CIRCULATING IMMUNE CELLS IN ATHEROSCLEROTIC DISEASE

Identification of patients at risk

John Meeuwsen

Circulating immune cells in atherosclerotic disease
Identification of patients at risk

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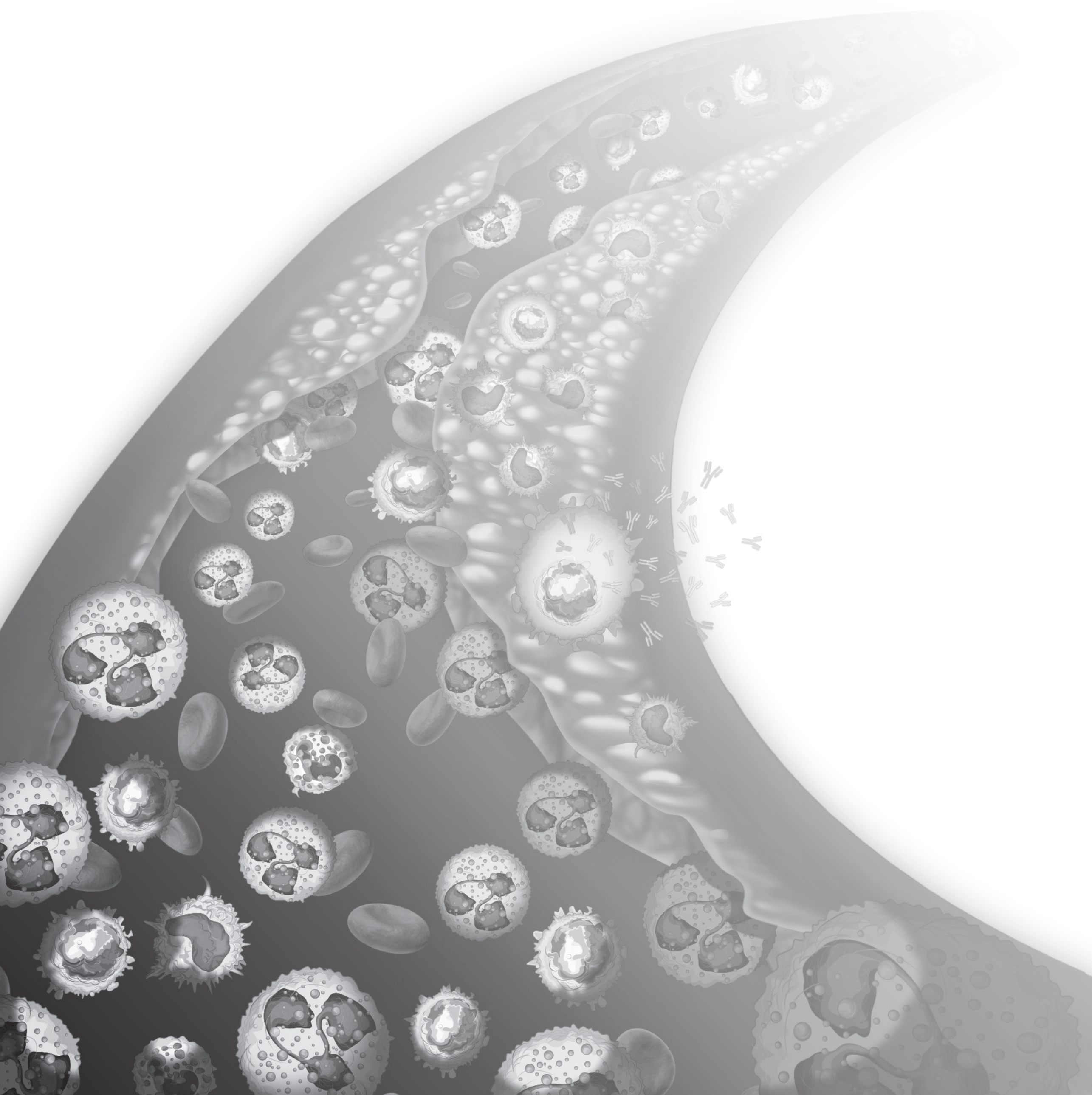

CHAPTER

1

Introduction and thesis outline

INTRODUCTION

Burden of cardiovascular disease

The leading causes of morbidity and mortality are cancer and cardiovascular disease. This thesis focusses on cardiovascular disease, which caused approximately 17.9 million deaths worldwide in 2015[1]. Deaths as a consequence of cardiac ischemia and cerebrovascular ischemia are the most prevalent within this category (8.9 and 3.6 million, respectively)[1]. Besides severe health problems for the patient, the economic burden on society is also a major challenge[2]. The estimated health care costs in the United States for cardiovascular disease and stroke were $189.7 billion in 2012 to 2013[3]. Despite improved treatment, it is expected that the burden of cardiovascular disease increases in the future, mostly in low- and middle-income countries[4]. Therefore, research on the major underlying pathology of CVD, atherosclerosis, is of great importance to obtain increased insights. These insights may eventually lead to novel biomarkers and therapeutic targets for patients with cardiovascular disease.

