate in situ in functional endothelium, contributing to endothelial injury recovery and limiting atherosclerotic plaque formation; in the light of their repairing effect, EPCs are biomarkers of endothelial health [62]. A reduced number of circulating EPCs has been described in a number of conditions associated with an increased cardiovascular risk, including RA [63, 64]. In patients with RA, different authors observed an inverse correlation between ADMA levels and the number of circulating EPCs which can be reversed by TNF inhibitors [64–67]. Since NO is a key regulator of EPC migration and differentiation, lowering endogenous production of NO by the endothelium, ADMA can markedly reduce the mobilization and function of EPCs, impairing the protective effect [67, 68]. Figure 2 summarizes the physiopathology of ADMA in ED development in patients with RA.

4. ADMA as Biomarker of Cardiovascular Risk in Rheumatoid Arthritis

In the last years, the potential role of ADMA as a biomarker of cardiovascular risk has been investigated in several conditions. Recently, a meta-analysis of about 20,000 nonoverlapping participants enrolled in 22 cohort studies and long-term follow-up demonstrated an association between circulating levels of ADMA and cardiovascular outcomes, including coronary heart disease and stroke [69]. ADMA was also correlated with noninvasive markers of subclinical atherosclerosis such as flow-mediated dilation (FMD) and intima-media thickness (IMT). Brachial artery FMD is a noninvasive method to evaluate NO-mediated flow response to subischemic stimuli. FMD is a useful marker of CVD risk since it correlates with more invasive measurement of ED, with cardiovascular risk, and with coronary artery vasodilatory function [70]. In healthy subjects, elevated ADMA levels are associated with a reduced FMD, suggesting that ADMA may represent a biomarker of ED [71, 72]. In RA patients, the decrease of the endothelium-dependent macrovascular function starts to be evident within the first year of the disease; some authors detected an association with disease activity, not confirmed by others, and with serology [73, 74]. Some reports suggested an inverse correlation between ADMA levels and FMD, not confirmed by other studies [66, 75–77] (Table 1).

Ultrasonographic evaluation of carotid IMT is a reliable marker of cardiovascular outcome correlating with traditional risk factors and with the incidence of clinical cardiovascular events [78, 79]. A meta-analysis of the literature published in 2015 reported an increased carotid IMT with a higher prevalence of carotid plaque in RA patients compared to control subjects [80]. A meta-analysis of over 6,000 patients showed a positive relation between carotid IMT and ADMA, suggesting a role for the latter as a serological biomarker of cardiovascular risk [81]. As for RA, literature data seems not to confirm the association between carotid IMT and ADMA levels [77, 82–84] (Table 1). A single recent study, investigating biomarkers of micro- and macrovascular function in 197 RA patients, demonstrated a significant correlation between ADMA levels and noninvasive markers of endothelial dysfunction, in those patients showing a high disease activity: the authors showed a positive correlation between ADMA levels and cIMT and between arterial stiffness and ADMA/SDMA ratio, especially in patients with high inflammatory markers [85].

The studies investigating a possible association between markers of disease activity and ADMA led to conflicting results. A few studies on RA patients demonstrated a positive correlation between ADMA levels and C-reactive protein and disease activity score (DAS28) values, suggesting a link between a high inflammatory state, ADMA levels, and CVD in active RA; however, other studies failed to replicate these results [77, 82, 86–89]. Similarly, some reports described an association between anticitrullinated peptide antibodies (ACPA) titer and ADMA levels, especially in patients with early disease [87, 90, 91]. In RA patients, ADMA showed a positive correlation with Hcy

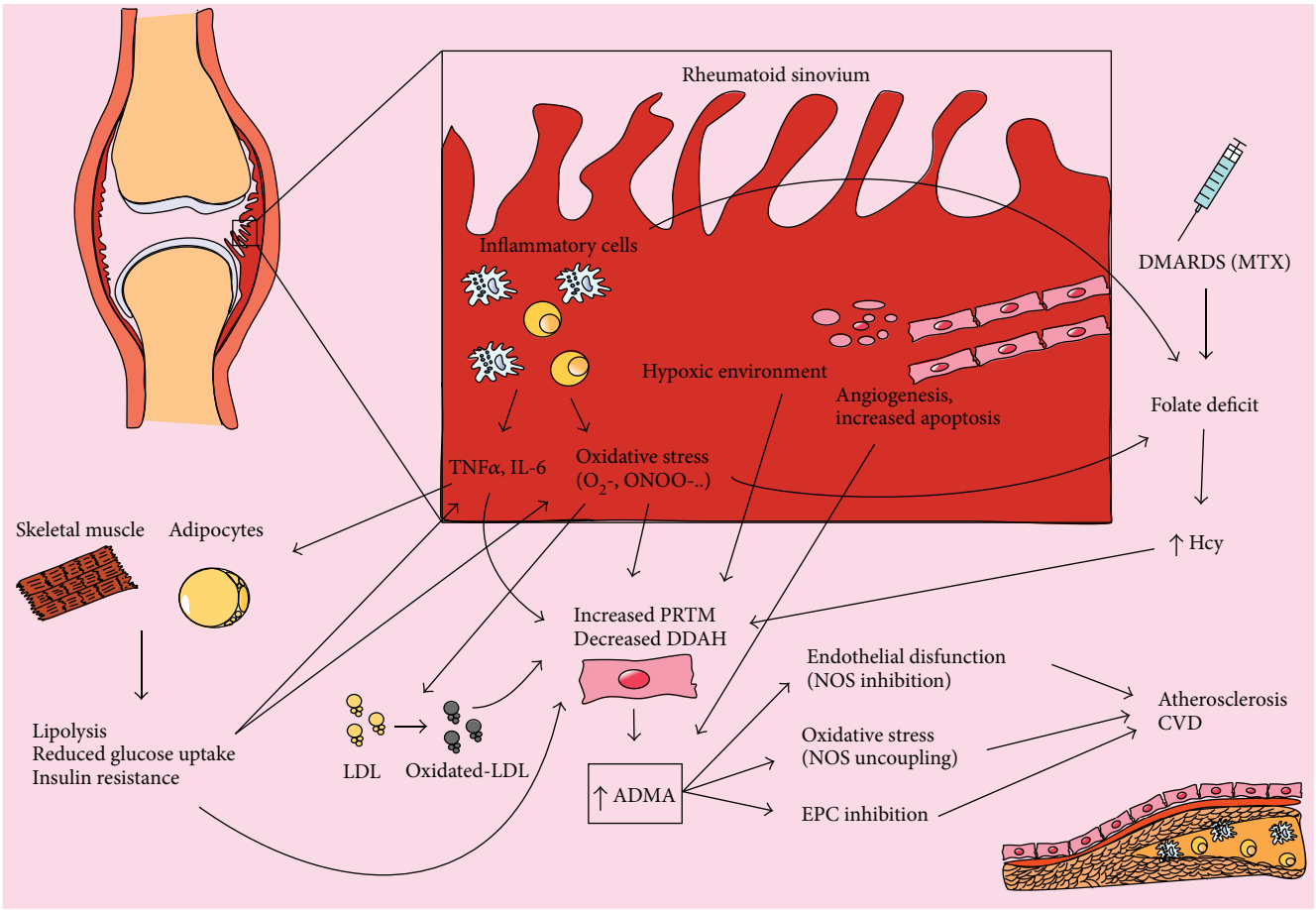

Figure 2: Mechanisms of ADMA-induced endothelial dysfunction in rheumatoid arthritis. The inflammatory microenvironment of the inflamed synovia produces cytokines and reactive oxygen species which directly stimulate PRTM and inhibits DDAH in endothelial cells, increasing ADMA production. Cytokines influence metabolically active tissues like skeletal muscle and adipocytes, inducing insulin resistance. This can generate a positive feedback loop, with an increased release of cytokines from macrophages and increased ADMA production in endothelial cells as well. The increased levels of oxidated lipoproteins, as a consequence of the oxidative stress linked to synovitis, furthermore contribute to ADMA synthesis, as well as the increased apoptosis of endothelial cells in inflamed synovium. At last, folate deficit, related to the increased cellular turnover, to the oxidation of folate from reactive oxygen species and to the methotrexate treatment, induces an increased generation of homocysteine, which contributes to ADMA increase. ADMA increase can induce endothelial dysfunction, oxidative stress, and EPCs inhibition, conducing atherosclerosis development and cardiovascular complications. PRMT: protein arginine methyltransferases; DDAH: dimethylarginine dimethylaminohydrolase; NOS: nitric oxide synthase; ADMA: asymmetric dimethyl arginine; EPCs: endothelial progenitor cells; MTX: methotrexate; Hcy: homocysteine; CVD: cardiovascular disease.

levels and it is associated with insulin resistance (the homeostasis model assessment (HOMA) being a strong predictor of ADMA serum levels) [92, 93]. Finally, ADMA serum levels correlate with other markers of endothelial health status, such as EPCs: the reduction in circulating EPCs, correlating with high plasmatic level of ADMA and high DAS28 values, was restored by a short course of TNF inhibitors [65]. In summary, these observations strongly suggest a possible role of ADMA as a reliable biomarker of early atherosclerosis in RA patients, especially in the context of an active disease.

ADMA levels start increasing in the early phase of disease, and the introduction of disease-modifying antirheumatic drug (DMARD) treatment seems to decrease the levels compared to those observed in the control group [77]. In a study on 20 early, untreated RA patients, our group demonstrated that therapeutic intervention with conventional synthetic DMARDs or TNF inhibitors significantly reduced ADMA serum levels [77]. Even in long-standing RA patients, the treatment with TNF inhibitors seems to reduce ADMA levels: this effect was shown in a study on 33 RA patients starting etanercept or adalimumab but was not confirmed by other authors [66, 83, 84, 91]. Table 1 summarizes the main findings of the studies investigating ADMA serum levels in the context of RA [65, 66, 75–77, 82–98].

The heterogeneity of methods used to assess subclinical atherosclerosis and the different contributions of traditional and disease-related risk factors in a complex disease such as RA may account for the lack of concordance of the results and limit the usefulness of ADMA as a marker for atherosclerotic risk stratification. In this regard, a cutoff level of ADMA defining a dysfunctional endothelium could be helpful.

Table 1: Main findings of the studies investigating ADMA in rheumatoid arthritis.

Number of RA patients (controls)	Main findings	Reference
91 (31)	No correlation between ADMA and subendocardial viability ratio	Anyfanti et al. [94]
201	No association between ADMA and genetic variants of the AGXT2 gene	Dimitroulas et al. [95]
197	Association between microvascular function, arterial stiffness, and cIMT and ADMA/SDMA levels in RA patients with high inflammatory marker	Dimitroulas et al. [85]
40 (29)	Inverse correlation between ADMA and FMD; positive correlation between ADMA and disease duration; no correlation with CRP	Sentürk et al. [88]
30 (30)	No relationship between ADMA concentration and aortic augmentation; no difference in ADMA levels between patients and controls	Erre et al. [96]
201	Difference in ADMA levels according to MTHFR; positive correlation between ADMA and Hcy and ESR	Dimitroulas et al. [92]
100	No correlation between ADMA and thCys at baseline and after omega-3 fatty acids, vitamin E, vitamin A, copper, and selenium, or placebo; correlation between ADMA and arginine	Kayacelebi et al. [98]
201	Positive correlation between ADMA and ESR and ADMA and CRP	Sandoo et al. [89]
33	Correlation between ADMA and DAS28; reduction of ADMA levels after 3 months of anti-TNF	Spinelli et al. [66]
201	No significant relationship between DDAH genetic variables and ADMA levels	Dimitroulas et al. [97]
17 (12)	Inverse correlation between ADMA levels and circulating EPC number	Spinelli et al. [65]
35 (35)	ADMA and RF have similar sensitivity and specificity in the detection of endothelial dysfunction	Spasovski and Sotirova [91]
67	HOMA, an indicator of insulin resistance, predicts elevated ADMA levels	Dimitroulas et al. [93]
48 (32)	Association between baseline PWV and ADMA but no correlation with cIMT; anti-TNF therapy increased L-arginine/ADMA ratio but not ADMA after 3 months	Angel et al. [84]
20 (20)	Significantly higher ADMA levels in RA than controls; significant reduction after 12 months of treatment	Di Franco et al. [77]
35	No change in ADMA levels after 2 weeks and 3 months of anti-TNF treatment	Sandoo et al. [75]
46 (50)	Higher ADMA levels in RA than in controls; correlation with CRP, DAS28, and 8-isoprostanes	Kwaśny-Krochin et al. [86]
60 (29)	Significantly higher ADMA levels in RA compared with controls; no correlation with demographic or disease characteristics	Sandoo et al. [83]
25	No change in ADMA levels and cIMT after treatment	Turiel et al. [82]
25 (25)	Higher ADMA levels in early RA than in controls. Significant negative correlation between ADMA levels and CFR; no correlation with IMT	Turiel et al. [90]
20	Positive correlation between ACPA and ADMA levels; no correlation with disease activity indices	Surdacki et al. [87]
36 (20)	Chronic low-dose prednisolone lower ADMA levels	Radhakutty et al. [109]

ADMA = asymmetric dimethyl arginine; AGXT2 = alanine-glyoxylate aminotransferase 2; SDMA = symmetric dimethyl arginine; cIMT = carotid intima media thickness; FMD = flow-mediated dilation; CRP = C-reactive protein; MTHFR = methylenetetrahydrofolate reductase; Hcy = homocysteine; ESR = erythrocyte sedimentation rate; thCys = total L-homocysteine; DAS28 = disease activity score 28; TNF = tumor necrosis factor; DDAH = dimethylaminohydrolase; EPCs = endothelial progenitor cells; RF = rheumatoid factor; HOMA = homeostasis model assessment; PWV = pulse wave velocity; CFR = coronary flow reserve; ACPA = anticitrullinated peptide antibodies.

5. Possible Therapeutic Intervention

Since methylarginines play a key role in the physiopathology of ED and ADMA levels have been strictly associated to cardiovascular risk, several pharmacological interventions have been investigated on the possible effect on ADMA levels and cardiovascular outcomes. However, taking into account the wide spectrum of indications of the drugs investigated and of the inter-study result variability, the actual relation between ADMA level reduction and cardiovascular benefits is still inconclusive [13]. Effect of statins on methylarginine metabolism has been investigated in different conditions such as diabetes, stroke, and hypercholesterolemia, demonstrated to effectively reduce plasmatic ADMA levels in recent controlled trials [99–101]. *In vitro*, statins increase the expression of DDAH genes and the bioavailability of tetrahydrobiopterin (BH4), which is a critical eNOS cofactor inhibiting NOS uncoupling phenomenon [102]. A recent

Table 2: ADMA lowering effect and possible pharmacodynamic mechanism of different drugs.

Drug	Investigated conditions	Hypothesized mechanism	Results	References
Statins	Diabetes mellitus, stroke, hypercholesterolemia	Increase DDAH expression, increased bioavailability of tetrahydrobiopterin	Decreased ADMA serum levels (18–50%)	[100, 109]
Fibrate	Hypertriglyceridemia	Increase DDAH activity through NF-kB suppression via PPAR-α receptors	Uncertain effect on ADMA serum levels, increase L-arginine/ADMA ratio	[111]
Niacine	Dyslipidemia	Depletion of methyl groups for niacine metabolism and consequent reduction in ADMA synthesis	Decreased ADMA serum levels (10%)	[112]
ACE inhibitors/ARB	Chronic glomerulonephritis, hypertension	Decreased NADPH oxidase upregulation by RAA system, with consequent reduced ROS-mediated DDAH inhibition	Decreased ADMA serum levels (10–16%)	[113, 114]
Thiazolidinediones	Diabetes mellitus	Through PPAR-γ receptor activation: reduced insulin resistance, increased expression of DDAH in renal tubules, suppressed activity of NF-kB	Controversial; from no reduction to reduction of ADMA serum levels (10%), possible protection against ADMA effect	[115]
Metformin	Diabetes mellitus Polycystic ovarian syndrome	Partially unknown, apparently not mediated by PRTM or DDAH Competitive antagonist of ADMA	Decreased ADMA serum levels (27%)	[116]
Nebivolol	Hypertension	Upregulation of DDAH, downregulation of PRTM	Decreased ADMA serum levels (37–44%)	[117, 118]
Acetylsalicylic acid	Coronary artery disease	Upregulation of DDAH and eNOS	Decreased ADMA serum levels (30%)	[119]
Estrogens	Postmenopausal women	Upregulation of DDAH via ERα	Decreased ADMA serum levels (18–20%)	[120, 121]
Folate and B group vitamins	Hypertension, hyperhomocysteinemia, chronic heart failure	Increased bioavailability of methylenetetrahydrofolate	Decreased ADMA serum levels (14%), acute decrease during e.v. infusion	[122, 123]
α-Lipoic acid	End-stage renal disease, diabetes mellitus	Activation and upregulation of DDAH via STAT3	Decreased ADMA serum levels (9%)	[124]
N-Acetylcysteine	End-stage renal disease	Partially unknown, direct activation DDAH, or ROS scavenging	Decreased ADMA serum levels (30%)	[125]

double-blind randomized study demonstrated that supplementation of oral tetrahydrobiopterin significantly improved the endothelial function measured by FMD in a small cohort of RA patients [103]. The authors did not investigate the effect on ADMA levels but, considering the implication of folate in methylarginine metabolism, an ADMA-lowering effect could be expected. This is also supported by the consolidated evidence of the role of folate supplementation on plasmatic Hcy lowering, in consideration of the interplay between HHcy and raised ADMA levels [47]. This suggests that larger and targeted studies, addressing the potential effect of tetrahydrobiopterin supplementation on ADMA levels in relation to ED and risk of CVD, are desirable.

In a small study on RA patients, atorvastatin effectively reduced arterial stiffness measured by pulse wave analysis, without affecting acute-phase reactants [104]. The lipid-lowering agent ezetimibe showed the ability to lower ADMA levels and to ameliorate renal function in patients with chronic kidney disease, probably by protecting DDAH enzymatic site from oxidative inactivation [105]. Besides the lipid-lowering effect, ezetimibe, as well as simvastatin, demonstrated to reduce disease activity and C-reactive protein levels and to improve the endothelial function and the arterial stiffness in patients with RA [106].

The evidence that lipid-lowering drugs couple an anti-inflammatory effect with an improvement of endothelial function, by modulation of ADMA metabolism, may suggest a role for these drugs in the management of cardiovascular risk associated to RA. The ADMA-lowering effect of several other agents have been investigated in conditions different from RA. Only few studies addressed the effects of therapeutic intervention for RA on ADMA levels. Treatment with DMARDs, especially anti-TNF agents, demonstrated a lowering effect on ADMA levels, more pronounced in high inflammatory conditions (patients with high levels of acute-phase reactants) [85]. A recent meta-analysis showed that treatment with TNF inhibitors improves endothelial function in patients with RA [107]. It is very likely that effect of TNF inhibitors on cardiovascular risk is multifactorial, acting on different steps of the atherosclerotic process. Longitudinal studies demonstrated a short-term effect of TNF inhibitors on ADMA levels, not confirmed in studies with different

follow-up [77, 83, 84]. Nevertheless, in a 12 month follow-up study, TNF inhibitors improved the arginine/ADMA ratio despite not impacting on ADMA absolute levels [84]. These results imply that the modulation of ADMA metabolism could partially account to the atheroprotective effect of TNF inhibitors.

The effect of folate supplementation on plasmatic Hcy is well known and some authors hypothesized an interplay between HHcy and raised ADMA [47]. A single study on a large population of RA patients ($n = 201$) demonstrated that Hcy levels are significantly related to serum ADMA, contrasting with previous data obtained in a smaller group of patients [92, 98]. The relationship between ADMA and Hcy levels is intriguing since the latter is affected by the use of methotrexate, a milestone in the RA treatment. In a recent study, Dimitroulas et al. demonstrated a trend of the MTHFR polymorphism to influence ADMA levels, with the C667T polymorphism associated to higher ADMA levels, only at the univariate analysis [92]. Interestingly, C677T polymorphism was associated with subclinical atherosclerosis and CVD risk in a study on 612 RA patients followed up for 5 and 10 years [108]. These evidences may support the protective, antiatherogenic effect of methotrexate.