Atherosclerosis

Atherosclerosis is a chronic lipid-driven inflammatory disease of the larger arteries. Atherosclerotic lesions in the coronary arteries are already present at a very young age, as demonstrated in a landmark study among young United States soldiers[5]. Atherogenesis is initiated by endothelial damage of the arteries. Upon endothelial damage, low-density lipid (LDL) cholesterol enters the vessel wall and will form a fatty streak in the inner wall of the vessel, the intima[6]. Over time, LDL cholesterol influx further increases and LDL particles are oxidized by local molecules or factors released by infiltrating cells, such as macrophages[6-8]. Macrophages take up the oxidized LDL and develop into foam cells. A combination of high LDL cholesterol and inflammation drives the chronic progression of atherosclerosis[7, 9, 10]. Atherosclerotic plaques in the body can remain silent for a long time, without presentation of clinical symptoms. Eventually, plaque erosion or rupture give rise to clinical manifestations, such as ischemic stroke and myocardial infarction. Among other risk factors as ageing, male sex, smoking and high blood pressure, inflammation plays an important role in plaque progression and plaque destabilization. Therefore, the inflammatory processes in atherosclerotic disease have been thoroughly investigated in the last decades. Investigation of circulating inflammatory cells, that are believed to actively infiltrate atherosclerotic lesions, is one of the possibilities to increase our understanding of specific immune cell subsets in cardiovascular disease. In addition, circulating cells are easily accessible and therefore interesting biomarker candidates. Finally, as circulating cells interact with atherosclerotic plaques, they hold potential as a therapeutic target for patients with cardiovascular disease.

THESIS OUTLINE

PART ONE - CIRCULATING IMMUNE CELLS IN ATHEROSCLEROSIS

Circulating cells in CVD

Several studies have addressed the question if circulating inflammatory cells hold prognostic value for the occurrence of primary or secondary cardiovascular events. **Chapter 2** provides an overview of the prognostic value of circulating inflammatory cells in patients with stable and acute coronary artery disease. Total white blood cell count, and in particular the neutrophil to lymphocyte ratio (NLR) appears to best predict secondary adverse CVD outcomes. Surprisingly, circulating monocyte numbers, which are major contributors in the initiation, progression and destabilization of atherosclerotic lesions, showed only limited associations with the occurrence of secondary cardiovascular manifestations. To clarify this apparent contradiction, more studies that consider the distinct pro- and anti-inflammatory cell subsets are necessary to better understand their role in atherosclerotic disease. Eventually, the identification of specific inflammatory atherogenic cell subsets might form a source of highly specific biomarkers or therapeutic targets.

Monocytes

In **chapter 3**, we investigated the association between different monocyte subsets and cardiovascular disease. For these analyses, we took advantage of the Athero-Express, a large cohort study that includes severe atherosclerotic patients undergoing carotid endarterectomy (CEA) in the Sint Antonius hospital and University Medical Centre in Utrecht[11]. Medical information, standardized plaque characterizations and follow-up information is available from these patients. In addition, we isolated peripheral blood mononuclear cells, and investigated specific subpopulations of monocytes and B cells in relation to atherosclerotic plaque phenotypes and the risk of secondary cardiovascular manifestations. Based on their pro-inflammatory and atherogenic role in atherosclerotic mouse studies[12-15], we investigated if circulating classical monocytes were associated with characteristics of vulnerable plaques, including low amounts of collagen and smooth muscle cells, and increased fat content, neovascularization, and intraplaque hemorrhage. In addition, we evaluated the prognostic value of $CD14^{++}CD16^{+}$ classical, $CD14^{+}CD16^{+}$ intermediate and $CD14^{+}CD16^{++}$ non-classical monocytes for secondary cardiovascular events during three-year follow-up.

B cells

In addition, we identified different B cell subsets and related them to the occurrence of secondary CVD manifestations in CEA patients from the Athero-Express in **chapter 4**. Until then, evidence for a role of B cells in atherosclerosis was primarily derived from experimental models, and their role in humans remained largely unexplored. First, B cells were generally thought to be atheroprotective[16, 17], but animal studies towards specific B

cell subsets revealed distinct atheroprotective (B1 and marginal zone B2 cells[18, 19]) and atherogenic ((follicular) B2 cells[20-24]) B cell subtypes. We described the prognostic value of different circulating B cell subtypes in patients with atherosclerosis, and in order to further unravel the role of these B cells in atherosclerosis, we looked into the antibody production of B cells, which is one of the three main functions of B cells besides antigen presentation and cytokine excretion[25]. Several antibodies have been described in atherosclerosis, of which antibodies against oxidized low-density lipoprotein (oxLDL) are the most prominent. For example, IgM antibodies directed against oxLDL have been suggested to play an atheroprotective role in animals as well as humans[18, 26]. More recently, the role of IgG4 in relation to atherosclerosis has been investigated[27-30].

Immunoglobulin 4

Immunoglobulin 4 (IgG4) is the antibody with the lowest prevalence (<5% of total IgG) in the human body. Earlier studies have indicated that high serum IgG4 levels were associated with atherosclerotic plaques in IgG4-related disease (IgG4-RD) patients and with the presence of CAD in patients undergoing coronary angiography[27-30]. However, longitudinal studies examining the role of IgG4 in relation to CVD were lacking. In **chapter 5**, we evaluated the association between circulating IgG4 levels and cardiovascular events during follow-up in males and females from the Athero-Express biobank. In addition, to explore if the number and presence of IgG4 positive cells associates with advanced plaque stages, we determined IgG4 and CD138 positive cells in carotid autopsy sections from the Maastricht Pathology Tissue Collection.

CD200 receptor

In 2017, an important breakthrough was achieved in the inflammatory field of atherosclerosis. So far, clinical treatment relied mainly on medication that inhibited coagulation and reduced LDL cholesterol. This has changed since the landmark study by Ridker and colleagues showed that inhibition of an excessive inflammatory reaction, using canakinumab on top of regular treatment, reduced the risk of recurrent cardiovascular events with 15% in patients with stable cardiovascular disease[31]. In different experimental settings, researchers have tried to dampen inflammation and thereby to reduce atherosclerotic lesion formation. For this purpose, several immune co-stimulatory and co-inhibitory molecules have been evaluated[32]. For example, inhibition of the co-stimulatory molecule CD40L, which is expressed on B and T lymphocytes among others, showed reduced atheroma formation[33]. In addition, co-inhibitory molecules including CTLA-4 and PD-1 have been associated with reduced inflammatory responses and decreased atherosclerotic lesion formation[34-36]. In **chapter 6**, we also investigated if stimulation of the inhibitory CD200 receptor (CD200R) could inhibit inflammation and reduce atherosclerotic plaque formation. CD200R is present on many cell types, including B and T lymphocytes, but mainly expressed by monocytes and macrophages[37]. Moreover, disruption of the CD200-CD200R pathway has been associated with enhanced development of auto-immune diseases such as auto-immune encephalomyelitis and

collagen induced arthritis[38]. Therefore, we hypothesized that treatment of atherosclerotic LDLR$^{-/-}$ mice with an agonistic antibody, OX110, which stimulates the immune inhibitory CD200 receptor would reduce inflammation and inhibit plaque progression.

PART TWO – INFLAMMATION AND CVD IN WOMEN WITH A HISTORY OF PREECLAMPSIA

Women in cardiovascular disease

The pathology and clinical presentation in cardiovascular disease differs between men and women[39-41]. For example, cardiovascular disease presents earlier in men than in women. In addition, it has been shown that males present more often with plaque rupture and that plaque erosion occurs more frequently in females[42]. This may suggest that optimal treatment for CVD in females differs as well. However, guidelines for CVD prevention and treatment rely heavily on randomized clinical trials (RCTs) in which women are often underrepresented, as shown by a recent meta-analysis[43]. Reasons for the underrepresentation of women in RCTs are difficult to ascertain, but likely result from exclusion criteria such as child-bearing potential and older age[43-45]. It is important to consider these sex differences in cardiovascular research. As underlying pathological mechanisms of CVD are likely to be different between men and women, investigation of female-specific risk factors, such as a history of preeclampsia, deserve increased attention in cardiovascular research.

Preeclampsia

Preeclampsia (PE) is a pregnancy related disorder, characterized by high blood pressure and proteinuria[46]. PE complicates the pregnancy and leads to substantial morbidity and mortality[46]. Besides the direct impact of PE during pregnancy, the future risk for many diseases, including CVD, is significantly increased[47-49]. Women with a history of PE have approximately 2.5 times increased risk of coronary heart disease[50].