A very recent study investigated the effect of low-dose glucocorticoids on arginine metabolisms by comparing patients who were chronically treated or not with prednisolone and demonstrated higher levels of ADMA and MMA in those patients who were not taking glucocorticoids; the authors conclude that long-term glucocorticoid treatment could help in protecting endothelial health in RA patients [109].

Table 2 summarizes potential therapeutic intervention with ADMA-lowering effect.

6. Conclusion

CVD risk reduction is still an unmet need in the long-term management of RA patients and, despite the great improvement of RA treatment, CVD is still the main cause of death. In 2016, the European League Against Rheumatism (EULAR) updated the recommendations for the management of CVD in rheumatic disease firstly published in 2009, suggesting the need for an aggressive and targeted risk management [110]. The research agenda still includes issues about the precise effect of antirheumatic drugs with different modes of action and the additional value of novel biomarkers for CVD risk prediction on CVD risk [110]. The physiopathology of ED in chronic inflammatory diseases such as RA is still largely unknown, and biomarkers to efficiently stratify patients according to their CV risk are scant. ADMA seems to have the potential to solve part of these issues. The apparent physiopathological role of ADMA in endothelial NO deficit as well as the correlation between the circulating ADMA levels and cardiovascular outcomes suggest that ADMA could be a good candidate for further basic research. Moreover, better understanding the role of ADMA in ED could also provide potential target of pharmacological intervention to lower the cardiovascular risk in RA.

Conflicts of Interest

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

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Hindawi
Mediators of Inflammation
Volume 2017, Article ID 3102737, 11 pages
https://doi.org/10.1155/2017/3102737

Review Article

Cigarette Smoking and Adipose Tissue: The Emerging Role in Progression of Atherosclerosis

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Received 9 June 2017; Revised 8 November 2017; Accepted 27 November 2017; Published 27 December 2017

Academic Editor: Arbi Pecani

Smoking is an established risk factor for atherosclerosis through several underlying pathways. Moreover, in the development of atherosclerotic plaque formation, obesity, defined as excess fat mass accumulation, also plays a vital role in dyslipidemia and insulin resistance. Substantial evidence shows that cigarette smoking induces multiple pathological effects in adipose tissue, such as differentiation of adipocytes, lipolysis, and secretion properties in adipose tissue. Therefore, there is an emerging speculation in which adipose tissue abnormality induced by smoking or nicotine is likely to accelerate the progression of atherosclerosis. Herein, this review aims to investigate the possible interplay between smoking and adipose tissue dysfunction in the development of atherosclerosis.

1. Introduction

Cardiovascular diseases (CVDs) are identified as the leading causes of death in many countries, in both developing world and industrialized regions [1], and these diseases include coronary artery diseases, ischemic stroke, and peripheral artery diseases. The basic pathology of the aforementioned diseases is the advancement of atherosclerosis (AS) leading to vascular stenosis and plaque rupture. Notably, it is estimated that approximately 11% of global cardiovascular deaths are attributed to smoking [1], indicating that smoking is one of the independent risk factors in AS. Recent papers have shown an increase in carotid artery intima-media thickness among currently smoking adolescents, which points to an early atherogenic remodeling of the vasculature in youth [2], further aggravating the global burden of disease. Therefore, a variety of studies have been dedicated to explore the underlying causes of smoking-induced atherogenesis.

Apart from cigarette smoking, obesity is another public health issue in that the worldwide prevalence of obesity has dramatically increased since 1980 [3]. Obesity, characterized as excessive adipose tissue, has a harmful effect on vascular function, and its associated comorbidities are prone to develop CVDs. Similar to smoking, obesity in childhood/adulthood and the long-term consequences of vascular injury can be clinically relevant [4]. The underlying mechanisms of adipose tissue in AS have been studied in recent years, while the accurate pathways remain to be elucidated.

In particular, in 2002, it has estimated that approximately 20% of US smokers, about 9 million people, were obese [5]. Several studies have reported that people with coexistence of obesity and current smoking habits show especially large risks for mortality related to CVDs and other diseases [6, 7]. Furthermore, a burgeoning body of studies has reported that cigarette smoking has a complicated effect on body weight and the function of adipose tissue [8–10]. Therefore, this specific interaction between cigarette smoking and adipose tissue in atherogenesis may represent a crucial target for future therapy. The objective of the present paper is to delineate the mechanisms through which exposure to chemicals in cigarette smoking affects the differentiated status and functions of adipocytes, which may contribute to AS.

2. Pathogenesis of Atherosclerosis: a Brief Overview

Accrued data have defined atherosclerosis as a chronic low-grade inflammation of the vasculature system characterized

by atherosclerotic plaque formation and rupture. Abnormal accumulation and retention of low-density lipoprotein (LDL) and lipoprotein remnants have been implicated as initial triggers [11]. Associated enzymes in the vessel wall have the ability to modify this LDL to oxidized LDL, which serve as inflammatory signals [12]. Inflammatory cells are subsequently recruited to the arterial wall, such as monocytes, which differentiate into macrophages and are, as a result, activated to engulf oxidized LDL via scavenger receptors, creating foam cells, which secrete chemokines and other kinds of cytokines that further create a vicious cycle, more immune cell infiltration, and activation [13]. Additionally, there is another possible pathway in which many of these lipid-rich macrophages undergo apoptosis and necrosis, releasing their contents into the extracellular space and then formatting a necrotic core. Proliferation and migration of vascular smooth muscle cells (VSMCs) also participate in the pathological process of plaques, resultantly stimulating the release of cytokines, such as interleukin-1, 8 (IL-1, 8) and interferon-γ (IFN-γ). Collectively, these various cascade reactions lead to fatty streak formation and followed advancement of plaques. Aside from immune cell entry into the plaque through the intima, immune cells are also observed in the outer part of the vessel wall, the adventitia, and perivascular adipose tissue (PVAT). Of note, collagenous conduits and vasa vasorum may bridge the communication between the intima and adventitia, which highlights the role of the adventitia in coordinating the immune response in AS [14]. Furthermore, due to absence of the fascia barrier between PVAT and adventitia, it seems possible that PVAT secretes various kinds of local adipokines and cytokines to interact with the adventitia [15]. As a result, PVAT and the adventitia have provided emerging insight on atherogenesis.

3. Smoking and Atherosclerosis: a Well-Known Mechanism

Cigarette smoke contains more than 4000 different components, which complicates the understanding of the potential mechanisms of tobacco-related diseases [16]. Among these constituents, nicotine has been identified as one of the most important ingredients that participate in vascular inflammation. Nicotine has been shown to increase physiological parameters, such as blood pressure and heart rate [17]. In addition, while binding with high-affinity nicotinic acetylcholine receptors (nAChRs), nicotine exerts several bioactive actions on different cellular effectors involved in plaque formation and progression [18]. According to a number of in vitro and clinical studies, there is strong evidence that exposure to cigarette smoking impairs the normal prosperity of endothelial cells, especially in the youth group [19]. Nicotine and the resulting increased oxidative stress induce vascular endothelial dysfunction via inhibition of the activation of endothelial nitric oxide synthase (eNOS) and decreasing the generation and bioavailability of nitric oxide (NO) [20]. Moreover, nicotine increases the expression of adhesion molecules on endothelial cells, namely, intracellular adhesion molecular-1 and E-selectin, as a result of enhanced attachment and transmigration of monocytes to the vessel wall [21]. Considering the types of inflammatory cells, a recent study has shown that nicotine upregulates CD36 expression in monocytes/macrophages via activation of nAChRs, facilitating to engulf the lipid particles by macrophages [22]. Macrophages stimulated by the treatment of nicotine secrete elevated inflammatory cytokines, namely, tumor necrosis factor-α (TNF-α), IL-1β, and chemokines, creating the proinflammatory microenvironment in the subendothelium [23]. Furthermore, it is shown that VSMCs undergo the contractile-to-synthetic transition, characterized by enhanced growth and migration of VSMCs, which contributes to foam cell formation [24]. Apart from the above alternations of cells, other substances and structures can be induced upon exposure of nicotine. For instance, under the treatment of nicotine, vasa vasorum has been shown to expand to intima plaque and the neovasculature is discovered in the plaque, one of the markers of the instable plaques [18]. Finally, exposure to smoking results in platelet activation, stimulation of a coagulation cascade, and impairment of anticoagulative fibrinolysis, which, in turn, promotes pathological thrombus formation [25]. Overall, cigarette smoking or nicotine has multiple actions on the advancement of AS (see Figure 1).

Polycyclic aromatic hydrocarbons (PAHs), another class of compounds in cigarette smoke, also induce atherogenesis [26]. PAHs binding with aryl hydrocarbon receptor can downregulate the cholesterol efflux [26]. Other cigarette-derived substances also participate in various pathways to promote AS [27].

4. Adipose Tissue and Atherosclerosis

Obesity is characterized by excessive or abnormal accumulation of adipose tissue. The traditional roles of adipose tissue are to store free fatty acids after eating and release them in fasting state, which is sensitive to the regulation of insulin. Notably, it is now widely recognized that adipose tissue is not only a storage depot but also an active source of bioactive factors, such as adipokines, which influence lipid levels, inflammation, oxidative stress, insulin resistance, and AS [28]. There are various kinds of adipokines participating in the advancement of atheromas, the bioactive actions of which are shown in Table 1.

Accumulating evidence indicates that obesity leads to adipose tissue dysfunction, including adipocyte hypertrophy, enhanced inflammation, and impaired vascular structure and function [29]. In light of the wide distribution of adipose tissue in human bodies, adipose tissue has the ability to exert systemic effects on the cardiovascular risk factors and associated CVDs. Blood lipid abnormalities caused by excess adipose tissue are classical features of metabolic syndrome, such as the presence of small dense LDL particles, which in part induce the proinflammatory place within the vascular wall [30]. More importantly, adipose tissue dysfunction leads to an imbalance in the production of adipokines. When obesity is present, the main characteristics of adipokine concentration are identified as elevated levels of leptin, resistin, and TNF-α [31–33], in conjunction with a decline in production of adiponectin and omentin [34, 35]. According to Table 1, the imbalance of adipokines relevant to obesity

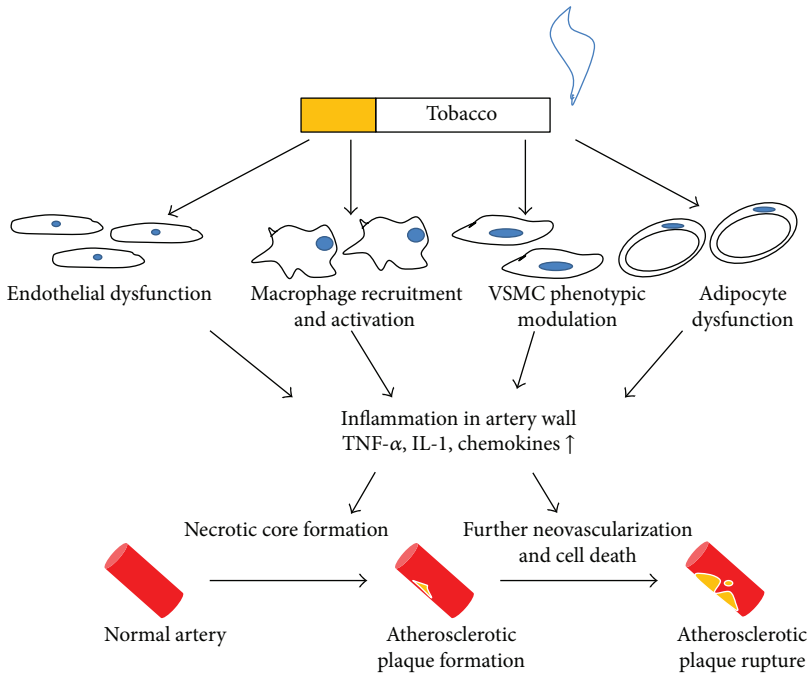

Figure 1: Smoking-associated inflammatory response in artery walls. Smoking causes endothelial dysfunction with VSMCs phenotypic modulation. In addition, more macrophages are recruited and activated to secrete cytokines and chemokines. The resultant inflammatory response leads to atherosclerotic plaque formation and subsequent plaque rupture. TNF-α: tumor necrosis factor-α; IL-1, interleukin-1.

Table 1: Adipokines and their bioactive functions associated with cardiovascular diseases.

Adipokine	Bioactive functions associated with cardiovascular diseases	Reference
Leptin	Increase heart rate and elevate blood pressure level	[82]
	Increase lipolysis in skeletal muscle and adipocytes	[83]
	Increase reactive oxygen species secretion in endothelial cells, such as H_2O_2 and HO generation	[84]
	Facilitate cholesterol accumulation in macrophages	[85, 86]
	Promote the expression of matrix metallopeptidase-2 in VSMCs	[87]
	Act on various types of immune cells to promote the release of proinflammatory cytokines	[81, 88]
Adiponectin	Reduce tissue triglyceride content and upregulate insulin sensitivity	[89]
	Suppress endothelial cell apoptosis	[90]
	Suppress TNF-α-induced NF-κB activation to decrease the recruitment of monocytes	[91]
	Inhibit the expression of scavenger receptors-A1 of macrophages and mediate polarization toward anti-inflammatory M2 phenotype	[80, 92]
	Attenuate proliferation and migration of VSMCs	[93]
TNF-α	Downregulate insulin resistance	[94]
	Increase expression of adhesion molecules	[95]
	Induce the migration and proliferation of VSMCs	[96]
Omentin	Proangiogenic property and inhibition of vascular inflammation	[97, 98]
	Promote NO production and its vasodilating effect of vascular	[99, 100]
Resistin	Subsclinical marker of atherosclerosis	[101]
	Increase the levels of endothelin-1, VCAM-1, and CCL2	[102]
	Promote foam cell formation by the dysregulation of scavenger receptors in macrophages	[103]
A-FEBP	The major mediator of vulnerable plaque formation	[104]
	Secrete more proinflammatory cytokines, such as TNF-α and CCL2	[105]
Chemrin	Magnify the functions of adhesion molecules	[106]

VSMCs, vascular smooth muscle cells; TNF-α, tumor necrosis factor-alpha; NF-κB, nuclear factor-kappa B; NO, nitro oxide; VCAM-1, vascular cell adhesion molecule-1; CCL2, CC-chemokine ligand 2; A-FABP, adipocyte fatty acid binding protein.

would, upon interaction with multiple vascular cells, deteriorate the formation and advancement of plaques. Additionally, adipose tissue inflammation appears to be of importance in AS. For example, patients with coronary artery diseases produce higher levels of proinflammatory cytokines (such as TNF-α, IL-6, and visfatin) in epicardial adipose tissue [36]. Additionally, obese adipose tissue contains more M1 type macrophages, mast cells, and neutrophils, through in situ proliferation and migration, thus augmenting proinflammatory responses [37]. Adipocytes seem to associate with certain immune cells through cytokine secretion and antigen presentation [37], which may synergistically accelerate the chronic inflammation in obese subjects.

Aside from systematic effects, adipose tissue may have crucial local actions in AS due to unique types of adipose tissue, namely, PVAT. Both visceral adipose tissue and PVAT are mainly made up of white adipose tissue, which is more relevant to metabolic syndrome. Functionally, it is widely accepted that PVAT has a mechanical role as a connective tissue to protect the vessels against adjacent tissue [38]. In addition, PVAT has been reported to produce a wide range of adipokines, similar to visceral adipose tissue to interact with correspondent intima and meditate the vascular inflammation. Of note, compared to adipocyte in other adipose tissues, PVAT adipocytes release more angiogenic factors including thrombospondin-1, CC-chemokine ligand 2 (CCL2), and hepatocyte growth factor to mediate vascular remodeling [39]. Finally, the vasa vasorum serves as a conducting tube that delivers blood components, local adipokines, and inflammatory cells from PVAT, which highlights the pathological characters of PVAT in the atherosclerotic process.

5. Link between Cigarette Smoking and Adipose Tissue in Atherogenesis

5.1. Nicotinic Receptors in Adipose Tissue. nAChRs are members of a family of ligand-gated, pentameric ion channels that are tightly arranged around a central pore [18]. nAChRs are divided into muscular (α1, β1, γ/ε1, and δ1) and neuronal AChRs (α2–α9 and β2–β4) [40]. Traditionally, the major biological force of these receptors is to mediate the effects of the endogenous neurotransmitter, acetylcholine, at neuromuscular junctions. In addition, nonneuronal cells may also express functional nAChRs. Apart from the above cells, several lines of evidence show that exogenous nicotine and other components of cigarette smoking have the ability to bind to high-affinity nAChRs on multiple cell types in the cardiovascular system, specifically endothelial cells and VSCMs, which thereby exert direct actions and other cellular effectors that participate in the atherosclerotic plaque formation and growth [18, 27]. Furthermore, the study by Liu et al. detected nAChR expression in adipocytes by a reverse transcriptase-polymerase chain reaction. Under the further analysis of subunits for nAChRs, they found α1-7, 9, 10, and β1-4 mRNAs expressed in adipocytes. Cancello et al. evaluated α7-nAChR expression levels in whole subcutaneous adipose tissue obtained from morbidly obese subjects and from normal weight healthy individuals, further indicating that this receptor modulates inflammatory gene expression in human adipocytes [41]. The existence of α7-nAChRs in adipose tissue may establish a potential connection between nicotine and adipose tissue in chronic low-grade inflammation, thereby affecting the course of AS. Accordingly, several basic studies are employed to clear out the accurate signal pathway underlying the bind with nicotine and nAChRs in adipocytes. For instance, Wu et al. suggested that nicotine has the capacity to react with white adipose tissue through α7-nAChRs and adenosine 5′-monophosphate-activated protein kinase (AMPK) [8]. It follows that α7-nAChRs play a fundamental role in the nicotine-induced abnormality within adipose tissue.

Apart from nAChRs, there is another receptor located on the surface of adipocytes, local β-adrenergic receptor, to stimulate lipolysis in adipose tissue by systemic infusion of nicotine [42]. Several chemicals mediate the nicotine effects on β-adrenergic receptor, such as the catecholamines epinephrine and norepinephrine and other metabolites. The nicotine-evoked catecholamine release in the brain tissue, such as the striatum and hypothalamus [43], is possibly mediated by β2 and β4 nAChRs [44]. The nicotine metabolite nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, for example, can directly bind with β-adrenergic receptor to induce lung cancer, pancreatitis, and endothelial cell injury [45–47]. In both brown and white adipocytes, Cao et al. have found that there is the β-adrenergic/cAMP/PKA signaling pathway, participating in brown fat thermogenesis [48]. Furthermore, data from Fasshauer et al. shows that in 3T3-L1 adipocytes, β-adrenergic stimulation exerts certain effects on the physical function of adipocytes [49]. Thus, the above interactions may bridge the connection between nicotine or nicotine metabolites and adipose tissue. However, the downstream effects of this signaling pathway remain elusive, lacking the direct evidence needed to determine the accurate mechanisms.