Interestingly, atherosclerosis and preeclampsia share several pathological mechanisms. Therefore, in **chapter 7**, we hypothesized that the pathological mechanisms in preeclamptic women might underlie the increased risk for cardiovascular disease later in life. Because of the high risk of cardiovascular disease, the presence of coronary calcification and coronary stenosis in women with a history of preeclampsia was compared to women with an uncomplicated pregnancy[51]. Women with a history of PE displayed more coronary calcification than women with an uncomplicated pregnancy, already at an age of 45 to 55 years[52]. These findings highlighted the need for biomarkers that could identify women with a PE history which are at high risk for cardiovascular disease in order to prevent clinical manifestations. As mentioned earlier, neutrophils play an important role in the pathology of cardiovascular disease. Several studies have shown that neutrophils are recruited to atherosclerotic plaques, and implicated in endothelial dysfunction and recruitment of monocytes to atherosclerotic lesions[53-57]. Moreover, neutrophils display

high activity during preeclampsia[58-62]. Therefore, in **chapter 8**, we hypothesized that neutrophil numbers and/or activity, being implicated in both CVD and PE, were associated with the presence of coronary calcification and stenosis in women with a history of preeclampsia.
Finally, **chapter 9** encompasses the summary and general discussion of this thesis.

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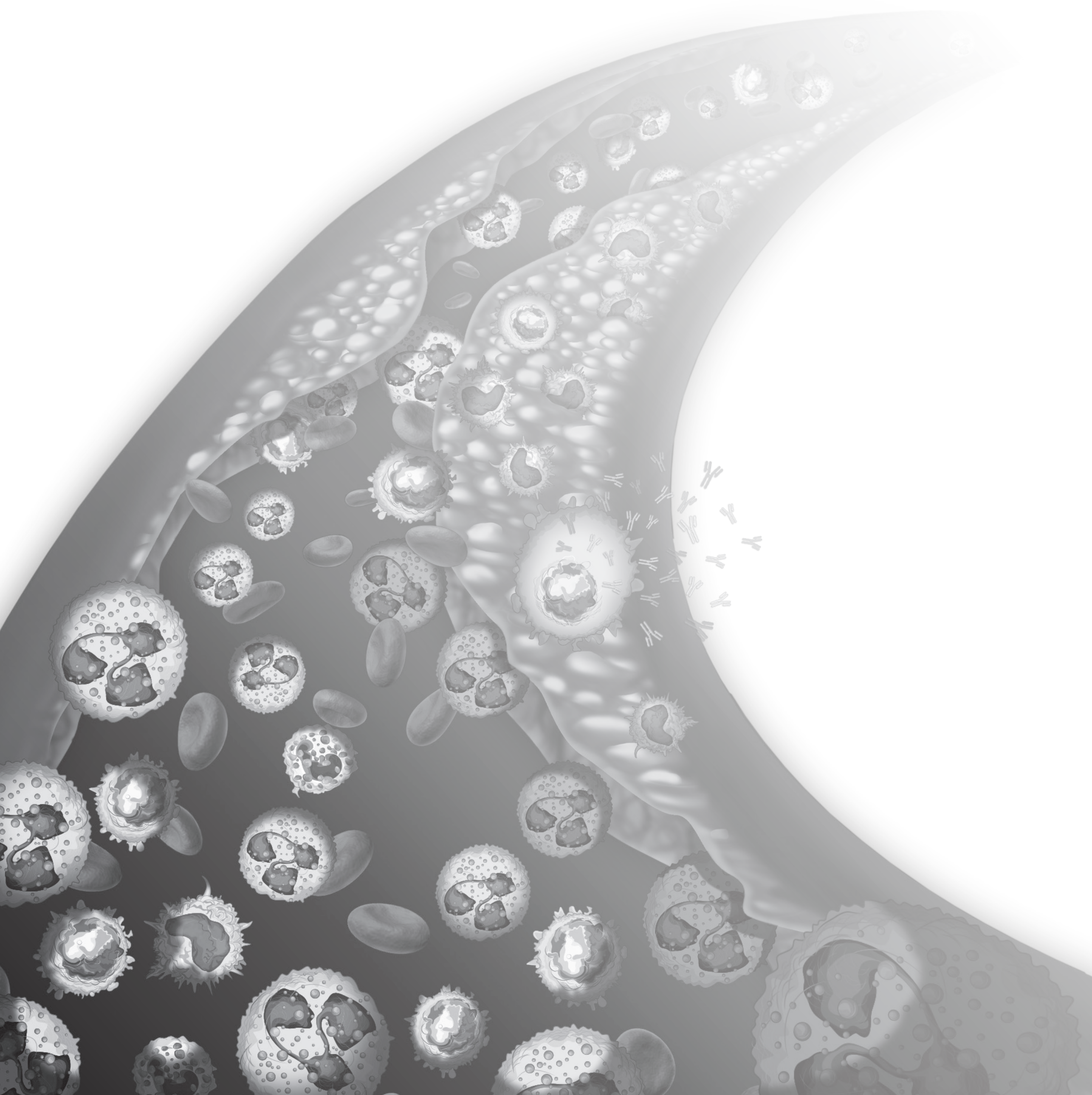
PART ONE

CIRCULATING IMMUNE CELL PROFILES IN ATHEROSCLEROTIC DISEASE



CHAPTER

2

Prognostic value of circulating inflammatory cells in patients with stable and acute coronary artery disease

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ABSTRACT

Atherosclerosis is a lipid driven chronic inflammatory disease underlying the majority of ischemic events such as myocardial infarction or stroke. Clinical management of ischemic events has improved considerably in the past decades. Accordingly, survival rates have increased. Nevertheless, 12% of patients die within 6 months after the initial event. To improve secondary prevention, appropriate risk prediction is key. However, up to date, there is no clinically available routine marker to identify patients at high risk for recurrent cardiovascular events. Due to the central role of inflammation in atherosclerotic lesion progression and destabilization, many studies have focused on the role of circulating inflammatory cells in these processes. This review summarizes the current evidence on the potential of circulating inflammatory cells as biomarkers for recurrent adverse manifestations in acute coronary syndrome and stable coronary artery disease patients.

INTRODUCTION

Cardiovascular disease (CVD) remains one of the most important causes of death worldwide. It represents a major challenge in healthcare and it is estimated that ~17.5 million people die from CVD annually. Although treatment and interventional options have significantly improved survival rates, consequently the incidence of chronic CVD is expected to increase in the next decades[1]. Currently, approximately 90% of patients that encounter a first myocardial infarction (MI) survive[2]. However, the risk of secondary cardiovascular manifestations is high. Roughly 5-15% of patients die during hospitalization or within 30 days after MI[3-5] and an additional 5% of patients die within 6 months upon hospital discharge[6]. The risk of re-infarction is most profound within the first year, occurring in approximately 17% of patients[7], and the risk for recurrent adverse events further increases over time[8].

Atherosclerosis is the culprit pathology driving coronary artery disease (CAD) and is characterized by the presence of lipid and inflammatory cells during initiation, progression and destabilization of an atherosclerotic plaque[9-11]. Monocytes and macrophages are well known for their role in plaque growth. Monocytes transmigrate through the disrupted endothelium and infiltrate the intimal area, where they mature into macrophages. By phagocytosis of oxidized LDL, they develop into foam cells which form an integral part of atherosclerotic plaques[12]. Next to monocytes, neutrophils, mast cells and (activated) lymphocytes massively accumulate in rupture-prone regions of the atherosclerotic plaques, suggesting an important role in plaque destabilization[13-15]. As the atherosclerotic lesion progresses, necrotic cell death occurs, a process that promotes further lesion progression as it contributes to the inflammatory response and enlargement of the necrotic core, which cumulatively can result in plaque rupture[16-18]. Whether the process of necrosis in the plaque directly alters the circulating inflammatory cells profile has, to the best of our knowledge, not been established. Acute coronary syndromes (ACSs) can result in obstructed blood flow, with myocardial necrosis and inflammation as a consequence. In response, the circulating cell profile is altered and inflammatory cells are actively requited to the damaged myocardial tissue[16].

Given their prominent role in cardiovascular disease, the sheer number of circulating inflammatory cells and their distribution into subpopulations may have predictive value for recurrent cardiovascular manifestations. As such, they can be used to improve risk stratification on top of existing prediction models (i.e. Global Registry of Acute Coronary Events (GRACE) risk score or the Thrombolysis in Myocardial Infarction (TIMI) risk score for ACS patients)[19]. Identifying patients at high risk may help to prevent the occurrence of re-infarction or death.

Many studies have studied circulating inflammatory cells as markers of recurrent adverse manifestations after MI. Therefore, we aimed to provide an overview on the value of circulating inflammatory cells as markers for secondary events and mortality in stable CAD and ACS patients.

THE PROGNOSTIC VALUE OF CIRCULATING INFLAMMATORY CELLS IN CORONARY ARTERY DISEASE

We have included studies that prospectively examine the predictive value of total WBC count, WBC subtype count or ratios in patients with CAD or ACS specifically. The primary endpoints include major adverse cardiovascular events (MACE) or mortality.

White Blood Cells

It is generally acknowledged that a high white blood cell (WBC) count roughly reflects an activated inflammatory status of an individual. It is well known that people with inflammatory or autoimmune disorders have an increased risk of CVD[20-22], which is most likely the consequence of inflammatory responses negatively affecting plaque stability[23]. Therefore, WBC count is well studied during the past decades in CAD patients. Although total WBC was not associated with plaque progression in one study[24], it has been associated with presence, severity and extent of coronary atherosclerosis in another study[25], as well as with in-stent restenosis in percutaneous coronary intervention (PCI) patients[26]. The prognostic value of total WBC count in CAD patients is not consistent (**Tables 1** and **2**). On one hand, an elevated WBC count (>8.2 x 10e9/L) has been independently associated with risk of adverse events and mortality (2.2-fold increase on average)[27-36], but on the other hand, others show no independent predictive value of total WBC count in patients with CAD[37-43]. These different findings might be explained by the fact that WBC count independently prognosticated outcome primarily in studies conducted on PCI patients, containing a mixed population of both acute and stable coronary artery disease (**Tables 1** and **2**). More specific prognostic information for risk of follow-up events or mortality might be derived from the WBC differential, since the different WBC subtypes have distinct roles in immune (dis)balance.