5.2. Alterations in Adipocyte Differentiation and Functions of Adipose Tissue

5.2.1. Smoking and Adipogenesis. In many populations, cross-sectional studies show that mean body mass (BMI) tends to be lower among smokers than nonsmokers. The underlying reason is the increase in metabolic rate induced by cigarette smoking. However, when using the waist circumference or waist-to-hip ratio instead of BMI, there is evidence suggesting that cigarette smoking is in favor of greater accumulation of visceral fat [50]. A rat experiment showed that maternal exposure to nicotine during lactation may promote obesity in adulthood, accompanied with higher central adiposity and hyperleptinemia [51]. An in vivo study using Sprague-Dawley rats shows that prenatal nicotine exposure led to an increase in epididymal white adipose tissue weight at weaning, and marked hypertrophy of adipocytes, with increased gene expression of proadipogenic transcription factors such as peroxisome proliferator-activated receptor-γ (PPAR-γ), resulting in increased body weight and fat deposition [52]. PPAR-γ is widely considered to be essential in inducing differentiation from preadipocytes to mature adipocytes. In

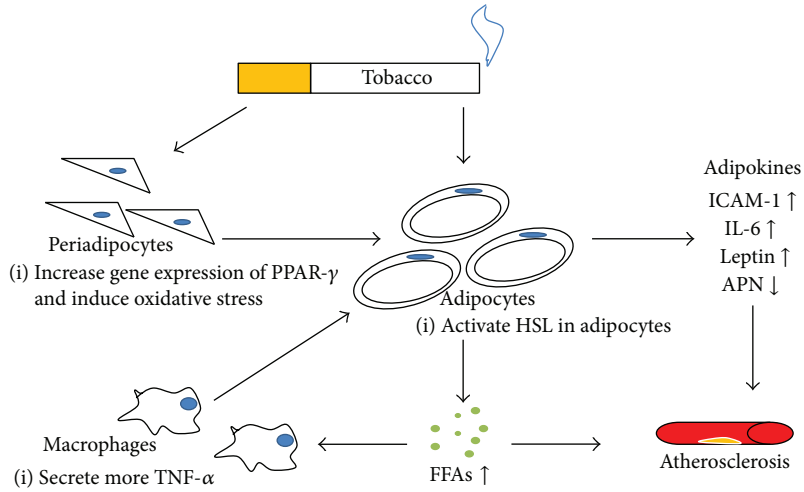

Figure 2: The direct effects of smoking on adipocytes. Smoking has direct actions on the differentiation of adipocytes. On the other hand, smoking can promote the release of FFAs through HSL. In turn, increased FFAs can stimulate macrophages to produce more TNF-α, which further induces adipocytes to secrete various kinds of adipokines, such as ICAM-1, IL-6, and leptin. In the above process, several products (FFAs and adipokines) can influence the artery walls. PPAR-γ, peroxisome proliferator activated receptor-γ; HSL, hormone-sensitive lipase; FFAs, free fatty acid; ICAM-1, intracellular adhesion molecule-1; IL-6, interleukin-6; APN, adiponectin.

one animal study, the supraphysiological activation of PPAR-γ by troglitazone, a kind of PPAR-γ agonist, increases the number of small adipocytes, which in turn promotes a flux of free fatty acids (FFAs) from the liver and muscle into WAT, leading to the upregulation of insulin sensitivity at the expense of increased WAT mass [53]. This is consistent with other studies that have reported that PPAR-γ-deficient mice are protected against adipocyte hypertrophy and obesity induced by high-fat diet and aging [54]. Of particular interest, cells with a reduction in PPAR-γ2 expression caused by artificial zinc finger repressor proteins are unable to undergo adipogenic differentiation, thereby suggesting that PPAR-γ2 plays a central role in orchestrating the adipogenesis process [55]. Of note, PPAR-γ has been reported to have anti-inflammatory activity, but the specific role in adipocyte remains unclear. Given that the possible elevated expression of PPAR-γ is induced by nicotine, more research is needed to elucidate the "nicotine-PPAR-γ" axis in the development of adipogenesis (Figure 2).

In light of the underlying pathways to influence the activity of PPAR-γ, it is plausible that oxidative stress may have a certain role in mature adipocyte formation. Exposure of 3T3-L1 adipocytes to concentrations of nicotine ranging from 60 nM to 6 μM significantly increased the generation of reactive oxygen species (ROS) [56]. Additionally, female C57BL/6 mice, upon exposure to acute smoking showed increased activity of glutathione peroxidase in the inguinal adipose tissue, potentially proving that oxidative stress was increased in adipose tissue [57]. Another model using α7-nAChR-specific lentivirus shRNA showed similar results [8], which emphasized the involvement of nAChRs on ROS production. Additionally, Lee et al. reported that ROS facilitate the cellular differentiation from 3T3-L1 preadipocytes to adipocytes by accelerating the mitotic clonal expansion, the second course of adipocyte differentiation, whereas an antioxidant treatment causes S-phase arrest [58]. Thus, nicotine may, in part, induce oxidative stress in adipose tissue contributing to the mature adipocyte.

Other substance from cigarette has also been investigated in several studies. In epididymal fat, chronic carbon monoxide also led to a significant decrease in adipocyte size and an increase in adipocyte number [59]. Additionally, through culturing the isolated adipocytes from mice adipose tissue, it was found that PAHs had the ability to increase the adipose tissue mass [60]. This may attribute to the PPAR activation of PAHs and their metabolites [61]. These adipogenesis disturbances may cause the abnormality of adipose tissue, such as enhanced lipolysis and dysfunction of adipokines secretion, which directly promotes cardiovascular diseases and worsen metabolic diseases and related risk factors, as Bays reviewed in detail [62].

5.2.2. Smoking and Lipid Metabolism. Exposure to cigarette smoke may break the balance of lipid levels through affecting the function of adipose tissue. This impaired state is commonly referred to as "dyslipidemia" and is considered to be the putative link between smoking and AS.

Considering smoking-induced dyslipidemia, the regulation of lipolysis in adipose tissue is the key event. Both clinical and animal studies found that nicotine can block phosphodiesterase activation to promote lipolysis and as a result increase the levels of circulating FFAs [8], which is consistent with the results from An et al. in 2007 [56]. Of great significance, intracellular enzyme hormone-sensitive lipase (HSL), which participates in the hydrolysis of triglyceride in adipocytes, is probably regulated by treatment of nicotine. β-Adrenergic receptor stimulation by catecholamines increases the level of cAMP and, ultimately, activates HSL [63]. Based on this, it is conceivable that via β-adrenergic receptors, smoking has an effect on the activation of HSL, producing elevated FFAs, which are synthesized into triglycerides through transportation into the liver and then secreted

back into circulation as triglyceride-rich lipoprotein, forming the profile of dyslipidemia (Figure 2). In contrast, one kind of PAHs, benzo[a]pyrene, has a significant inhibitory effect on epinephrine-induced FFAs release [60]. It meant the complexity of the compounds of cigarette smoking, which need further studies to explore out.

5.2.3. Smoking and Endocrine Function of Adipose Tissue. As previously discussed, adipokines and cytokines secreted by adipose tissue participated in the inflammation within the artery wall. Recently, the aim of many studies is to investigate how cigarette smoking affects the secretion of adipokines, thus resulting in a deeper understanding of the relationship between smoking and adipose tissue mediated by adipokines in the advancement of atherosclerotic plaques. Next, we will explore the alterations of many crucial adipokines under exposure to cigarette smoke, such as adiponectin, TNF-α, and leptin, as well as adipose tissue inflammation.

(1) Adiponectin and TNF-α. Hypoadiponectinemia was often detected in smokers without sexual difference, which is an independent risk factor for diabetes and AS [64, 65]. In accordance with this, adiponectin levels in healthy Greek smokers were elevated 9 weeks after smoking cessation [66]. In 3T3-L1 adipocytes, Iwashima et al. found that incubation with nicotine significantly reduced adiponectin mRNA expression and adiponectin secretion with dose dependence [64]. Moreover, cultured adipocytes and the adipose tissue of wild-type mice exposed to cigarette smoke extract high-molecular-weight adiponectin and subsequently block its release [67], suggesting an inverse association between smoking and the level of adiponectin.

An increasing body of studies further investigates the potential mechanisms underlying smoking-induced low adiponectin concentration. Of great interest, data from $\beta 2^{-/-}$ mice models have shown that the $\beta 2$ nAChR subunit may reduce the expression levels of AdipoQ genes under chronic nicotine administration [68], thus decreasing the generation of adiponectin. The exact signaling pathway involved in this process, however, has yet to be fully elucidated. Moreover, the activation of β-adrenergic receptors might have similar effects on the production of adiponectin, via a marked depletion of tissue adiponectin mRNA and elevated secretion of immature 30 kDa form [69]. Research has shown that cAMP, the second messenger of β-adrenergic receptors, may act indirectly through enhanced synthesis of inhibitory protein to destabilize adiponectin mRNA [69].

Of note, it is widely recognized that there is a negative interaction between adiponectin and TNF-α, investigated by in vivo study from Maeda et al. [70]. In human epicardial adipose tissue, tobacco smoking induces elevated levels of TNF-α and IL-6, creating the proinflammatory profile [71]. In vitro experiments have shown that TNF-α stimulates nuclear factor-kappa B (NF-κB), PI-3 kinase, and jun-N-terminal kinase cascades, subsequently enhancing lipolysis in periadipocytes [72]. In contrast, nicotine was detected to reduce TNF-α production from rat adipocytes in a dose-dependent manner via nAChRs, while the underlying mechanism remains to be clarified [68, 73]. Consequently, further research should pay more attention to the nAChRs in adipocytes and adipose tissue macrophages, both of which are the main source of TNF-α and adiponectin.

(2) Leptin. Results from several studies show that nicotine has paradoxical effects on the secretion of leptin among smokers. As a result of maternal exposure to nicotine during lactation, offspring rats displayed the hyperleptinemia phenotype and higher visceral and total body fat mass [51, 74]. In contrast, previous studies reported a negative trend between cigarette smoking and plasma leptin levels in both diabetic subjects and healthy subjects [75]. After BMI adjustment, smokers were found to have lower leptin concentrations, which is probably attributed to the indirect effects of elevated catecholamine levels, rather than nicotine [76]. Of particular significance, Perkins et al. found no difference in leptin concentration on smoking status after controlling for BMI and age, while leptin levels only in women increased after smoking cessation [77]. One putative cause for these different conclusions may be the lack of attention to ethnic differences and the varying quantity of cigarettes. More research is needed to elaborate on the association between leptin and smoking/nicotine.

(3) Adipose Tissue Inflammation. As previously discussed, adipose tissue inflammation plays a fundamental role in the progression of atherosclerotic plaques. Both adipose tissue macrophages and T lymphocytes also contribute to formation of the inflammatory microenvironment in adipose tissue.

Interestingly, a large number of studies focus on the interplays between smoking and lymphocytes, so there is an emerging notion that smoking may mediate the numbers and functions of lymphocytes and the inflammation status within adipose tissue. According to existing research, it primarily utilizes the following pathways in the aforementioned pathological process.

First, certain cytokines and adipokines induced by smoking might influence the number and function of resident lymphocytes in adipose tissue, as well as local inflammation. For example, after activation of the NLRP3 inflammasome, enhanced CCL2 expression in perivascular adipocytes of smokers is previously discussed [78]. Notably, the stimulation by smoking or nicotine on macrophages was shown to further trigger adipose tissue inflammation. Through the Toll-like receptor 4 (TLR4), cigarette smoke induces phosphorylation of NF-κB in cultured macrophages to activate inflammatory signaling, resulting in more cytokines secretion [79]. Aside from these direct effects, increased FFAs on exposure to smoking can be recognized by TLR-4 to exert indirect actions. As shown in Table 1, adiponectin and leptin also participate in the phenotype transition, such as macrophage polarization and T lymphocytes [80, 81]. Hence, adipose tissue inflammation will in turn exacerbate the adventitia response and eventually deteriorate intima injury. Given these smoking-induced changes in adipose tissue, it is important to identify whether smoking is directly involved in the recruitment and activation of lymphocytes or not and what the potential mechanisms of involvement are.

6. Conclusion

There is a complex yet significant interaction between cigarette smoking exposure, adipose tissue, and atherosclerotic plaque formation and rupture. Smoking or nicotine appears to affect the differentiation and functions of adipocytes, as well as the inflammatory status in adipose tissue. The current literature has always shed light upon the multiple molecular mechanisms by which components of tobacco smoke can initiate endothelial injury. Of great interest is the complicated interaction between smoking and adipose tissue and how this could establish a better understanding of the course of smoking-related AS. However, there are few studies that provide direct evidences responsible for the effects on adipose tissue by nicotine. Therefore, as the knowledge of nicotine-induced dysfunction in adipose tissue has advanced, it has become clear that there exists a "nicotine-adipose tissue-AS" axis, which paves the way for the development of further targeted therapy.

Abbreviations

AS: Atherosclerosis
AMPK: 5′-Monophosphate-activated protein kinase
BMI: Body mean index
CVDs: Cardiovascular diseases
cAMP: Cyclic adenosine monophosphate
FFAs: Free fatty acids
HSL: Hormone-sensitive lipase
IL: Interleukin
IFN: Interferon
LDL: Low-dense lipoprotein
nAChRs: Nicotinic acetylcholine receptors
NF-κB: Nuclear factor-kappa B
PPAR-γ: Peroxisome proliferator activated receptor-γ
PVAT: Perivascular adipose tissue
ROS: Reactive oxygen species
TLR4: Toll-like receptor 4
TNF: Tumor necrosis factor
VSMCs: Vascular smooth muscle cells.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

All authors contributed equally to the conception and design of the review, the analysis and interpretation of the literature, drafting the article, and approving the final version of the manuscript.

Acknowledgments

This work was supported by 81470471 from the Natural Science Foundation of China (NSFC).

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Hindawi
Mediators of Inflammation
Volume 2017, Article ID 9632846, 11 pages
https://doi.org/10.1155/2017/9632846

Review Article

Protective Effects of Methotrexate against Proatherosclerotic Cytokines: A Review of the Evidence

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Received 20 July 2017; Revised 2 November 2017; Accepted 26 November 2017; Published 21 December 2017

Academic Editor: Sandra Helena Penha Oliveira

There is good epidemiological evidence that patients with autoimmune rheumatic disease states, particularly rheumatoid arthritis, have an increased risk of cardiovascular morbidity and mortality when compared to the general population. The presence of a chronic systemic proinflammatory state in this patient group disrupts the structural and functional integrity of the endothelium and the arterial wall, favouring the onset and progression of atherosclerosis. A significant role in the detrimental effects of inflammation on endothelial function and vascular homeostasis is played by specific proatherosclerotic cytokines such as tumour necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6). Recent systematic reviews and meta-analyses have shown that treatment with methotrexate, a first-line disease-modifying antirheumatic drug (DMARD), is associated with a significant reduction in atherosclerosis-mediated cardiovascular events, such as myocardial infarction and stroke, and mortality, when compared to other DMARDs. This suggests that methotrexate might exert specific protective effects against vascular inflammation and atherosclerosis in the context of autoimmune rheumatic disease. This review discusses the available evidence regarding the potential antiatherosclerotic effects of methotrexate through the inhibition of TNF-α, IL-1, and IL-6 and provides suggestions for future experimental and human studies addressing this issue.

1. Introduction

Autoimmune rheumatic diseases such as rheumatoid arthritis (RA) are characterized by the presence of a chronic inflammatory state affecting the joints as well as a number of other organs and tissues [1]. The prevalence of RA ranges between 0.1 and 5%, depending on specific ethnic groups and geographic locations, and is higher in females than in males [1]. Patients with RA have an increased risk of death [2]. The increased mortality in this patient group, as well as in other autoimmune rheumatic conditions, is primarily due to a relatively high prevalence of cardiovascular disease and its clinical consequences, mainly acute atherosclerosis-related events such as myocardial infarction and stroke [3–5]. The risks of myocardial infarction and stroke are, respectively, 38% and 24% higher in RA patients when compared to the general population [4, 5]. This suggests that RA favours the onset of vascular damage and atherosclerosis either through conventional cardiovascular risk factors, such as diabetes, hypercholesterolaemia, or cigarette smoking, or through alternative mechanisms [6, 7]. One possible alternative mechanism is represented by the autoimmune- and inflammation-mediated disruption of the structural and/or functional integrity of the endothelium. This

leads to significant alterations in vascular homeostasis, driven by an impairment of nitric oxide (NO) synthesis by endothelial NO synthase (eNOS), that include a reduced endothelium-dependent vasodilatation, an increased leukocyte and monocyte adhesion to, and deposition in, the arterial wall, an increased intima-media thickness and arterial stiffness, and a prothrombotic tendency [8–13]. Notably, these abnormalities have been reported both in animal models of autoimmune rheumatic disease and in patients with RA [8, 14–18]. The "inflammatory theory" of atherosclerosis highlights the key role of specific cytokines, particularly tumour necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6), in disrupting endothelial integrity and vascular homeostasis [19, 20]. Given the presence of a chronic inflammatory state, and therefore a sustained vascular insult, it is likely that the pathophysiological role of proatherosclerotic cytokines is further augmented in RA. This might explain the increased cardiovascular risk reported in this patient group [7].

Over the last few years, an increasing number of *in vitro* and *in vivo* studies have sought to identify pharmacological strategies targeting inflammatory pathways for cardiovascular disease prevention and management [20–22]. A number of therapeutic agents commonly prescribed to combat immune activation and inflammation in RA might also exert salutary effects on endothelial function and vascular homeostasis. Recent epidemiological evidence suggests that methotrexate, an established first-line disease-modifying antirheumatic drug (DMARD), might exert protective effects against cardiovascular disease. This review discusses the role of cytokines and inflammation in the pathophysiology of endothelial dysfunction and atherosclerosis, the pharmacology of methotrexate, the reduced cardiovascular risk associated with its use, and the available evidence regarding the effects of this DMARD on proatherosclerotic cytokines and endothelial function.

2. Cytokines, Endothelial Dysfunction, and Atherosclerosis

The endothelium plays a key role in maintaining vascular homeostasis and protecting the vascular wall from a number of endogenous and exogenous proatherosclerotic insults [23]. A key role, in this context, is played by nitric oxide (NO), an endogenous messenger synthesised by the enzyme endothelial NO synthase (eNOS) [10]. NO regulates several important physiological processes, including vasodilation of arteries and arterioles, vascular tone, arterial stiffness, wave reflection, peripheral vascular resistance, blood pressure, and platelet function (Figure 1) [10]. A reduced NO synthesis by eNOS has been shown to be associated with virtually all cardiovascular risk factors [24]. Furthermore, clinical measures of endothelial dysfunction independently predict cardiovascular morbidity and mortality in several patient groups [25–27]. Therefore, the available evidence suggests that endothelial dysfunction is a key pathophysiological step in the sequence of events linking the presence of one or more cardiovascular risk factors with the development of atherosclerosis. Furthermore, endothelial dysfunction is useful in stratifying the risk of cardiovascular events at the population level and might represent an important target of therapies designed to mitigate such risk [28].

A significant number of cytokines, a group of low-molecular weight proteins, are produced in several cell types, such as endothelial cells, monocytes, and vascular smooth muscle cells, that play a key role in maintaining vascular homeostasis. Amongst them, the cytokines TNF-α, IL-1, and IL-6 have been extensively investigated not only from a pathophysiological point of view but also as therapeutic targets for novel cardioprotective therapies [21, 22, 29]. Both TNF-α and IL-1 are known to stimulate the synthesis of IL-6 during the process of immune activation and inflammation [30]. This, in turn, triggers several pathways that result in endothelial dysfunction, vascular inflammation and damage, and atherosclerosis [31]. The cellular effects of TNF-α and IL-1 are primarily mediated by the p38 mitogen-activated protein kinase (p38MAPK)/nuclear factor kappa-light-chain-enhancer of the activated B-cell (NF-κB) pathways [32]. By contrast, the effects of IL-6 are mediated by the IL-6 receptor and the signal transducer protein gp130 [33].

Experimental studies have demonstrated the deleterious effects of TNF-α, IL-1, and IL-6 on endothelial function, vascular homeostasis, and cardiovascular risk and the protective effects of pharmacological agents targeting these cytokines on surrogate vascular markers (Table 1).

2.1. TNF-α. Treatment of endothelial cells with TNF-α has been shown to increase the expression of the inducible form of NO synthase (iNOS), decreasing at the same time the expression of the endothelial constitutive isoform eNOS [34]. While the maintenance of eNOS activity provides adequate NO synthesis for the regulation of several physiological and antatherosclerotic effects, an excessive NO synthesis by iNOS leads to the intracellular formation of reactive oxygen species with consequent development of endothelial dysfunction, apoptosis, and vascular damage [35]. Not surprisingly, several studies have also reported that TNF-α significantly impairs endothelium-dependent vasodilation, through an increase in reactive oxygen species, promotes the adhesion of leukocytes to the endothelium, and favours endothelial cell apoptosis [36–38]. Furthermore, TNF-α inhibits the activity of the enzyme dimethylarginine dimethylaminohydrolase, with consequent accumulation of the endogenous eNOS inhibitor asymmetric dimethylarginine (ADMA) [39, 40]. This effect is clinically relevant as higher plasma concentrations of ADMA have been shown to independently predict cardiovascular events in patients with a wide range of cardiovascular risk at baseline (Table 1) [41, 42].