Monocytes

Plaque macrophages derive from infiltrating monocytes and are well known for their role in atherosclerotic initiation and development[24, 44, 45]. Distinct monocyte subsets exist and differ in function[46]. Classical CD14^{++}CD16^{-} monocytes are predominantly phagocytic, non-classical CD14^{+}CD16^{++} monocytes display mainly inflammatory characteristics, and a small transitional subset of intermediate CD14^{+}CD16^{+} monocytes displays both phagocytic and inflammatory function[47]. In a few studies exploring the prognostic power of total monocytes no evidence for monocytes as independent predictors of recurrent cardiovascular events could be established[38, 39]. However, others report that elevated monocyte count is a significant predictor for secondary cardiovascular manifestations and mortality increasing the average risk by 50%[30, 31, 40, 42, 48]. This has been shown to be primarily associated with the CD14^{+}CD16^{++} intermediate monocyte subtype[48] and to be dependent on follow-up time[40] (**Tables 1 and 2**). Another study investigating the role of monocyte activation showed that patients with high percentages of Urokinase-type Plasminogen Activator Receptor positive monocytes, indicative of increased monocyte infiltration and activation, had increased risk of recurrent cardiovascular events[49].

Neutrophils

Both under homeostatic and acute inflammatory conditions, neutrophils are the major component of the total WBC count (~60-70%). Although rarely found in human atherosclerotic plaques, experimental atherosclerosis mouse studies have shown a role for neutrophils in the initiation of atherosclerosis[50]. Elevated neutrophil levels are associated with rupture prone plaques[51, 52] and in-stent restenosis after PCI[26]. However, there is conflicting evidence about the prognostic value of neutrophils in stable CAD patients. Some reports show that elevated neutrophil count (>6 x 10e9/L) is associated with an approximately doubled risk of mortality or MACE[30, 31, 33]. Others could not establish this prognostic value of neutrophils[38-40, 42] (**Tables 1 and 2**). The studies that showed an independent prognostic value of neutrophils were more likely to include PCI and angina pectoris (AP) patients instead of CAD patients, and to have MACE as endpoint rather than mortality. This suggests that, also in the setting of cardiovascular disease, neutrophils are mostly related to an acute inflammatory reaction upon cardiac injury rather than the chronic inflammatory response in plaque development.

Lymphocytes

The role of lymphocytes in CVD has been extensively investigated in experimental atherosclerosis models. A plethora of different B- and T-cell subtypes with both atherogenic or atheroprotective roles has been reported (reviewed in Ref.[53]). However, in the human setting the evidence for a lymphocyte specific role in the presentation of atherosclerosis is limited and the outcome remains inconclusive. The majority of studies showed that high lymphocyte counts were not independently associated with plaque progression, in-stent restenosis, CVD events or mortality[24, 26,30, 38, 40], while there is only 1 study that reports on high levels of lymphocytes being protective for future events[31] (**Tables 1 and 2**). Another group observed a role for a specific T lymphocyte subset, as the increase of $CD4^{+}CD28^{null}$ T cells was associated with increased rate of CVD events[54] (**Table 2**). These $CD4^{+}CD28^{null}$ cells have profound effector functions and are considered to be pro-atherogenic as their levels are high in unstable, but not in stable plaques[55]. When assessing lymphocytes as a percentage of the total white blood cell count, high lymphocyte percentages were protective of 1-year MACE in PCI patients[28]. In contrast, high lymphocyte percentages showed to be detrimental in stable CAD patients for long-term follow-up (3 and 13.2 years) only[56]. It can be debated on whether using the percentage of white blood cells is the best parameter as the relative contribution of inflammatory cell subtypes may be similar in patients with or without follow-up events. However, due to an increase in total white blood cell counts, the total amount of cell subtypes may very well differ between patients.

Neutrophil to Lymphocyte Ratio

The neutrophil to lymphocyte ratio (NLR) is an emerging biomarker that may better reflect the immune status of an individual as compared to a single inflammatory cell subtype. In a recent study using multidetector computed tomography, NLR was shown to be associated

with severity and plaque morphology in CAD patients[57]. The NLR prognosticates outcome consistently in the majority of the studies performed in stable CAD patients (**Tables 1 and 2**). Risk of MACE or mortality increased approximately by 150% in patients with elevated NLR (average cut-off >2.5) [31, 33, 37, 38, 40, 43, 58-60]. The exact underlying mechanisms of NLR in relation to recurrent events in CAD patients are poorly understood. Low lymphocyte counts might result from increased cortisol levels that induce apoptosis specifically in lymphocytes (and eosinophils), but increase total WBC count[61]. A rise in neutrophil counts is often accompanied with increased neutrophil activity, thereby leading to the release of proteolytic enzymes, for example myeloperoxidase, which can induce tissue injury[62]. In addition, vascular obstruction as a consequence of neutrophil aggregates, a process also involving platelets and endothelial cells, increases the risk of recurrent events[63].

Table 1. Mortality predicted by total WBC and WBC differential in CAD patients categorized by follow-up time.

Study characteristics			WBC		Monocytes		Neutrophils		Lymphocytes		Neutrophil to Lymphocyte Ratio		Ref
Population	FU	Size (n)	Risk [95% CI]	*p*	Risk [95% CI]	*p*	Risk [95% CI]	*p*	Risk [95% CI]	*p*	Risk [95% CI]	*p*	
PCI	< 1	309	1.2 [1.0 - 1.3][b]	*									27
PCI	1 - 6	1425	4.8 [1.3 - 16.8]	n.s.	1.7 [1.0 - 2.9]	n.s.	5.7 [1.6 - 19.6]	n.s.			5.6 [1.6 - 19.8]	**	40
PCI	> 6 - 36	83	14.7 [2.7 - 80.7]	**									29
UAP/NSTEMI	> 6 - 36	280	1.4 [0.6 - 2.9]	n.s.									41
CAD	> 6 - 36	1246	2.0 [1.3 - 3.1]	**									36
CAD	> 6 - 36	422	5.3 [1.2 - 24.1]	n.s.	6.4 [1.4 - 28.8]	n.s.	5.5 [1.2 - 24.7][c]	n.s.	4.2 [1.4 - 12.4][a]	n.s.	8.1 [1.4 - 46.6]	*	38
PCI	> 6 - 36	1046									1.9 [1.3 - 3.0]	*	59
UAP/NSTEMI	> 36	275	1.7 [1.1 - 2.5]	*									34
PCI	> 36	1425	1.7 [1.0 - 2.7]	n.s.	2 .0[1.2 - 3.4]	*	2.0 [1.2 - 3.3]	n.s.	0.6 [0.4 - 0.9]	n.s.	3.0 [1.8 - 5.0]	***	40

The risk of mortality in CAD patients categorized by follow-up time. The indicated risk can be a relative risk, odds ratio or hazard rate. Unless otherwise stated, the indicated risk regards the risk of patients in the group with the highest cell count compared to patients with low cell counts. FU indicates follow-up time in months.

[a]Risk regarding cell percentage.

[b]Risk increase per 1 x 10e9 cells per liter of blood.

[c]Risk was also not significant for neutrophil percentage.

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Table 2. Cardiovascular events predicted by total WBC and WBC differential in CAD patients categorized by follow-up time.

Study characteristics			WBC		Monocytes		Neutrophils		Lymphocytes		Neutrophil to Lymphocyte Ratio		Ref
Population	FU	Size (n)	Risk [95% CI]	*p*	Risk [95% CI]	*p*	Risk [95% CI]	*p*	Risk [95% CI]	*p*	Risk [95% CI]	*p*	
AP	1 - 6	1125	2.8 [1.9 - 4.3]	***			2.5 [1.6 - 3.7]	***			1.7 [1.1 - 2.5]	**	33
CAD	> 6 - 36	389	1.3 [0.6 - 2.8]	n.s.	1.6 [1.1 - 2.5]	*	1.3 [0.7 - 2.3]	n.s.	0.8 [0.5 - 1.4]	n.s.			42
IS / MI / PAD	> 6 - 36	18558	1.4 [1.3 - 1.6]	***	1.2 [1.1 - 1.4]	**	1.5 [1.3 - 1.7]	***	1.0 [0.9 - 1.2]	n.s.			30
AP	> 6 - 36	1125	2.5 [1.7 - 3.7]	***			2.3 [1.6 - 3.4]	***			1.6 [1.1 - 2.3]	*	33
PCI	> 6 - 36	140	34.0 [4.1 - 281]	**					37.5 [4.5 - 311][a,d]	***			28
PCI	> 6 - 36	83	10.9 [2.4 - 49.7]	**									29
PCI SVG	> 6 - 36	530	1.2 [1.1 - 1.3][c]	***									32
UAP / NSTEMI	> 6 - 36	280	1.3 [0.7 - 2.3]	n.s.									41
ACP neg	> 6 - 36	975	NR	n.s.	NR	n.s.	NR	n.s.	2.5 [1.3 - 4.8][d]	**			39
Stable CAD	> 6 - 36	422	2.1 [1.0 - 4.4]	n.s.			1.8 [0.9 - 3.8][e]	n.s.			NR	n.s.	38
Angiography	> 6 - 36	951			3.0 [1.3 - 6.9][g]	**							48
CAD	> 6 - 36	263			4.0 [1.3 - 12.1][b]	*							49
Stable CAD	> 6 - 36 [h]	141							1.7 [0.8 - 3.4][a]	n.s.			56
UA	> 6 - 36	120							3.0 [1.1 - 8.3][f]	*			54
Angiography	> 6 - 36	3005									1.6 [1.1 - 2.2]	*	60
(U)AP	> 36	3227	1.4 [NR]	*	1.3 [NR]	*	1.8 [NR]	***	0.5 [NR]	***	2.2 [NR]	***	31
CHD	> 36	942	0.9 [0.9 - 1.0]	n.s.							1.1 [1.1 - 1.2][c]	***	43