Higher serum TNF-α concentrations have been associated with an increased risk of ischaemic stroke and recurrent coronary events in epidemiological studies [43, 44]. The key role of TNF-α in mediating the detrimental effects of inflammation on endothelial dysfunction and vascular homeostasis is also supported by the results of animal and human studies investigating the effects of specific TNF-α inhibitors. For example, treatment with adalimumab significantly reduced the adhesion of human leukocytes to endothelial cells and the expression of vascular cell adhesion molecule-1

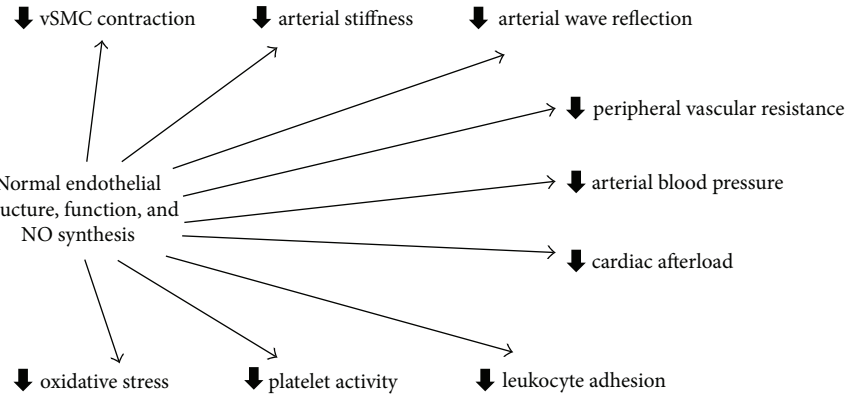

Figure 1: Endothelium, nitric oxide, and vascular homeostasis. NO: nitric oxide; VSMC: vascular smooth muscle cell.

Table 1: Effects of the cytokines TNF-α, IL-1, and IL-6 on endothelial function and vascular homeostasis.

Cytokine	Reported effects
Tumour necrosis factor-alpha	Endothelial nitric oxide synthase activity ↓
	Inducible nitric oxide synthase activity ↑
	Reactive oxygen species ↑
	Endothelium-dependent vasodilation ↓
	Leukocyte adhesion ↑
	Endothelial cell apoptosis ↑
	Asymmetric dimethylarginine ↑
Interleukin-1	Endothelium-dependent vasodilation ↓
	Vasoconstrictor response to pharmacological challenge ↑
	Endothelin-1 ↑
	Leukocyte adhesion ↑
	Vascular smooth muscle cell growth ↑
	Intima thickness ↑
	Arterial stiffness ↑
	Coagulation ↑
Interleukin-6	Expression of angiotensin II type-1 receptor ↑
	Endothelium-dependent vasodilation ↓
	Arterial stiffness ↑
	Oxidative stress ↑
	Thrombosis ↑

↑: Increase; ↓: decrease.

(VCAM-1), intracellular adhesion molecule-1 (ICAM-1), and E-selectin [45]. Other studies have shown that treatment with adalimumab or etanercept improved clinical measures of endothelium-dependent vasodilation in patients with psoriasis, reduced the plasma concentrations of ADMA, and increased circulating endothelial progenitor cells in patients with RA (Table 1) [46–48].

2.2. IL-1. Increased production of IL-1 has been shown to induce leukocyte adhesion to the endothelium, exert procoagulant activity, and stimulate the growth and chemotaxis of vascular smooth muscle cells, key steps in the pathogenesis of atherosclerosis [49–52]. In animal studies, exposure to exogenous IL-6 causes an increase in coronary vasospastic responses to pharmacological challenge and intima thickening (Table 1) [53]. Moreover, multiple factors known to associate with atherosclerosis, such as cholesterol crystals, atheroprone oscillatory flow, hypoxia, and neutrophil extracellular traps, have recently been found to activate the critical IL-1beta producing NLRP3 inflammasome [22].

Polymorphisms in the IL-1 receptor antagonist (IL-1Ra) gene have been shown to have significant associations with the presence of single-vessel coronary artery disease, assessed by coronary angiography, in patients with ischaemic heart disease [54]. Conversely, pharmacological inhibition of IL-1 with anakinra reduces the concentrations of the potent endogenous vasoconstrictor endothelin-1 and arterial stiffness and improves clinical measures of endothelial function in patients with RA [55]. Similar effects of anakinra on endothelium-dependent vasodilation have been reported in animal models of diabetes (Table 1) [56]. In a recently completed randomized placebo-controlled trial in 10,061 participants with a previous myocardial infarction and C-reactive protein concentrations $\geq$2 mg/L, canakinumab, a monoclonal antibody targeting IL-1beta, significantly reduced the primary end-point of nonfatal myocardial infarction, nonfatal stroke, or cardiovascular death after a median follow-up of 3.7 years. Of the three subcutaneous doses of canakinumab studied (50 mg, 150 mg, and 300 mg every three months), only the 150 mg dose met the prespecified multiplicity adjusted threshold for statistical significance. Furthermore, there were no significant differences in all-cause mortality between treatment with canakinumab and placebo [57].

2.3. IL-6. IL-6 has been shown to upregulate the expression of the angiotensin II type-1 receptor in vascular smooth muscle cells, responsible for vasoconstriction, cell apoptosis, and proinflammatory effects, and to impair endothelium-dependent vasodilation in animal models [58]. Increased plasma IL-6 concentrations are significantly associated with a reduced endothelium-dependent vasodilation both in healthy subjects [59] and in patients with hypertension [60] or hypercholesterolaemia [61]. A significant association between plasma IL-6 concentrations and clinical markers of increased arterial stiffness has been reported in patients with

hypertension (Table 1) [62]. A meta-analysis performed by the Emerging Risk Factors Collaboration has also shown that higher plasma IL-6 concentrations predict the risk of nonfatal myocardial infarction and coronary artery disease-related death, with a 25% increase in the risk of future vascular events for each increase in log IL-6 concentrations (RR 1.25, 95% CI 1.19 to 1.32) [63].

Furthermore, treatment with the IL-6 receptor inhibitor tocilizumab caused an increase in endothelium-dependent vasodilatation, and a concomitant reduction in arterial stiffness, in patients with RA [64, 65]. In another study, tocilizumab improved endothelial function and reduced markers of oxidative stress, inflammation, and thrombosis in patients with RA (Table 1) [66].

3. Methotrexate Pharmacology

Methotrexate, an analogue of the B vitamin folic acid, is a first-line synthetic DMARD for the management of RA and other autoimmune diseases that is normally administered once a week, either orally, subcutaneously, or intramuscularly, with doses ranging between 5 and 25 mg [67, 68]. Notably, methotrexate is the only DMARD that has demonstrated significant survival benefits in patients with RA [69–71]. After being transported into the cytoplasm, through the reduced folate carrier, methotrexate is converted into intracellular polyglutamates. The polyglutamate forms ensure the intracellular retention of methotrexate, allowing weekly administration despite the relatively short plasma elimination half-life (5–8 hours) [72]. The polyglutamates also mediate the immunomodulating and anti-inflammatory effects of methotrexate, by reducing the synthesis of purines, pyrimidines, and DNA through the inhibition of dihydrofolate reductase, thymidylate synthase, and aminoimidazole carboxamide ribonucleotide (AICAR) transformylase (ATIC, Figure 2) [73–75]. Furthermore, the inhibition of ATIC causes the accumulation of the substrate AICAR which, in turn, inhibits the enzymes adenosine deaminase and adenosine monophosphate (AMP) deaminase, involved in the catabolism of adenosine (Figure 2) [75]. Adenosine per se exerts significant anti-inflammatory effects, primarily through the A_{2A} and A_3 receptors [76], and mediates the anti-inflammatory effects of methotrexate. In animal models of inflammation, methotrexate has been shown to increase AICAR and adenosine concentrations and to inhibit the accumulation of leukocytes, in exudates from carrageenan-inflamed air pouches. The administration of AMP deaminase partly reversed the methotrexate-mediated reduction in leukocyte accumulation. Additionally, the administration of 3,7-dimethyl-1-propargylxanthine, an A_{2A} receptor antagonist, but not 8-cyclopentyl-dipropylxanthine, an A_1 receptor antagonist, suppressed the methotrexate-mediated reduction in leukocyte accumulation [77]. Furthermore, both AICAR and AMP activate the 5′ adenosine monophosphate-activated protein kinase (AMPK) [78]. AMPK activation is protective towards endothelial cell function and vascular homeostasis, by ensuring physiological NO synthesis, maintaining mitochondrial structure and function, and preventing oxidative stress and apoptosis [79]. Moreover, a role for

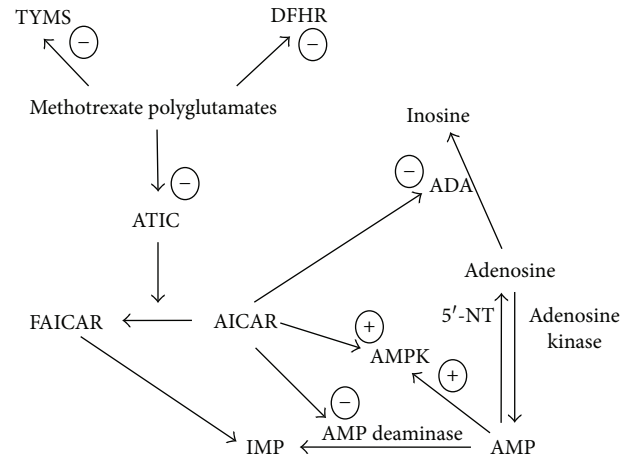

Figure 2: Intracellular effects of methotrexate. DHFR: dihydrofolate reductase; TYMS: thymidylate synthase; ATIC: aminoimidazole carboxamide ribonucleotide (AICAR) transformylase; FAICAR: 5-formamidoimidazole-4-carboxamide ribotide; IMP: inosine monophosphate; AMP: adenosine monophosphate; AMPK: 5′ adenosine monophosphate-activated protein kinase; ADA: adenosine deaminase; 5′-NT: 5′-nucleotidase; −: inhibition; +: activation.

AMPK in inhibiting vascular smooth muscle cell proliferation, a key proatherosclerotic event both in autoimmune rheumatic diseases and in the general population, has been proposed [80, 81]. There is experimental evidence that AMPK is a key mediator of the effects of methotrexate on inflammation and endothelial function. *In vitro* studies have shown that methotrexate, at concentrations between 0.1 and 0.5 μM, increased AMPK phosphorylation and AMPK activity in perivascular adipose tissue cells. In these cells, the administration of palmitic acid, a proinflammatory agent, decreased AMPK phosphorylation; however, these effects were significantly reduced by pretreatment with either methotrexate or AICAR. Furthermore, methotrexate significantly reduced the phosphorylation of NF-κB p65 and prevented the palmitic acid-mediated increase in the expression of TNF-α and IL-6, indicating anti-inflammatory effects. Silencing AMPK with AMPKa1/2-specific siRNA significantly decreased the inhibitory effects of methotrexate on palmitic acid-mediated NF-κB p65 phosphorylation and on the expression of TNF-α and IL-6. Pretreatment with methotrexate prevented the impairment of acetylcholine-induced, endothelium-dependent vasodilation in rat aorta following palmitic acid administration. Notably, the protective effects of methotrexate on endothelial function were attenuated by cotreatment with compound C, an AMPK inhibitor [82].

4. Methotrexate and Cardiovascular Risk

Recent systematic reviews and meta-analyses have investigated the associations between methotrexate treatment and cardiovascular risk. Micha et al. identified observational studies in 66,334 patients with either RA (9 studies), psoriasis (1 study), or polyarthritis (1 study), that reported 6235 cardiovascular events. The median duration of follow-up in

these studies was 5.8 years. Six studies compared methotrexate users versus never users, three compared current versus noncurrent users, and two compared initiators versus noninitiators. Cardiovascular endpoints included total or fatal cardiovascular disease (7 studies; two studies also provided separate estimates for myocardial infarction and ischaemic/haemorrhagic stroke), myocardial infarction (3 studies), and ischaemic stroke (1 study). Combined assessment of the included studies showed that methotrexate treatment was associated with a significant reduction in cardiovascular events (RR 0.79, 95% CI 0.73 to 0.87). Assessment of specific cardiovascular endpoints showed a similar effect size (overall cardiovascular disease events: RR 0.76, 95% CI 0.69 to 0.84; myocardial infarction: RR 0.82, 95% CI 0.71 to 0.96; stroke: RR 0.70, 95% CI 0.56 to 0.87) [83]. In a subsequent systematic review and meta-analysis, Roubille et al. identified 34 studies (28 studies in 236,525 RA patients, reporting 5410 cardiovascular events, and 6 studies in 220,209 patients with either psoriasis or psoriatic arthritis, reporting 2701 cardiovascular events). In studies conducted in RA patients, methotrexate use was associated with a reduced risk of all cardiovascular events (RR 0.72, 95% CI 0.57 to 0.91, $P = 0.007$) and myocardial infarction (RR 0.81, 95% CI 0.68 to 0.86, $P = 0.01$) when compared to other synthetic DMARDs. Although there was no significant effect of methotrexate on the risk of either stroke (RR 0.78, 95% CI 0.40 to 1.50) or major adverse cardiovascular events (RR 0.38, 95% CI 0.05 to 2.84), the number of studies assessing these endpoints was relatively low (one study for stroke and two studies for major adverse cardiovascular events, resp.) [84]. Therefore, the results of observational studies support the hypothesis that methotrexate exhibits specific protective cardiovascular effects when compared to other DMARDs.

5. Methotrexate and Proatherosclerotic Cytokines

In order to review the available evidence on the direct or indirect, mediated by adenosine, AICAR or AMPK activation, effects of methotrexate on proatherosclerotic cytokines, a PubMed literature search was conducted from inception to June 2017, using the following terms: methotrexate, adenosine, AICAR, AMPK, endothelium, inflammation, atherosclerosis, TNF-α, IL-1, and IL-6.

5.1. TNF-α. In *in vitro* studies, clinical concentrations of methotrexate (2×10^{-8} M and 2×10^{-7} M) have been shown to increase the release of soluble TNF receptor p75 from the cell surface [85]. This phenomenon is able to significantly inhibit the proinflammatory effects of TNF-α [86]. Adenosine is a known inhibitor of TNF-α expression through stimulation of the A_3 receptor (Table 2) [87, 88]. There is recent evidence that methotrexate stimulates AMPK phosphorylation and activity, induces manganese superoxide dismutase mRNA and protein, and increases the expression of the cytoprotective genes haem oxygenase-1 and Bcl-2-related protein in human umbilical vein endothelial cells (HUVECs) and arterial endothelial cells (HAECs). The pretreatment of endothelial cells with TNF-α did not affect the methotrexate-mediated upregulation of manganese superoxide dismutase and haem oxygenase-1 mRNA [89]. Methotrexate (10^{-8} M to 10^{-6} M) and/or adenosine treatment has also been shown to prevent the TNF-α-induced (a) expression of ICAM-1 and VCAM-1 in human

Table 2: Effects of methotrexate, adenosine, AICAR, and AMPK activation on endothelial function and vascular homeostasis.

Mediator	Reported effects
Methotrexate	Release of soluble TNF-α receptor p75 ↑
	TNF-α expression/concentrations ↓
	IL-6 expression/concentrations ↓
	ICAM-1 expression ↓
	VCAM-1 expression ↓
	eNOS activity ↑
	Endothelium-dependent vasodilatation ↑
	Mitochondrial mass, membrane potential, and intracellular ATP concentrations ↑
Adenosine	TNF-α expression/concentrations ↓
	IL-6 expression/concentrations ↓
	ICAM-1 expression ↓
	VCAM-1 expression ↓
	E-selectin expression ↓
	eNOS activity ↑
	Blood pressure ↓
	Mitochondrial mass, membrane potential, and intracellular ATP concentrations ↑
	Formation of atherosclerotic lesions ↓
	Cholesterol concentrations ↓
	Triglyceride concentrations ↓
AICAR/AMPK	IL-1 expression/concentrations ↓
	IL-6 expression/concentrations ↓
	ICAM-1 expression ↓
	VCAM-1 expression ↓
	NO synthesis ↑
	Endothelium-dependent vasodilation ↑
	Endothelium-independent vasodilation ↑
	Blood pressure ↓
	Oxidative stress ↓
	Endoplasmic reticulum stress ↓
	Manganese superoxide dismutase induction ↑
	NF-κB ↓
	Monocyte adhesion to endothelial cells ↓
	Restenosis ↓
	Cholesterol efflux capacity ↑
	Cellular glucose uptake ↑
	Glycolysis ↑

AICAR: aminoimidazole carboxamide ribonucleotide; AMPK: 5′ adenosine monophosphate-activated protein kinase; TNF-α: tumour necrosis factor-alpha; IL-1: interleukin-1; IL-6: interleukin-6; ICAM-1: intercellular adhesion molecule-1; VCAM-1: vascular cell adhesion molecule-1; ATP: adenosine triphosphate; eNOS: endothelial nitric oxide synthase; NF-κB: nuclear factor kappa-light-chain-enhancer of the activated B-cell; NO: nitric oxide; ↑: increase; ↓: decrease.

umbilical vein endothelial cells (HUVEC) [90] and (b) reduction of mitochondrial mass, membrane potential, and intracellular ATP concentrations, in endothelial cells through increased activity of eNOS [91]. Furthermore, adenosine has been shown to reduce the circulating concentrations of TNF-α and IL-6 in a mice model of sepsis (Table 2) [92]. By contrast, therapeutic concentrations of methotrexate, 0.1, 0.25, and 0.5 μM, significantly increased the expression of the TNF-α receptor-associated factor 1 gene (~threefold change) and of the TNF-α receptor superfamily member 9 gene (~threefold change) in EA.hy 926 cells, derived from the fusion of primary endothelial cells with an epithelial tumour cell line [93].

5.2. IL-1. In an *in vitro* model of palmitate-induced endothelial dysfunction, AICAR-mediated AMPK activation has been shown to significantly reduce IL-1, IL-6, and VCAM-1 synthesis, by preventing the activation of the NLRP3 inflammasome [94]. Similarly, pharmacological activation of AMPK with oestradiol prevented the IL-1-induced expression of VCAM-1 and ICAM-1 in cultured human endothelial cells (Table 2) [95]. By contrast, therapeutic concentrations of methotrexate, 0.1, 0.25, and 0.5 μM, significantly increased the expression of the IL-1 alpha gene (~threefold change) and of the IL-1 receptor-like 1 gene (~fourfold change) in EA.hy 926 cells [93].

5.3. IL-6. In db/db mice fed with Western diet, methotrexate treatment (4 mg/kg) caused a significant reduction in the circulating concentrations of IL-6 and TNF-α. These effects were associated with a significant increase in endothelium-dependent vasodilatation and a reduction in VCAM-1 [96]. In another study, methotrexate treatment (0.1–0.5 μM) significantly reduced the expression of IL-6 and TNF-α. These effects were mediated by the activation of AMPK and were associated with an improvement in eNOS activity and endothelium-dependent vasodilation in rat aorta (Table 2) [82]. Adenosine has been shown to prevent the thrombin-mediated increased expression of IL-6, VCAM-1, ICAM-1, and E-selectin in HUVECs. These effects are mediated by the activation of the adenosine receptor A_{2A} [97]. Similarly, adenosine inhibited, in a dose-dependent fashion, the release of IL-6, VCAM-1, and ICAM-1 in HUVECs pretreated with either IL-1, TNF-α, or lipopolysaccharide (Table 2) [98].

6. Additional Vascular Effects of Adenosine, AICAR, and AMPK Activation

A number of studies have shown that adenosine, AICAR, and AMPK activation provide beneficial effects on endothelial function and vascular homeostasis that are independent of those on TNF-α, IL-1, or IL-6.

6.1. Adenosine Accumulation. There is good evidence that adenosine, through the activation of the A_{2A} and A_{2B} receptors, lowers blood pressure as a result of increased NO synthesis in the endothelium and direct vasodilation [99, 100]. Studies have also demonstrated the presence of central nervous system-mediated hypotensive effects through the activation of the A_3 receptor [101]. Furthermore, the pharmacological activation of the A_{2B} receptor prevents the formation of atherosclerotic lesions and reduces the plasma concentrations of cholesterol and triglycerides, possibly through the reduced activation of the transcription factor sterol regulatory element-binding protein 1 in the liver [102, 103]. Activation of adenosine receptors seems also to modulate glucose homeostasis. However, the pathophysiological and clinical relevance of this finding is yet to be determined (Table 2) [104].

6.2. AICAR and Activation of 5′ Adenosine Monophosphate-Activated Protein Kinase (AMPK). There is increasing evidence that AICAR and or/AMPK activation stimulates NO synthesis in the endothelium, enhances endothelium-dependent and endothelium-independent vasodilation, reduces blood pressure, prevents vessel restenosis, and increases cholesterol efflux capacity [105–110]. Furthermore, AMPK stimulates cellular glucose uptake, through GLUT-1 and GLUT-4 transporters, and glycolysis, through phosphorylation of two isoforms of the enzyme 6-phoshofructo-2-kinase: fructose-2,6-biphosphatase, with beneficial effects on glucose homeostasis [111–114]. There is also evidence that AMPK activation protects endothelial cells from the deleterious effects of chronic exposure to high concentrations of glucose and fatty acids and consequently reduces oxidative stress, inflammation, and endoplasmic reticulum stress (Table 2) [115, 116].

7. Discussion

The available evidence from systematic reviews and meta-analyses of observational studies in patients with either RA or other autoimmune rheumatic disease states suggests that the use of methotrexate is associated with a significant reduction in cardiovascular morbidity and mortality when compared to other DMARDs. Furthermore, a relatively small number of experimental studies have shown that methotrexate can exert beneficial effects on endothelial function and vascular homeostasis by preventing or blocking the effects of key proatherosclerotic cytokines such as TNF-α, IL-1, and IL-6. The reported effects are mediated either by methotrexate directly or through the activation of adenosine receptors, AICAR, or AMPK (Table 2). Pending further *in vitro* and *in vivo* studies investigating the exact mechanisms involved in such effects, a number of limitations need to be considered when interpreting the available data:

(1) The dose of methotrexate used in some studies [96] and its consequent local concentrations in target cells and tissues are quite different from those normally observed in patients with autoimmune disorders.

(2) No study has assessed the intracellular concentrations of methotrexate polyglutamates, as a factor mediating the effects of the drug on the study end-points.

(3) The effects of methotrexate, adenosine, AICAR, or AMPK activation on proatherosclerotic cytokines

were not compared to those of other synthetic or biologic DMARDs.

(4) Other studies, not specifically investigating TNF-α, IL-1, and IL-6, have shown that methotrexate can also exert antiproliferative effects in human umbilical vein endothelial cells and EA.hy 926 cells [93, 117, 118] and reduce NO synthesis, possibly as a result of reduced availability of tetrahydrobiopterin, an essential cofactor for endothelial nitric oxide synthase, secondary to dihydrofolate reductase inhibition [119].

(5) There is no direct evidence that the methotrexate-induced inhibition of TNF-α, IL-1, or IL-6 leads to sustained beneficial effects on endothelial function, atherosclerosis, arterial structure and function, and cardiovascular risk in human studies.

These issues should be accounted for in future studies investigating the effects of methotrexate on proatherosclerotic cytokines. In particular, the use of other DMARDs as comparator should help to determine whether methotrexate treatment exerts specific antiatherosclerotic effects that might help to explain its superiority, in terms of cardiovascular risk reduction, reported in observational studies. Furthermore, a comprehensive assessment of proatherosclerotic and antiatherosclerotic cytokines, such as transforming growth factor-β, IL-10, and IL-35 [120], as well as measures of endothelial cell proliferation, apoptosis, and nitric oxide synthesis, might provide additional mechanistic insights regarding the possible vasculoprotective effects of methotrexate and the potential rationale for combining methotrexate treatment with other interventional strategies targeting specific cytokines. In this context, the results of the Cardiovascular Inflammation Reduction Trial (CIRT, clinicaltrials.gov identifier NCT01594333), investigating the effects of methotrexate on myocardial infarction, stroke, and cardiovascular death in patients with type 2 diabetes or metabolic syndrome and stable coronary artery disease, will provide additional knowledge regarding the potential repurposing of methotrexate for cardiovascular risk management and prevention in different patient populations [121].

8. Conclusions

The evidence recently generated from experimental studies suggests that methotrexate, an anchor drug in the treatment of RA and other autoimmune disorders, might exert significant beneficial effects on endothelial function and vascular homeostasis by targeting key cytokines regulating the immune responses and inflammation pathways responsible for the development of atherosclerosis and thrombosis. However, further studies are required to fully establish the role of this DMARD as an antiatherosclerotic and cardioprotective agent.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Hindawi
Mediators of Inflammation
Volume 2017, Article ID 3824276, 9 pages
https://doi.org/10.1155/2017/3824276

Research Article

Overexpression of Cholesteryl Ester Transfer Protein Increases Macrophage-Derived Foam Cell Accumulation in Atherosclerotic Lesions of Transgenic Rabbits

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Received 27 May 2017; Revised 13 October 2017; Accepted 2 November 2017; Published 28 November 2017

Academic Editor: Fabio Cacciapaglia

High levels of plasma high-density lipoprotein-cholesterol (HDL-C) are inversely associated with the risk of atherosclerosis and other cardiovascular diseases; thus, pharmacological inhibition of cholesteryl ester transfer protein (CETP) is considered to be a therapeutic method of raising HDL-C levels. However, many CETP inhibitors have failed to achieve a clinical benefit despite raising HDL-C. In the study, we generated transgenic (Tg) rabbits that overexpressed the human CETP gene to examine the influence of CETP on the development of atherosclerosis. Both Tg rabbits and their non-Tg littermates were fed a high cholesterol diet for 16 weeks. Plasma lipids and body weight were measured every 4 weeks. Gross lesion areas of the aortic atherosclerosis along with lesional cellular components were quantitatively analyzed. Overexpression of human CETP did not significantly alter the gross atherosclerotic lesion area, but the number of macrophages in lesions was significantly increased. Overexpression of human CETP did not change the plasma levels of total cholesterol or low-density lipoprotein cholesterol but lowered plasma HDL-C and increased triglycerides. These data revealed that human CETP may play an important role in the development of atherosclerosis mainly by decreasing HDL-C levels and increasing the accumulation of macrophage-derived foam cells.

1. Introduction

Epidemiological studies have clearly shown that a low high-density lipoprotein cholesterol (HDL-C) level is a strong and independent risk factor for the development of cardiovascular disease (CAD) [1]. Cholesteryl ester transfer protein (CETP) transfers the cholesteryl esters from HDL to apolipoprotein B- (apoB-) containing particles in exchange for triglycerides (TG) [2] and has been considered to be a new drug target for increasing HDL-C levels. Pharmaceutical CETP inhibitors such as Torcetrapib [3] and Dalcetrapib [4] have been shown to raise HDL-C levels effectively, but research into their clinical efficacy was unfortunately terminated due to off-target effect or lack of clinical benefit. A meta-analysis suggested that Evacetrapib, either as a monotherapy or in combination with a statin, reduces low-density lipoprotein cholesterol (LDL-C) and increases HDL-C levels without affecting TG concentrations [5] but has no clinical benefit [6]. The newer CETP inhibitors, Anacetrapib and TA-8995, have shown promising effects on the lipid profile and metabolism (increase in HDL-C and reduction in LDL-C levels), but their cardiovascular effects and safety profile have not yet been confirmed in large outcome trials [7]. Despite an increase in HDL-C and a reduction in

LDL-C, treatment with Torcetrapib and Dalcetrapib was aborted due to an increase in the risk of major cardiovascular events and mortality [8]. Studying common CETP gene variants has not yet led to a consensus on the connection between CETP and atherosclerosis, and the relationship between reduced CETP function and susceptibility to atherosclerosis has proven complex and confusing [9–13]. Most but not all studies in transgenic (Tg) mice have shown that CETP inhibition reduces atherosclerosis development [14–18], and the role of CETP in atherosclerosis requires further deep investigation because of the differences in the lipid metabolism of mice and humans. Rabbits have plasma LDLs and are more susceptible to atherosclerosis than rodents, which are relatively resistant to atherosclerosis [19]. Inhibition of CETP in cholesterol-fed rabbits led to increased HDL-C levels and reduced atherosclerotic lesions but had no effect on aortic cholesterol content [20–23]. However, whether the overexpression of CETP will affect plasma lipoproteins, atherosclerotic lesions and plaque composition in cholesterol-fed rabbits is unclear. In our study, we created Tg rabbits expressing human CETP (hCETP) transgene to investigate the effect of CETP on atherosclerotic lesions and lipoprotein metabolism. Our results showed that increased expression of hCETP increased the accumulation of macrophage-derived foam cells in atherosclerotic lesions.

2. Materials and Methods

2.1. Generation and Identification of Human CETP Transgenic Rabbits. Japanese white rabbits were supplied by the Laboratory Animal Center of Xi'an Jiaotong University. The generation of Tg rabbits expressing human CETP (hCETP) was conducted in our laboratory by microinjection as previously described [24]. For hepatic expression of hCETP, a 1717 bp cDNA of the (NM_000078) hCETP gene was cloned into EcoRV and SacII sites 3′ of the human apoE promoter and 5′ of the human apoE poly A signal and liver element. The resultant fragment was isolated by digestion with Sal I (Figure 1(a)), injected into fertilized rabbit zygotes, and then transplanted into recipient rabbits. Through polymerase chain reaction (PCR) of the genomic DNA extracted from the blood, founder Tg was identified and then bred into F1 progeny. Four-month-old Tg and non-Tg rabbits were used for the current study. Both Tg rabbits ($n = 12$) and non-Tg rabbits ($n = 12$) were fed a chow diet containing 0.3% cholesterol and 3% soybean oil for 16 weeks. All animals were sacrificed by an overdose of pentobarbital sodium and xylazine hydrochloride. All animal experiments were approved by the Laboratory Animal Administration Committee of Xi'an Jiaotong University and performed according to the Guidelines for Animal Experimentation of Xi'an Jiaotong University and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH, Publication number 85–23, revised 2011).

2.2. Biochemical Analyses. Blood samples were collected via the auricular artery using an EDTA anticoagulant tube after overnight fasting and then centrifuged (3000 rpm, 15 min, 4°C) to obtain the plasma. The plasma TG, total cholesterol (TC), LDL-C, and HDL-C were analyzed every 4 weeks using commercial kits (Biosino Bio-Technology & Science Inc., Beijing, China). Plasma CETP activity was determined as previously described [25]. The plasma CETP concentrations were measured using a human cholesteryl ester transfer protein ELISA kit (Cusabio Co. Ltd., Hubei, China) according to the manufacturer's instructions.

2.3. Measurement of Blood Pressure. The blood pressure of the rabbits was measured as previously described [24]. First, rabbits were anesthetized with pentobarbital sodium. Then, an artery catheter was inserted into the ear artery with a pressure transducer and amplifier attached to a digital PowerLab data acquisition system (ML870 PowerLab) (AD Instruments, Bella Vista, NSW, AUS). The data were collected 10 minutes after the rabbits became calm and there were no blood pressure fluctuations. The blood pressure measurements were calculated using Chart 5 Pro v5.5 software (AD Instruments).

2.4. Quantitative PCR Analysis. Total RNA was isolated from the liver, heart, spleen, lung, kidney, adrenal gland, fat, muscle, testis, aortic arch, macrophage, brain, marrow, and intestine of rabbits using TRIzol reagent (Invitrogen, CA, USA) and reverse-transcribed into cDNA using a reverse transcription kit (Takara, Shiga, Japan). The quantitative real-time PCR reactions were composed of SYBR® Premix Ex Taq™ II (10 μl), total primer pairs (2 μl), cDNA template (1 μl), and RNase-free water (7.0 μl). The primers used for real-time PCR were as follows: human CETP primers: forward, 5′-TCAGCCACTTGTCCATCGC-3′; reverse, 5′-GGCATC GGTCCGCACTCTA-3′and rabbit GAPDH primers: forward, 5′-ATCACTGCCACCCAGAAGAC-3′; reverse: 5′-G TGAGTTTCCCGTTCAGCTC-3′. The cycling conditions were 95°C for 30 s, followed by 40 cycles of 95°C for 30 s, and 55°C for 40 s.

2.5. Western Blotting. Protein samples were extracted from the fresh livers of both Tg rabbits and non-Tg littermates ($n = 3$) incubated in a lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, and protease inhibitor, pH 7.4) for 30 minutes in ice and then centrifuged for 10 minutes at 12000g to discard the cell debris. Total protein concentrations were determined to ensure that the equal loading of proteins was separated on 10% SDS-PAGE and transferred onto PVDF membrane. Antibodies against rabbit CETP (1 : 400; Abcam, Cambridge, UK), human CETP (1 : 400; Abcam, Cambridge, UK), and GAPDH (1 : 500; Beyotime, Beijing, China) were used for Western blot analysis. The blots were developed using HRP-conjugated secondary antibodies (1 : 2000; Thermal, MA, USA) and the ECL-plus system.

2.6. Atherosclerosis Quantification. The entire "aortic tree" fixed in 10% neutral buffered formalin was stained with Sudan IV for evaluation of the gross atherosclerotic lesions as previously described [26]. The area of the atherosclerotic lesion (sudanophilic area) was measured using image analysis software (Mitani, Tokyo, Japan) [27]. For the microscopic

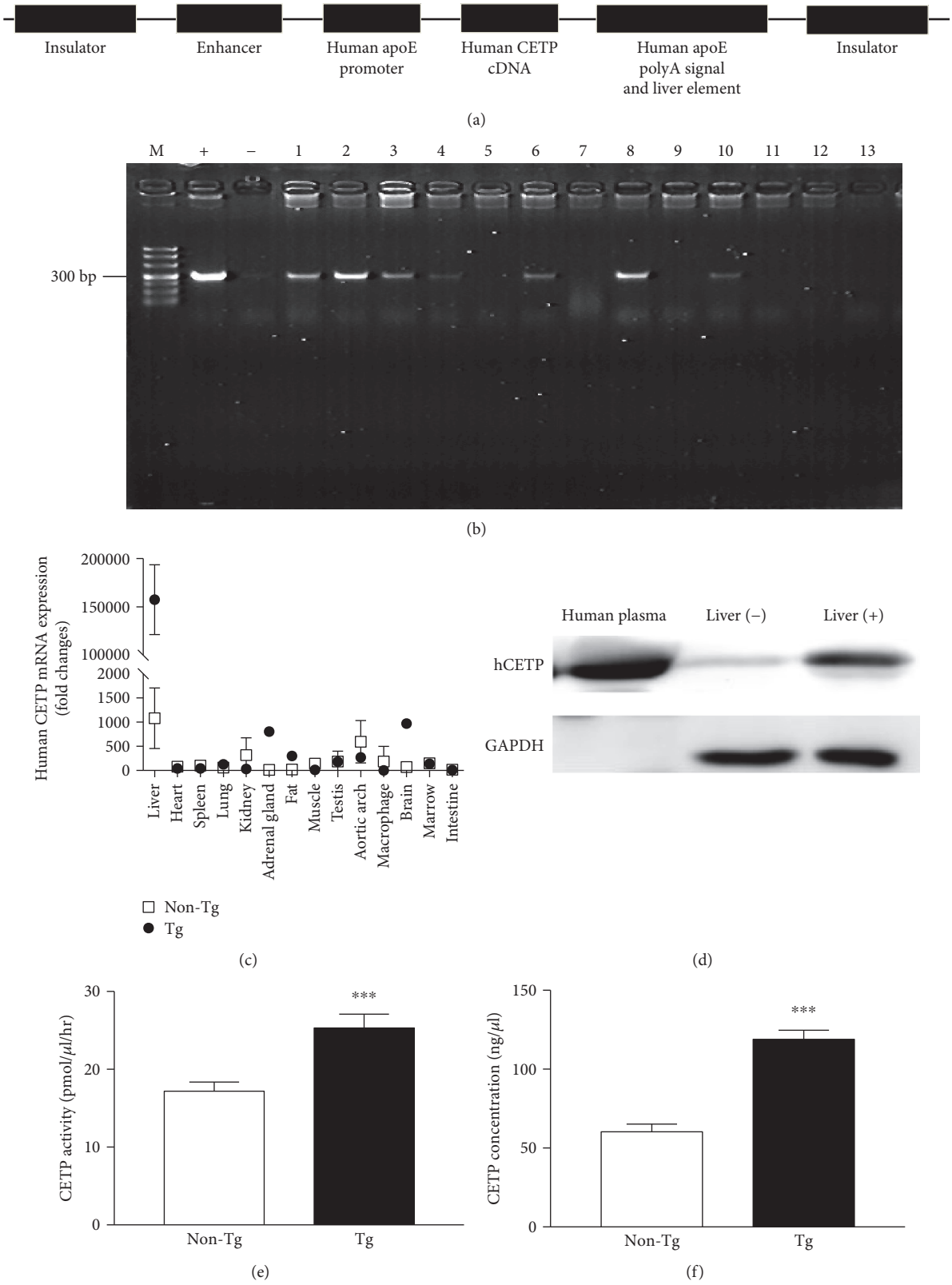


Figure 1: Generation and identification of human CETP Tg rabbits. (a) Tg construct for the microinjection. (b) Identification of the integration of the human CETP transgene in the rabbit genome by PCR (M: DNA marker; +: positive control plasmid; −: negative control; lanes 1–13: rabbit DNA sample). (c) Tissue distribution of human CETP mRNA in Tg and non-Tg rabbits ($n = 3$ for each group). (d) Western blotting analysis of CETP from liver and plasma ($n = 3$ for each group). (e) Plasma CETP activity ($n = 4$ for each group). (f) Plasma human CETP concentrations ($n = 7$ for each group). Data are expressed as the mean ± SEM. $^{***}P < 0.001$ versus non-Tg littermates.

quantification of the lesion area, the aortic arch was processed through routine steps of desiccation followed by clearing, dipping and embedding in wax, and serial sectioning (4 μm). The sections were then stained with hematoxylin and eosin (H&E) and Elastica van Gieson (EVG). The lesion composition of the atherosclerosis plaque was evaluated after immunostaining with the anti-rabbit α-actin antibody (1 : 500; Dako, CA, USA) for the identification of smooth muscle cells and the anti-rabbit RAM11 antibody (1 : 100; Dako, CA, USA) for the identification of macrophages as previously described [28]. The sections for microscopic quantification were examined and photographed under a light microscope equipped with a digital camera (Nikon, Tokyo, Japan) and measured with image analysis software (WinROOF ver. 6.5, 130 Mitani. Fukui, Japan).

2.7. Statistical Analysis. In a total, 24 rabbits ($n = 12$ for each group) were used for the current study to examine the effect of increased plasma CETP on plasma lipids and atherosclerosis. For lipid analysis and atherosclerosis evaluation, all rabbits were used. For other analyses, only some of the rabbit specimens were collected and used: CETP levels ($n = 7$ for each group) by ELISA, CETP activity ($n = 4$ for each group), CETP mRNA, and protein expression by RT-PCR and Western blotting analysis ($n = 3$ for each group) were quantitated for a comparison. Data are expressed as the mean ± SEM. Statistical analysis was performed using Student's t-test with an equal F value or Welch's t-test when the F value was not equal. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Identification of Human CETP Tg Rabbits. In this study, we successfully generated Tg rabbits (Tg) expressing hCETP confirmed by PCR genotyping (Figures 1(b) and 1(c)). Founder Tg rabbits were mated with non-Tg rabbits, and the germline transmission was confirmed. As shown in Figure 1(c), human CETP transgene was almost exclusively expressed in the liver whereas no expression in non-Tg rabbits. The plasma and hepatic CETP expression was evaluated by Western blotting analysis (Figure 1(d)) and Tg rabbits expressed two-fold higher levels of CETP concentrations and activity (Figures 1(e)–1(f)) than non-Tg rabbits.

3.2. Plasma Biochemical Parameters. Plasma levels of lipids were measured every four weeks as shown in Figure 2(a). For calculating lipid levels during the experiment, plasma lipids were also expressed by the area under the curve (AUC) shown in Figure 2(b). Both mean values and AUC of the plasma TC and LDL-C after high cholesterol diet (HCD) were not significantly different between two groups. Throughout the experiment, the TGs (Figure 2(c)) were maintained at higher levels in Tg group than in non-Tg group, while the HDL-C levels (Figure 2(d)) were significantly lower in Tg rabbits.

3.3. Body Weight, Organ Weight, and Blood Pressure. The effects of hCETP on the body weight, organ weight, and blood pressure are shown in Table 1. There was no obvious difference in the body weight between two groups, either at the start or at the end of the experiment. Neither the weight of the major organs, including the heart, kidneys, and liver, nor the blood pressure was significantly different between two groups.

3.4. Quantification of Atherosclerotic Lesions. Compared to the atherosclerotic lesions of the control group, increased expression of hCETP did not significantly affect the gross atherosclerotic lesions in Tg rabbits (Figure 3(a)). In addition, there was no significant difference in all parts of the rabbit aorta, including the aortic arch and the thoracic and abdominal aortas (Figure 3(b)). Representative micrographs of the aortic arch lesions of each group stained with EVG and H&E or immunohistochemically stained with Abs against SMC a-actin and RAM11are shown in Figure 3(c). Apparently, Tg rabbits showed increased tendency of intimal lesions along with enhanced SMCs and macrophage accumulation compared with that in non-Tg littermates (Figures 3(d) and 3(e)). However, only macrophage-positive areas were statistically significantly increased by 2.8-fold ($P < 0.001$) in Tg rabbits (Figure 3(e)).

4. Discussion

The potential atherogenicity of CETP relates to its ability to transfer cholesteryl esters from the antiatherogenic HDLs to the proatherogenic very low-density lipoproteins and LDLs [29–32]. However, there is also evidence that CETP may be involved in reverse cholesterol transport (transfer of cholesterol from peripheral cells through the plasma to the liver) [33]. Rare mutations leading to reduced function of CETP have been linked to accelerated atherosclerosis [34]. Genetic deficiency of CETP in rabbits has beneficial effects on enhancing HDL function and reducing atherosclerosis [35]. Thus, theoretically, CETP may be either proatherogenic or antiatherogenic. In this study, we successfully created Tg rabbits that expressed hCETP in the liver. Our present study showed that increased hepatic hCETP enhanced macrophage-derived foam cell accumulation in the lesions in Tg rabbits fed an HCD, even though there was no significant difference in the gross atherosclerotic lesions. Because the rabbits were fed with a cholesterol diet for 16 weeks, the main lesions are those of fatty streaks which are composed of macrophage-derived foam cells with a small number of SMCs. In human patients, those complicated lesions (such as plaque stenosis and rupture) leads to myocardial infarction. Therefore, it is necessary to investigate whether increased CETP can also affect plaque vulnerability in the future. For such a purpose, we need to feed the rabbits with a cholesterol diet for a longer time such as 28 weeks [28]. Atherosclerosis is a chronic disease process characterized by the focal subendothelial accumulation of apolipoprotein-B-containing lipoproteins, immune and vascular wall cells, and extracellular matrix [36]. The lipoproteins acquire features of damage-associated molecular patterns and trigger first an innate immune response, dominated by monocyte-macrophages, and then an adaptive immune response [37]. There are many studies showing that

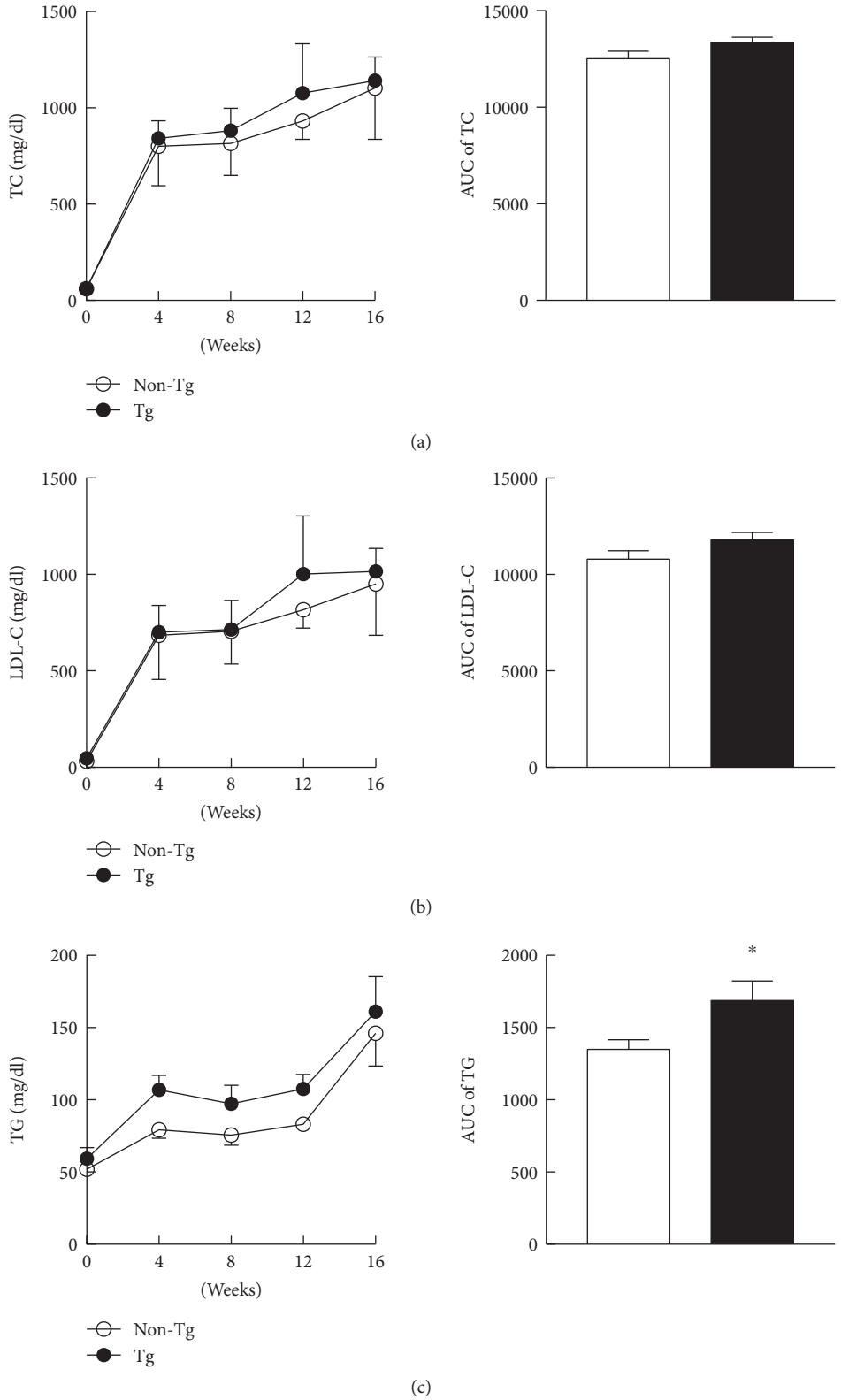
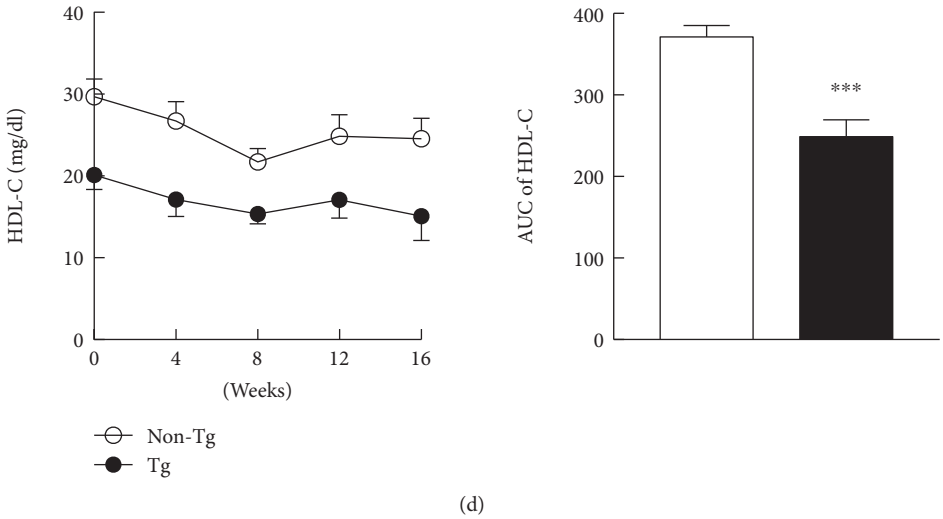


Figure 2: Continued.



(d)

Figure 2: The plasma levels of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), triglyceride (TG), and high-density lipoprotein cholesterol (HDL-C) (a–d). Data are expressed as the mean ± SEM, $n = 12$ for each group. $^{*}P < 0.05$, $^{***}P < 0.001$ versus non-Tg littermates.

Table 1: The body weight, weight of the major organs, blood pressure, and heart rate in Tg and non-Tg littermates at the end of the experiments. Data are expressed as the mean ± SEM, $n = 7$ for each group.

	Non-Tg	Tg
Body weight (Kg)	3.40 ± 0.23	3.55 ± 0.21
Heart weight (g)	5.88 ± 1.05	6.44 ± 0.88
Kidney weight (g)	14.70 ± 1.94	15.78 ± 3.14
Liver weight (g)	96.92 ± 13.95	107.62 ± 9.07
SBP (mmHg)	99.5 ± 5.8	96.7 ± 7.8
DBP (mmHg)	87.6 ± 5.4	85.7 ± 6.6
Heart rate (BPM)	299.2 ± 13.4	248 ± 14.6

SBP: systolic blood pressure; DBP: diastolic blood pressure; BMP: beats per minute.

there are a number of autoantibodies existed in either plasma or atherosclerotic lesions that may initiate and participate in the development of atherosclerosis. High levels of antiphospholipid autoantibodies, antiphosphorylcholine autoantibodies, anti-LDL autoantibodies, and anticyclic citrullinated protein autoantibodies have been shown to be associated with increased cardiovascular risk [38]. Although our studies showed that increased CETP expression increased subendothelial accumulation of macrophages, pathophysiological significance of this finding in terms of macrophage infiltration and/or proliferation or autoantibody formation during the progression of atherosclerosis remains to be addressed in the future study. It was reported that dyslipidemic patients would have an elevated CETP concentration and/or an accelerated rate of net transfer of cholesteryl esters from HDL to apoB-containing lipoproteins as well as accelerated atherosclerosis [39]. CETP may be deleterious for atherosclerosis, but it is also likely that high levels of CETP are the result rather than the cause of dyslipidemia [40]. In our study, Tg rabbits with a higher CETP concentration had low levels of HDL-C and high levels of TGs but did not exhibit a significant effect on gross lesion area of aortic atherosclerosis. These observations may have implications for research into CETP inhibitors and the role of HDL-C in atherosclerosis.

Therapeutic intervention targeting HDL was once a major focus of research on the treatment of atherosclerotic disease [41]. The HDL-mediated removal of excess free cholesterol from macrophage foam cells is thought to play a major role in the protection against the development of atherosclerosis, which may have a possible beneficial effect on macrophage foam cell formation [42]. Large cholesteryl esters-rich HDL particles from 4 subjects with complete CETP deficiency showed an increased ability to promote cholesterol efflux from macrophage foam cells [43]. Inhibition of CETP by Torcetrapib increases macrophage cholesterol efflux to HDL [44]. In our study, high expression of the CETP gene was inborn, and the HCD was used as an arteriosclerotic auxiliary to further explore the roles of the CETP gene in the development of atherosclerosis and plaque formation. Immunohistochemical staining was performed to analyze the plaque components. We found that increased expression of the CETP promoted macrophage-derived foam cell formation in Tg rabbits. A high level of CETP mRNA and CETP concentration did not lead to a significant increase in the plasma LDL-C levels but did cause an obvious reduction in the HDL-C levels. In spite of this, there are several limitations in the current study. For example, it is not known whether CETP promotes accumulation of macrophage-derived foam cells in atherosclerotic lesions through inhibiting cholesterol efflux from macrophages, and other molecular mechanisms should be examined in the future. Furthermore, the number size of Tg and non-Tg rabbit size used in the current study was rather limited therefore whether these results can be directly translated into humans required more vigorous investigation. Finally, it remains to

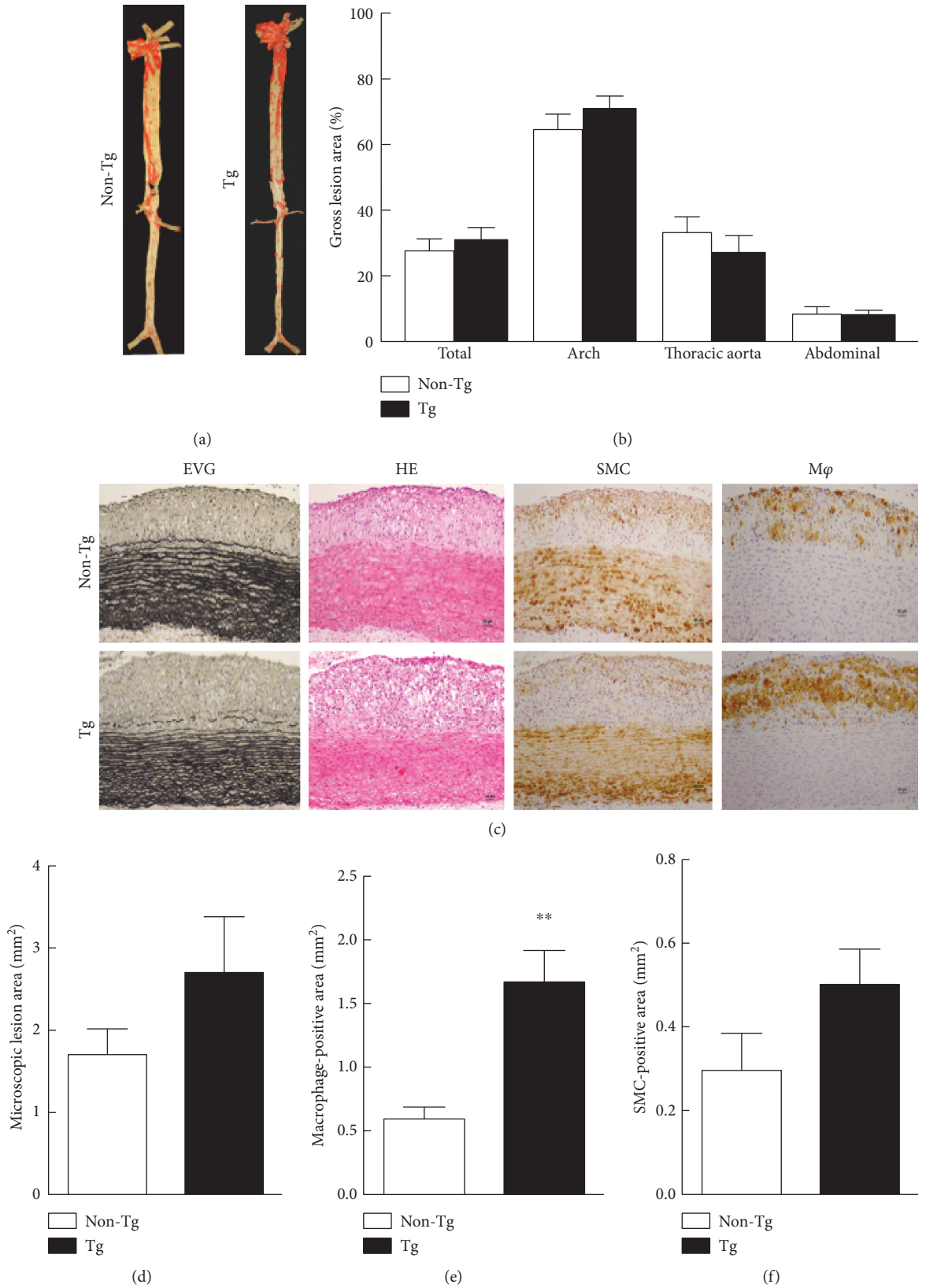


Figure 3: Representative aortic atherosclerosis lesions and their quantitative analysis. (a) "Aortic trees" were stained with Sudan IV. (b) Quantitative analysis of the atherosclerotic arterial lesions. (c) Aortic sections were stained with Elastica van Gieson (EVG) and hematoxylin and eosin (H&E) or immunohistochemically stained with Abs against macrophages (Mφ) or smooth muscle cells (SMCs). The quantitative analysis of the aortic arch lesion area (d), the cellular composition of the Mφ, and SMCs are shown at the bottom (e, f). $n = 12$ for each group. Data are expressed as the mean ± SEM. $^{**}P < 0.01$ versus non-Tg.

be established whether increased CETP expression can be used for treating atherosclerosis in humans. In conclusion, increased hepatic expression of hCETP in Tg rabbits increased macrophage-derived foam cell accumulation potentially via the reduction of HDL-C levels. The present studies may have implications for CETP inhibition in atherosclerosis.

Abbreviations

CETP: Cholesteryl ester transfer protein
EVG: Verhoeff van Gieson
HCD: High cholesterol diet
H&E: Hematoxylin and eosin
HDL-C: High-density lipoprotein cholesterol
LDL-C: Low-density lipoprotein cholesterol
Mφ: Macrophages
RCT: Reverse cholesterol transport
SMC: Smooth muscle cell
TC: Total cholesterol
TG: Triglyceride.

Conflicts of Interest

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

Acknowledgments

This work was supported in part by the National Natural Science Foundation of China (no. 8107025 and 81370379), the Fundamental Research Fund for the Central Universities and the Natural Science Foundation of Shaanxi Province (2012KJXX-07, 2014PT013, and 2016JM8061), Wuzhong Innovation and Entrepreneurship Talent Project (WC201526) of Suzhou, and the study on the development and evaluation of Tg rabbit model, the Science and Technology Key Projects of Ningxia, 2013.

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Hindawi
Mediators of Inflammation
Volume 2017, Article ID 1070959, 11 pages
https://doi.org/10.1155/2017/1070959

Research Article

Proportions of Proinflammatory Monocytes Are Important Predictors of Mortality Risk in Hemodialysis Patients

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Received 7 May 2017; Revised 6 August 2017; Accepted 13 September 2017; Published 22 October 2017

Academic Editor: Arbi Pecani

Despite the continuous progression in dialysis medicine, mortality and the burden of cardiovascular disease (CVD) among hemodialysis patients are still substantial. Substantial evidence suggests that proinflammatory (CD16+) monocytes contribute to the development of atherosclerosis. A cohort of 136 stable hemodialysis patients (follow-up: 6.25 year) was assessed to investigate the association between the proportion of CD16+ monocytes for all-cause and CVD mortalities. The CD16+ monocytes were associated with both mortalities after adjusting for a preexisting CVD history. Compared to the reference group (CD16+ monocytes within [15.6–18.6], the first and second quartile), patients with CD16+ monocytes above the highest quartile level (>21.5) had an adjusted hazard ratio (HR) of 30.85 (95% confidence interval [CI]: 7.12–133.8) for CVD mortality and 5.28 (2.07–13.49) for all-cause mortality, and those with CD16+ monocytes below the lowest quartile ≤15.6), had significantly elevated death risks after 3.5-year follow-up (HR [95% CI]: 10.9 [2.42–48.96] and 4.38 [1.45–13.24] for CV and all-cause mortalities, respectively). The hemodialysis patients with CD16+ monocyte level in a low but mostly covering normal range also portended a poor prognosis. The findings shed some light for nephrologists on future prospects of early recognizing immune dysfunction and improving early intervention outcomes.

1. Introduction

It has been established beyond any doubt that cardiovascular (CV) events are an important cause of death, accounting for up to 40–50%, in end-stage renal disease (ESRD) patient population. In the early 70s, Foley et al. have reported that mortality from cardiovascular disease (CVD) is 10–20 times higher in ESRD patients compared with the general population [1]. Interestingly, some authors found that mortality from non-CV disease in dialysis patients was also increased to the same extent as mortality from CVD [2, 3]. Over these years, the potential link between CV and non-CV mortality was explored. Ishani et al. [4] showed that septicemia or bacteremia in dialysis patients was associated with subsequent CV-related events such as myocardial infarction, heart failure, and stroke. On the other hand, the risk of myocardial infarction and that of stroke were substantially higher after a diagnosis of systemic respiratory tract infection [5]. These studies suggested that both CV and infectious causes of death are linked to inflammation, and possibly, these two events may aggravate each other.

Mounting evidence shows that disturbed endothelial function may be an early marker of atherosclerotic process [6]. Clinical and experimental data support a link between endothelial dysfunction and inflammation [7–10]. Chronic systemic inflammation, a common feature in dialysis

patients, has been identified as an epidemiologically important risk factor for CV morbidity and mortality in dialysis patients [11–13]. Of 30 prevalent patients, 50% had elevated serum levels of inflammatory markers such as C-reactive protein, IL-6, and procalcitonin [12, 14, 15]. In addition, a shift towards proinflammatory monocyte subsets [16] and monocyte dysfunction [17] is also noted in these patients. Available evidence showed that even low-grade systemic inflammation has been found to be associated with devastating prognosis of dialysis patients [18–21].

Monocytes can be subdivided into three phenotypically and functionally distinct subpopulations based on the expression of the lipopolysaccharide (LPS) receptor (CD14) and the CD16 (Fcgamma receptor III) [22, 23]. In healthy individuals, approximately 80–90% of monocytes are highly CD14 positive and CD16 negative (CD14++CD16−): classical monocytes. The remaining 10–20% of monocytes are CD16 positive, which are further subdivided into CD14++CD16+ and CD14+CD16++ cells, intermediate and nonclassical monocytes, respectively [23]. Compared with CD16 negative conventional monocytes, CD16 positive monocytes, also called proinflammatory monocytes, express higher levels of major histocompatibility complex (MHC) class II antigens, adhesion molecules, chemokine receptors, and proinflammatory cytokines such as TNF-α, but lower levels of the anti-inflammatory cytokine, that is, IL-10 [24, 25]. CD16 positive monocytes are elevated in various pathologic conditions, including inflammatory and infectious diseases [26], cancer [27], and in coronary heart disease as ESRD [16, 28, 29]. However, to date, the mechanism by which CD16 positive monocytes increase remains unclear.

Here, we examined the interrelationships between the proportion of proinflammatory monocytes (CD16+ monocytes) and all-cause mortality as well as CV mortality in a cohort of stable ESRD patients on hemodialysis. This study might shed more light on the potential mechanisms that link microinflammation with future CV events.

2. Methods

2.1. Patients and Study Sample. Adult outpatients on hemodialysis at the Tungs' Taichung MetroHarbor Hospital (TTMHH) in June 2009 were enrolled. A total of 136 patients were eligible. All the enrolled patients signed informed consents. This study was conducted in full compliance with the provisions of the Personal Information Protection Act and the Human Subjects Research Act of Taiwan and was approved by the institutional review board (number: 102011).

All the patients were dialyzed three times a week with a high-flux polysulfone membrane (FX80 and FX100; Fresenius Medical Care, Bad Homburg, Germany) and bicarbonate dialysate solutions. The median blood flow rate was 280 ml/min (range 250–300 ml/min). All dialysate flows were 800 ml/min, and treatment time was 240 minutes for each patient. All patients were dialyzed through a native arteriovenous (AV) fistula. Blood samples were obtained just before the midweek dialysis session. The dialysate revealed concentrations of bacterial and endotoxin contamination below the detection limit (100 colony-forming units/ml and <0.25 endotoxin units). Systolic and diastolic blood pressures (SBP and DBP) were measured in a supine position and after at least a 10-minute rest using a full automatic noninvasive sphygmomanometer.

Each patient's medical chart prior to study enrollment was thoroughly reviewed, and data pertaining to underlying kidney disease, history of CVD, and common comorbid conditions were extracted. The causes of renal failure were diabetic nephropathy ($n = 68$), chronic glomerulonephritis ($n = 30$), polycystic kidney disease ($n = 3$), hypertensive nephrosclerosis ($n = 15$), or unknown ($n = 20$). Patients who had started on hemodialysis for less than 3 months had history of chronic liver diseases, neoplasm, or inflammatory diseases, and those on long-term corticosteroids were excluded. A preexisting history of CVD was defined as a history of coronary artery disease (CAD, including a history of myocardial infarction, coronary artery angioplasty/stenting/bypass surgery, and carotid endarterectomy/stenting), cerebrovascular disease (CeVD, e.g., stroke), nontraumatic lower extremity amputation, and lower limb artery bypass surgery/angioplasty/stenting. Diabetes mellitus (DM) cases were ascertained if a patient had a history of DM diagnosis, a spontaneous plasma glucose level of >200 mg/dl, and/or received hypoglycemic treatment. The survival data were then retrieved in September 2016.

2.2. Laboratory Methods. All blood samples were collected during the midweek dialysis from the AV fistula, immediately after the insertion of the dialysis cannula but before the administration of heparin. Blood was sampled in 4 c.c. Venoject II tubes and centrifuged (10 min, 3000 rpm) and stored at −70°C pending analyses, if not analyzed immediately. Serum albumin, urea, creatinine (Cr), total cholesterol, and triglyceride (TG) were determined according to standard methods. The serum levels of high-sensitivity C-reactive protein (hsCRP) were measured using a Behring Nephelometer II (Dade Behring, Tokyo, Japan).

2.3. Determination of CD14 and CD16 Mononuclear Phenotype. Peripheral blood was collected by venipuncture using ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. For cytometric analysis, monoclonal antibodies against CD14 (fluorescein isothiocyanate (FITC) conjugated; clone RMO52; Beckman Coulter, Miami, FL, USA), CD16 (phycoerythrin (PE) conjugated; clone 3G8; Beckman Coulter, Miami, FL, USA), CD45 (phycoerythrin cyanin-5 (PC5); clone J33; Beckman Coulter, Miami, FL, USA), and CD56 (clone IM2073; Beckman Coulter, Miami, FL, USA) were used. Briefly, 100 l of the whole blood was stained with saturating amounts of the abovementioned monoclonal antibodies and corresponding isotype controls. After incubation for 15 min at room temperature in the dark according to the manufacturer's recommendations, OptiLyse C (Beckman Coulter, Miami, FL) was added to lyse RBC and the samples were fixed. Fixed cells were analyzed by flow cytometry within 6 hours.

Determination of leukocyte and monocyte subset distribution was performed using a FC500-Cytometer (Beckman

Coulter), and CXP analysis software (version 2.2) was used (Schroers et al., 2005). Monocytes were identified as CD45 positive and CD56 negative cells exhibiting a specific forward and sideward scatter profile. Monocytes were then gated in an SSC/CD dot plot, identifying monocytes as CD86 cells with monocyte scatter properties. Subsets of CD14 monocytes with and without CD16 were defined according to the surface expression pattern of the lipopolysaccharide receptor CD14 and the CD16 (Fcgamma receptor III). One million cells were analyzed from each sample, and the percentage of CD16 positive mononuclear cells (CD14+/CD16+ and CD14++/CD16+) and the number of cells out of the total monocytes were compared using fluorescent microbeads (Flow-Count, Beckman Coulter). The CD86 antibody (clone HA5.2B7; Beckman Coulter, Miami, FL, USA) was used in this study.

2.4. Statistical Analysis. The sample characteristics were summarized using frequencies and percentage for categorical variables and using median (i.e., the second quartile, q_2), interquartile interval (IQI, an interval bounded by the first and the third quartiles, q_1 and q_3), mean, and standard deviation (SD) for continuous variables. Spearman's correlation analysis was applied to evaluate bivariate associations between CD16+ monocytes and other observed variables. The Cox regression was applied to evaluate the association of mortality with CD16+ monocytes and with other variables. Two types of mortalities were investigated in this study: CV and all-cause mortalities. The starting point of the survival time was designed at 2009/06/01. Cases who survived till 2016/09/01, transferred to other centers or transplanted during study observation period, were censored at the date. The raw CD16+ monocytes were categorized according to its three quartiles (q_1, q_2, and q_3) into a variable of four levels (from the lowest to the highest level: Q1, Q2, Q3, and Q4). The Cox regression analysis results were displayed in hazard ratio (HR), its associated 95% confidence interval (CI), and p value. The crossover pattern of hazards among the four-level CD16+ monocytes was modeled using time-dependent effect in the Cox regression model. Throughout this study, tests for statistical associations were evaluated at a significance level of 0.05. The analyses were all performed in SAS version 9.1.

3. Results

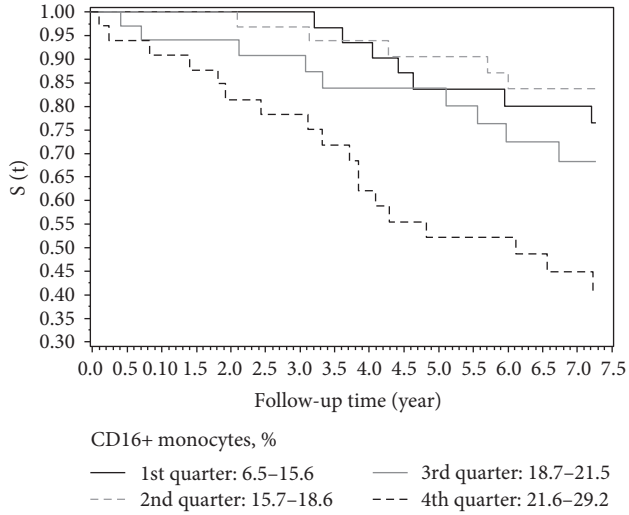
The descriptive statistics of the whole study sample were summarized in Table 1. Of the 136 patients, 39 died in CVD, 18 died in other causes, 8 censored because of transplantation or transferred to another center, and 71 survived till the follow-up ends. The mean (minimum–maximum) follow-up time was 5.57 (0.10–7.25) years for the overall sample and was 7.03 (2.66–7.25) years for the 79 non-death cases.

The Kaplan-Meier curves of CV death and of all-cause death by CD16+ monocyte level were displayed in Figure 1, where follow-up details on the observed case numbers were listed below the figures. The curves overall appeared that patients with CD16+ monocytes in the fourth quarter had the worst survival rate (the black dotted line in Figure 1)

Table 1: The summary of the sample characteristics ($n = 136$).

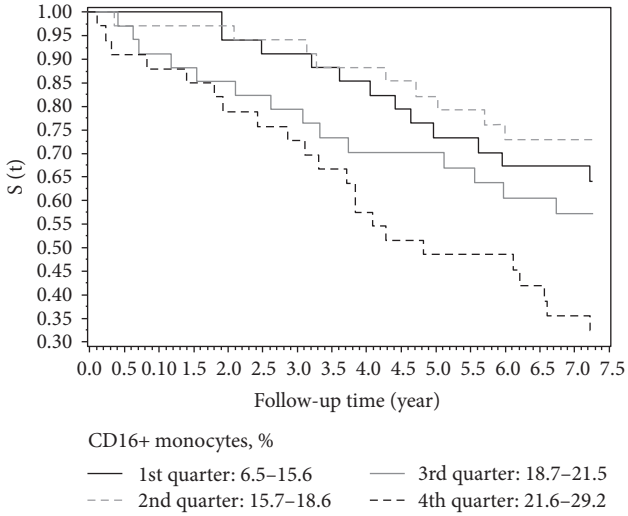
Variables	Missing number	n (%)
Sex, female versus male	0	62 (45.59) versus 74 (54.41)
DM, no versus yes	0	68 (50) versus 68 (50)
Hypertension, no versus yes	0	38 (27.94) versus 98 (72.06)
Preexisting CVD, no versus yes	3	62 (46.62) versus 71 (53.38)
HD vintage, year	0	
0–3		20 (14.71)
>3, ≤5		37 (27.21)
>5, ≤8		37 (27.21)
>8		42 (30.88)
		Median (IQI)
CD16+ monocytes, %	1	18.6 (15.6, 21.5)
HD vintage, year	0	6.04 (3.92, 9.04)
Age, year	0	59 (51.5, 69)
BMI, kg/m^2	0	23.62 (21.08, 25.32)
WBC, 10^3/mm^3	1	6.6 (5.4, 7.5)
Monocyte, $10^3/\mu$l	1	5.9 (4.8, 7)
AbsoMono, cells/μl	1	367.2 (291.2, 482.4)
HsCRP, mg/l	2	2.8 (1.5, 3.8)
Hb, g/dl	1	11.2 (10.1, 12.2)
PLT, 10^4/cm^3	1	160 (54, 218)
FBS, mg/dl	1	96 (82, 139)
HbA1c	2	6 (5, 7.1)
Albumin, g/dl	1	4.2 (4, 4.4)
Ferritin, μg/dl	2	679 (467, 838)
TG, mg/dl	1	115 (81, 187)
HDL, mg/dl	2	43.5 (34, 56)
Cholesterol, mg/dl	3	163 (140, 189)
rTG	3	0.77 (0.48, 1.13)
rHDL	3	0.28 (0.21, 0.36)
cHDL	3	116 (94, 142)
BUN, mg/dl	2	66.5 (58.1, 75.7)
Cr, mg/dl	2	10.5 (9.2, 11.9)
UA, mg/dl	2	7.6 (6.7, 8.5)
Ca, mg/dl	2	9.4 (9, 9.8)
P, mg/dl	3	4.6 (3.8, 5.6)
SBP, mmHg	0	138.5 (121.5, 154.5)
DBP, mmHg	0	77 (69, 84.5)

Note: the numbers in the second column indicated the missing numbers of each variable. DM: diabetes mellitus; CVD: cardiovascular diseases including CAD and CeVD; CAD: coronary artery diseases; CeVD: cerebrovascular disease; BMI: body mass index; WBC: white blood cell count; AbsoMono: absolute monocyte; HsCRP: high-sensitivity C-reactive protein; Hb: hemoglobin/haemoglobin; PLT: platelet count; FBS: fasting blood sugar; HbA1c: glycated hemoglobin; TG: triglyceride; HDL: high-density lipoprotein cholesterol; cholesterol: total cholesterol; rTG: ratio of TG to cholesterol; rHDL: ratio of HDL to total cholesterol; cHDL: the value resulted from subtracting the level of HDL from the level of total cholesterol; BUN: blood urea nitrogen; Cr: creatinine; UA: uric acid; Ca: serum calcium; P: serum phosphorus; SBP: systolic blood pressure; DBP: diastolic blood pressure; IQI: interquartile interval which is bounded by the first and the third quartiles (q_1 and q_3) of the variable listed in the first column.



Follow-up years	0	0.5	1	1.5	2	2.5	3	3.5	4	4.5	5	5.5	6	6.5	7	7.5
Number death by CVD history																
Present	0	3	2	1	2	2	0	6	4	5	2	1	3	2	1	1
Nonpresent	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	1
Number of survivor	136	133	131	130	128	125	125	119	115	110	108	107	103	101	99	97

(a) Estimation for CV death (log-rank p value = 0.0004)



Follow-up years	0	0.5	1	1.5	2	2.5	3	3.5	4	4.5	5	5.5	6	6.5	7	7.5
Number death by CVD history																
Present	0	5	2	2	4	3	2	7	5	5	3	2	3	3	3	1
Nonpresent	0	0	1	0	1	1	1	0	2	0	2	1	2	1	1	2
Number of survivor	136	131	128	126	121	117	114	107	100	95	90	87	82	78	74	71

(b) Estimation for all-cause death (log-rank p value = 0.0032)

Figure 1: The Kaplan-Meier curves by CD16+ monocyte level. The Kaplan-Meier curves for CD16+ monocyte level within the lowest to the highest quarters were indicated by black solid line, gray dashed line, gray solid line, and black dashed line. The CD16+ monocyte ranges of the four quarters were the same as those listed in the second row of Table 1. The numbers of death by baseline CVD status and survivor during follow-up were listed below the figures.

and those in the second quarter had better survival rate (the gray dashed line in Figure 1) compared to others. Patients without preexisting CVD history accounted a minor proportion in the overall death numbers: 10.26% (4/39) for CV death and 23.08% (15/65) for all-cause death. Such numbers were reduced to 5.41% (2/37) and 12.73% (7/55) in subsequent Cox regression analyses because of missing covariates. Since the survival curves for CD16+ monocytes in the first and second quarters were a crossover at 3.5 years, the time-dependent effect between the two levels of CD16+ monocytes was incorporated in the later Cox regression for CVD and all-cause mortalities.

The bivariate analysis for CD16+ monocytes and other variables was displayed in Table 2. Most variables were not significantly associated with CD16+ monocyte level, except for age, ferritin, and preexisting CVD history at baseline.

Table 2: The results of association analysis for CD16+ monocytes.

Variables	CD16+ monocyte level				p value
	6.5~15.6	>15.6, ≤18.6	>18.6, ≤21.5	>21.5, ≤29.2	
n	34	34	34	33	
	n (%)	n (%)	n (%)	n (%)	
Sex					
Female	15 (44.12%)	14 (41.18%)	15 (44.12%)	18(54.55%)	0.374
Male	19 (55.88%)	20 (58.82%)	19 (55.88%)	15(45.45%)	
DM					
No	20 (58.82%)	18 (52.94%)	16 (47.06%)	13(39.39%)	0.083
Yes	14 (41.18%)	16 (47.06%)	18 (52.94%)	20(60.61%)	
Hypertension					0.806
No	8 (23.53%)	11 (32.35%)	11 (32.35%)	8(24.24%)	
Yes	26 (76.47%)	23 (67.65%)	23 (67.65%)	25(75.76%)	
Preexisting CAD or CeVD					
No	22 (64.71%)	20 (58.82%)	13 (40.63%)	7(21.21%)	<0.0001*
Yes	12 (35.29%)	14 (41.18%)	19 (59.38%)	26(78.79%)	
HD vintage (year)					
0–3	7 (20.59%)	4 (11.76%)	6 (17.65%)	3(9.09%)	0.849
>3, ≤5	8 (23.53%)	10 (29.41%)	8 (23.53%)	11(33.33%)	
>5, ≤8	6 (17.65%)	11 (32.35%)	14 (41.18%)	6(18.18%)	
>8	13 (38.24%)	9 (26.47%)	6 (17.65%)	13(39.39%)	
	Mean ± *SD*	*Mean* ± *SD*	*Mean* ± *SD*	*Mean* ± *SD*	
CD16+ monocytes, %	12.77 ± 2.32	16.96 ± 0.87	20.28 ± 0.87	24.77 ± 2.07	—
HD vintage, year	7.99 ± 5.74	6.8 ± 3.81	6.29 ± 3.82	6.89 ± 3.59	0.753
Age, year	56.03 ± 12.03	59.91 ± 11.35	61.59 ± 13.15	63.45 ± 10.67	0.004*
BMI, kg/m^2	23.1 ± 3.38	23.31 ± 3.64	23.1 ± 3.27	25.03 ± 3.88	0.238
WBC, 10^3/mm^3	7.08 ± 2.12	6.28 ± 1.43	6.53 ± 1.58	6.32 ± 1.35	0.245
Monocyte, $10^3/\mu$l	5.71 ± 1.77	5.89 ± 1.58	6.27 ± 1.74	6.06 ± 1.47	0.192
AbsoMono, cells/μl	413.94 ± 214.01	366.77 ± 117.41	408.65 ± 149.62	380.39 ± 120.19	0.961
HsCRP, mg/l	2.57 ± 1.42	2.42 ± 1.46	2.49 ± 1.41	2.96 ± 1.34	0.367
Hb, g/dl	11.47 ± 1.59	11.39 ± 1.31	11.15 ± 1.57	10.97 ± 1.27	0.135
PLT, 10^4/cm^3	143.21 ± 90.39	124.96 ± 82.61	163.69 ± 88.49	153.32 ± 89.62	0.311
FBS, mg/dl	110.85 ± 42.25	111.86 ± 43.94	126.56 ± 59.31	114.76 ± 45.17	0.732
HbA1c, %	6.38 ± 1.72	5.96 ± 1.29	6.29 ± 1.33	6.59 ± 1.71	0.198
Albumin, g/dl	4.24 ± 0.22	4.43 ± 1.45	4.18 ± 0.32	4.1 ± 0.29	0.163
Ferritin, μg/dl	657.41 ± 256.8	594.68 ± 271.22	745.94 ± 326.2	717.58 ± 288.81	0.041*
TG, mg/dl	144.56 ± 107.83	132.05 ± 73.32	154.29 ± 105.66	141.42 ± 83.4	0.782
HDL, mg/dl	47.18 ± 17.99	48.82 ± 17.11	48.03 ± 19.79	43.48 ± 14.52	0.877
Cholesterol, mg/dl	168.5 ± 37.08	165.7 ± 37.16	161.24 ± 37.81	164.12 ± 41.12	0.767
rTG	0.86 ± 0.6	0.82 ± 0.39	0.9 ± 0.52	0.88 ± 0.49	0.805
rHDL	0.29 ± 0.11	0.31 ± 0.12	0.3 ± 0.11	0.27 ± 0.09	0.757
cHDL, mg/dl	121.32 ± 36.73	116.58 ± 37.82	113.21 ± 34.76	120.64 ± 38.17	0.689
BUN, mg/dl	67.6 ± 14.69	67.32 ± 12.52	66.88 ± 12.84	65.98 ± 15.42	0.59
Cr, mg/dl	10.84 ± 2	10.66 ± 2.24	10.72 ± 2.3	10.07 ± 2.12	0.149
UA, mg/dl	7.56 ± 1.49	7.85 ± 1.35	7.65 ± 1.25	7.17 ± 1.93	0.622
Ca, mg/dl	13.32 ± 22.21	9.43 ± 0.39	9.26 ± 0.65	9.38 ± 0.79	0.225
P, mg/dl	4.65 ± 1.46	4.75 ± 1.35	4.41 ± 1.2	4.76 ± 1.4	0.55
SBP, mmHg	134.91 ± 22.34	138.85 ± 19.65	135.79 ± 15.02	140.88 ± 25.02	0.446
DBP, mmHg	78 ± 10.53	76.38 ± 10.38	76.15 ± 8.33	75.79 ± 9.69	0.479

Note: the abbreviations are the same as those denoted in Table 1. *p value of Spearman's association test using the raw data value of CD16+ monocytes and observed variables.

Table 3: The univariate Cox regression analysis results for death risk.

Outcome types	CVD				All causes			
Covariates	HR	95% CI		p value	HR	95% CI		p value
CD16+ monocytes, %								
$Q1_{(\leq 3.5\ y)}$ versus Q2	0.45	0.05	3.96	0.469	0.92	0.27	3.09	0.888
$Q1_{(>3.5\ y)}$ versus Q2	5.16	0.56	47.86	0.149	2.00	0.53	7.63	0.308
Q3 versus Q2	2.18	0.73	6.52	0.161	1.86	0.81	4.31	0.145
Q4 versus Q2	5.13	1.90	13.84	0.001*	3.44	1.58	7.48	0.002*
Sex, male versus female	0.96	0.51	1.79	0.888	0.91	0.54	1.53	0.72
Age, years	1.03	1.01	1.06	0.011*	1.04	1.02	1.06	<0.001*
HD vintage, years	0.97	0.9	1.04	0.42	0.95	0.89	1.01	0.132
DM, yes versus no	2.18	1.12	4.25	0.021*	1.87	1.09	3.2	0.023*
Hypertension, yes versus no	3.14	1.23	8.04	0.017*	2.16	1.09	4.27	0.027*
Preexisting CAD, yes versus no	7.99	3.63	17.59	<0.001*	5.66	3.11	10.3	<0.001*
Preexisting CeVD, yes versus no	2.11	1.04	4.29	0.038*	2.05	1.14	3.67	0.016*
BMI, kg/m^2	1.03	0.94	1.12	0.535	0.98	0.91	1.05	0.547
WBC, $10^3/\mu l$	1.16	0.97	1.4	0.112	1.13	0.97	1.32	0.113
Monocyte,$10^3/\mu l$	1.06	0.87	1.28	0.558	1.05	0.89	1.23	0.583
HsCRP, mg/l	1.49	1.15	1.91	0.002*	1.38	1.12	1.68	0.002*
FBS, mg/dl	1.01	1	1.01	0.019*	1.01	1	1.01	0.01*
rHDL	4.98	0.31	80.71	0.259	4.95	0.5	48.94	0.172
cHDL, mg/dl	4.98	0.31	80.71	0.259	4.95	0.5	48.94	0.172
HbA1c, %	1.26	1.05	1.52	0.015*	1.21	1.04	1.42	0.015*
P, mg/dl	1.06	0.84	1.34	0.606	1.01	0.83	1.22	0.945
SBP, mmHg	1.01	1	1.03	0.171	1.01	1	1.02	0.181
DBP, mmHg	1.01	0.98	1.05	0.43	1.01	0.98	1.04	0.521
AbsoMono, cells/μl	1	1	1	0.121	1	1	1	0.127
PLT, $10^3/\mu l$	1	1	1.01	0.042*	1	1	1.01	0.133
Ferritin, $\mu g/dl$	1	1	1	0.155	1	1	1	0.761
TG, mg/dl	1	1	1	0.675	1	1	1	0.334
HDL, mg/dl	1	0.98	1.02	0.978	1	0.98	1.01	0.84
Cholesterol, mg/dl	0.99	0.98	1	0.121	0.99	0.98	1	0.029*
rTG	0.86	0.44	1.68	0.654	0.82	0.46	1.43	0.48
Hb, g/dl	0.89	0.7	1.13	0.348	0.92	0.76	1.12	0.405
Albumin, g/dl	0.23	0.06	0.85	0.028*	0.13	0.04	0.41	<0.001*
BUN, mg/dl	1	0.97	1.02	0.802	0.99	0.97	1.01	0.165
Cr, mg/dl	0.83	0.71	0.97	0.019*	0.77	0.67	0.88	<0.001*
UA, mg/dl	0.89	0.72	1.1	0.274	0.86	0.73	1.01	0.073
Ca, mg/dl	0.97	0.8	1.17	0.734	0.96	0.76	1.21	0.732

Note: the abbreviations are the same as those denoted in Table 1. Q1, Q2, Q3, and Q4 denoted the four ascending classes of the categorical variable which was derived from categorizing each covariate by its three quartiles. The values of the three quartiles were listed in Table 1. Rows indicated as $Q1_{(\leq 3.5\ y)}$ versus Q2 and $Q1_{(>3.5\ y)}$ versus Q2 listed the time-varying effect of CD16+ monocytes below the lowest quartile for follow-up time before and after 3.5 years. * indicates p values of less than 0.05.

Patients having preexisting CVD tended to have a high-level CD16+ monocytes ($p < 0.0001$). The Spearman correlation coefficients (p value) for CD16+ monocytes with ferritin and with age was, respectively, 0.18 (0.0412) and 0.25 (0.0039). Both manifested that higher CD16+ monocytes were correlated to higher ferritin and older age.

The univariate Cox regression analysis results in Table 3 showed that patients with medical conditions such as DM, hypertension, and preexisting CAD or CeVD history tended to have higher risk in both CV death and all-cause death. In specific, patients with DM at baseline had significantly higher CVD death risk (HR = 2.18, 95% CI: 1.12–4.25) and those with a preexisting CeVD event had significantly higher all-cause death risk (HR = 2.05, 95% CI: 1.14–3.67). Patients of old age and with high level of hsCRP, fasting blood sugar (FBS), and glycated hemoglobin (HbA1c) tended to have

Table 4: The multiple Cox regression analysis results for death risk.

Outcome types	CVD death				All-cause death			
Covariates	HR	95% CI		p value	HR	95% CI		p value
CD16+ monocytes, %								
Q4 versus Q3	12.81	3.72	44.09	<0.001*	3.26	1.49	7.14	0.003*
Q4 versus Q2	30.85	7.12	133.8	<0.001*	5.28	2.07	13.49	<0.001*
Q3 versus Q2	2.41	0.58	10.03	0.227	1.62	0.61	4.29	0.333
Follow-up time ≤ 3.5 years								
Q1 versus Q2	1.63	0.15	17.33	0.685	0.9	0.26	3.16	0.875
Q4 versus Q1	18.92	2.17	164.9	0.008*	5.84	1.71	19.98	0.005*
Q3 versus Q1	1.48	0.16	13.47	0.73	1.79	0.52	6.17	0.358
Follow-up time > 3.5 years								
Q1 versus Q2	10.9	2.42	48.96	0.002*	4.38	1.45	13.24	0.009*
Q4 versus Q1	2.83	0.81	9.86	0.102	1.21	0.44	3.29	0.716
Q3 versus Q1	0.22	0.05	0.91	0.037*	0.37	0.13	1.07	0.067
Baseline medical condition								
CeVD history, yes versus no	6.98	2.18	22.3	0.001*	2.74	1.41	5.32	0.003*
CAD history, yes versus no	44.57	13.1	151.7	<0.001*	9.4	4.53	19.48	<0.001*
CAD history versus CeVD history	6.39	1.79	22.8	0.004*	3.43	1.34	8.75	0.01*
Age, $>q_2$ versus others	2.88	1.14	7.28	0.025*	2.36	1.24	4.52	0.009*
Cholesterol, $>q_2$ versus others	2.98	1.19	7.44	0.02*	—	—	—	—
Platelet, $>q_2$ versus others	7.23	2.66	19.67	<0.001*	3.99	2.04	7.79	<0.001*
Cr								
$\leq q_1$ versus others	4.26	1.82	10	<0.001*	—	—	—	—
$\leq q_2$ versus others	—	—	—	—	4.49	2.31	8.75	<0.001*
UA, $\leq q_1$ or $>q_3$ versus others	7.38	2.47	22.01	<0.001*	2.5	1.35	4.62	0.003*
SBP, $>q_2$ versus others	5.11	1.92	13.59	0.001*	—	—	—	—
DBP, $\leq q_1$ or $>q_3$ versus others	7.43	2.62	21.06	<0.001*	4.08	1.98	8.4	<0.001*
rTG, $>q_3$ versus others	0.18	0.07	0.47	<0.001*	0.21	0.09	0.47	<0.001*
Ferritin, $>q_3$ versus others	—	—	—	—	0.45	0.23	0.87	0.018*

Note: the abbreviations are the same as those indicated in Table 1. q_1, q_2, and q_3 denoted the three quartiles—the first quartile, median, and the third quartile—of each covariate and the values were listed in Table 1. Q1, Q2, Q3, and Q4 here denoted the four ascending categories derived from the categorized CD16+ monocyte levels by its three quartiles. * indicates p values of less than 0.05.

higher risk in both mortalities (all these variables had HRs > 1 in Table 3). Those with high level in albumin and Cr tended to have lower risk in both mortalities (all these variables had HRs < 1 in Table 3). For instance, a CVD death risk HR value of 1.03 (95% CI: 1.01–1.06) for the variable age in Table 3 indicated that a 1-year increment in age is significantly associated with 3% increase in CVD death risk. An all-cause death risk HR value of 0.13 (95% CI: 0.04–0.41) for the variable albumin indicated that 1 g/dl increment in albumin is significantly associated with 87% decrease in all-cause death risk. Patients who had CD16+ monocyte level lying in the second quarter (i.e., CD16+ monocyte level $> q_1$ and CD16+ monocyte level $\leq q_2$) expressed the lowest risks in both CVD and all-cause death (a J-shaped relationship).

Both the Kaplan-Meier curve and the univariate Cox regression analysis demonstrated a J-shaped relationship between CD16+ monocytes and patients' death risks, especially after the time of follow-up exceeds 3.5 years. Considering the possible effect of the lowest quartile of CD16+ monocytes and the risk of death, any possible reverse causation was adequately addressed in the analyses by maintaining a varying reference category. A multiple Cox regression analysis (Table 4) demonstrated that the J-shaped relationship between CD16+ monocytes and hemodialysis patients' death risks persisted after accounting for baseline conditions, for the hazard crossover effect between the two lowest quarters of CD16+ monocytes, and for a range of covariates. Patients with CD16+ monocytes in the fourth quarter manifested significantly higher death risks as compared to all other quarters; the HRs ranged from 2.83 to 30.85 for CV death and from 1.21 to 5.84 for all-cause death after adjusting other covariates (see Table 4 for detailed results). Further, patients with CD16+ monocytes below q_1 had an elevated adjusted HR for both CV death (HR = 10.9, $p = 0.002$) and for all-cause death (HR = 4.38, $p = 0.009$) in the fully adjusted model.

In analysis regarding CV death risk, preexisting CeVD and CAD history had a significant effect after adjusting the effect of CD16+ monocytes (HR = 6.98 and HR = 44.57, resp., both $p \leq 0.001$). Interestingly, a preexisting CAD history appeared to be associated with higher CV death risk than a preexisting CeVD (HR = 6.39, $p = 0.004$). Patients of old age (above median), with PLT, SBP, and cholesterol above median, with Cr below the first quartile, with uric acid (UA) and DBP out of the IQI, and with ratio of TG to total cholesterol (rTG) below the third quartile, were associated with higher CV death risk. For all-cause death risk, the presence of preexisting CeVD and CAD had a significant effect (HR = 2.74 and HR = 9.4, resp., $p = 0.003$ and $p < 0.001$) after adjusting the effect of CD16+ monocytes. Patients of age above median, with PLT above median, with Cr below median, with UA and DBP out of the IQI, and with rTG and ferritin below the third quartile, were associated with higher all-cause death risk. The detailed results were listed in Table 4.

4. Discussion

Our data accord with some previous findings of increased mortality in dialysis patients with higher percentages of nonclassical CD16 positive monocytes. Recent studies have established that phenotypic variations in the surface of monocytes are associated with the occurrence of CVD in both chronic kidney disease (CKD) and non-CKD patients. While Berg et al. found that classical CD16 negative monocytes can predict future CV risk in nonuremic population [30], some authors found that intermediate CD14++CD16+ monocytes predict CV events in CKD patients [31, 32]. Differences in study design and studied populations may account for some of the discrepancies regarding the correlation of monocyte subsets and adverse cardiac events in these studies. Nevertheless, flow cytometry is a powerful technique and its use has obviously allowed for risk stratification in a wide variety of diseases.

The innate immune system plays a major role in the initiation and propagation of atherosclerosis, with monocytes/macrophages being the key component in this process [33]. Apart from being responsible for counteracting exogenous bacterial, viral, and fungal infections [34], they are also involved in endogenous inflammatory processes. They contribute to atherogenesis through promoting leukocyte recruitment to plaques, and their roles are also mediated by activation of downstream signaling pathways, such as nuclear factor kappa-B pathway [35]. Monocyte involvement in the development of atherosclerotic plaques was reported in the 1970s, with monocyte accumulation demonstrated in porcine atherosclerotic lesions [36]. In recent years, we became aware of the role of different monocyte subsets in the pathogenesis of atherosclerosis, particularly specific monocyte subpopulations with their diverse phenotypes and sentinel roles in both the innate and adaptive immune system. Our understanding of how monocyte subsets participate in this process is largely based on mouse models of atherosclerosis [37, 38].

In non-CKD populations, many cohort and case-control studies have documented an association of monocytosis with cardiovascular diseases [39–42]. Elevated monocyte counts were also identified as an independent predictor of total and CV mortality in hemodialysis patients [43]. In a cohort of 951 patients, Rogacev et al. [31] found that nonclassical CD14+CD16+ monocytes independently predicted cardiovascular events in subjects referred for elective coronary angiography. Numbers of CD16 positive monocytes but not overall monocyte counts positively correlate with body mass index and insulin resistance as well as diabetes and intima-media thickness [44]. In patients with symptomatic CAD compared to healthy controls, the percentage of CD16 positive monocytes was found to be increased after adjustment for common risk factors [45, 46]. Assessment of plaque vulnerability in patients with both stable and unstable angina pectoris found that more vulnerable plaques were associated with an increase in percentage of CD16 positive monocytes.

CKD had been shown to alter the number, subset distribution, and function of circulating monocytes [47, 48]. In previous studies [16, 31, 32], patients with CKD have an increased percentage of CD16 positive monocytes in the circulation. In our study, we further observed that in patients with preexisting CVD, the presence of higher percentage of CD16 positive monocytes was found to be associated with increased CV and all-cause death.

More interestingly, we found that a subset of dialysis patients with CD16+ monocytes falling within the normal range tends to suffer great risk of CV death. Advanced CKD is characterized by the dynamic coexistence of the generalized immune depression that contributes to the high prevalence of infections among these patients and systemic inflammation that may contribute to CVD. Accumulation of proinflammatory cytokines may be due to decreased renal elimination and/or increased generation following induction by various factors such as uremic toxins, oxidative stress, volume overload, and comorbidities [49, 50]. ESRD is associated with immunosuppression due to the impact of the uremic milieu and a variety of associated metabolic disorders on the other. Impaired monocyte function, including defects in chemotaxis, phagocytosis, and a decrease in the production of cytokines, had been reported [50]. Frequent exposure to these diverse external stimuli might lead to a state of chronic low-grade activation, and a high-percentage monocytic primed cell was found in hemodialysis patients [51].

The prevailing and continuous antigenic stimulation might result in exhaustion in the downstream signaling cascade, and this might subsequently impair the innate and adaptive components of the immune system's response to microbial challenge. The presence of a subgroup of our patients with functional monocyte deactivation may be due to LPS tolerance. This state of "immune paralysis" in these patients may be related to downregulation of toll-like receptor, especially toll-like receptor-2 (TLR-2) and toll-like receptor-4 (TLR-4) expression on monocytes [49, 52]. TLR-2 and TLR-4 are involved in innate immunity, and activation of these receptors leads to systemic inflammation in the host. Several authors found that a decrease of TLR-4 was found on unstimulated monocytes in CKD patients compared with

healthy controls [52–54]. The etiological factor of "immune paralysis" may be related to chronic endotoxemia [55], frequent blood membrane interaction, or other toxic metabolites related to uremic milieu. It seems possible that continuous activation of monocytes suppresses the expression of TLR-4, contributing to immune deficiency and increased incidence and severity of infections in ESRD population. Clearly, the J-shaped effect of low CD16+ monocytes on CV death risk observed in this study needs further research for it to be clarified.

Lastly, the analysis results of this study, namely, the association between CD16+ monocytes and mortalities in hemodialysis patients, were obtained mostly in patients with a preexisting CVD history. In the multiple Cox regression analysis, we found that those without CVD history at baseline had just 2 nonmissing cases suffered from CV death and 7 nonmissing cases from all-cause death. Moreover, the association pattern shown in Table 4 remained unchanged when the analyses were performed on the sample composed of those with CVD history at baseline.

We acknowledge several limitations of this study. One of them is the relatively small number of patients, particularly the relatively small number of patients without preexisting CAD. Thus, caution should be exercised in the interpretation of our results. Besides, our patient population was also limited to those on hemodialysis and may not be generalizable to the broader population.

Taken together, the results of this study indicated that high level in CD16+ monocytes was associated with significantly higher risks in CV and all-cause death in hemodialysis patients with preexisting CAD. Overall, nonclassic monocytes were detrimental, whereas the minor subset of the relatively low CD16-expressing monocytes was associated with an unfavorable clinical outcome. In spite of the limited study sample, we highlight the current facts and future perspectives of how the assessment of microinflammation can assist clinicians in early and efficient recognition of inappropriate performance of the immune system to reduce mortality. Nevertheless, more studies based on large-scale cohort are still desired to elucidate this issue further.

Disclosure

The funder had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflicts of Interest

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

Acknowledgments

This study was funded by a grant from Tungs' Taichung MetroHarbor Hospital, Taichung, Taiwan, Grant no. TTMHH-102C0013.

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