CHD	> 36	4535	1.4 [1.0 - 1.9]	NR					35
Stable CAD	> 36	2370	1.4 [0.7 - 2.5]	n.s.			1.7 [1.3 - 2.2]	***	37
stable CAD	> 36	141			2.3 [1.3 - 3.8][a]	NR			56
PCI	> 36	798					2.3 [1.3 - 4.3]	**	58

The risk of major adverse cardiovascular events (MACE) in CAD patients categorized by follow-up time. The indicated risk can be a relative risk, odds ratio or hazard rate. Unless otherwise stated, the indicated risk regards the risk of patients in the group with the highest cell count compared to patients with low cell counts. FU indicates follow-up time in months.
[a]Risk regarding cell percentage.
[b]Risk regarding >15% Urokinase-Type Plasminogen Activator Receptor positive monocytes.
[c]Risk increase per 1 x 10e9 cells per liter of blood or increase of 1 NLR.
[d]Lowest tertile or quartile compared to highest.
[e]Besides neutrophil count, the neutrophil percentage was also not associated with MACE risk.
[f]Risk regarding CD4+CD28null T cell frequency >4%.
[g]$CD14^{+}CD16^{++}$ monocytes, total monocyte count or other subsets were not associated with MACE.
[h] Risk score was also not related for 3 years of follow-up.
The level of significance in multivariate analyses is indicated by * p<0.05; ** p<0.01; *** p<0.001; n.s. indicates not significant. NR indicates not reported.

THE PROGNOSTIC VALUE OF CIRCULATING INFLAMMATORY CELLS IN ACUTE CORONARY SYNDROMES

In patients with acute coronary syndrome, the rate of recurrent cardiovascular events and mortality is higher as compared to stable CAD patients. Because of the increased event rate and the differences in plaque composition[64, 65], the prognosis in these patients is likely to be different as well. Therefore, we consider the prognostic value of circulating cells in ACS patients separately in this chapter.

White blood cells

Similar to more general CAD populations, also in specific ACS populations contrasting results are reported for the prognostic value of white blood cells. Several reports show that increased leukocyte count (on average >12 x 10e9/L) predicted increased MACE and mortality risk independently (approximately 2.5 times risk increase)[3, 66-76]. However, these findings were not corroborated by others[77-84] (**Tables 3 and 4**). In general, WBC count was a better predictor in patients with short follow-up than long follow-up. In addition, the studies which showed a prognostic value for the WBC count had a larger sample size, which might explain the different observations in prognostic value of WBCs. How the different WBC subtypes may relate to the risk for MACE or mortality during follow-up will be discussed below.

Monocytes

Although monocytes play a central role in atherosclerosis, their role in risk prediction has been scarcely investigated in ACS patients (**Tables 3 and 4**). In one study, the three monocyte subsets were investigated over time after MI, and monocyte numbers peaked at day 2 post MI[85]. Total, CD14^{++}CD16^{+} classical monocytes and CD14^{++}CD16^{-} non-classical monocytes, but not CD14^{+}CD16^{++} intermediate monocytes, were predictive for MACE when assessed 2 days post MI. Of five different measurements over time in this study, CD14^{++}CD16^{+} classical monocytes were most consistently (four out of five) associated with recurrent CVD events[85]. In addition, the classical CD14^{++}CD16^{+} monocyte was significantly associated with carotid atherosclerosis and intraplaque neovascularization[86]. In two other studies the total monocyte count did not independently prognosticate mortality[74, 83]. Thus, there is limited and inconclusive evidence for a prognostic value of circulating monocytes for recurrent CVD events and mortality, despite the fact that monocytes are important in plaque development and rupture. The lack of a clear association may have different explanations. It can be due to the fact that mainly total monocyte numbers are used, while it has been clearly established that different monocyte subtypes exist[47]. Indeed, in line with the findings in stable CAD patients, a significant association with recurrent cardiovascular events was observed in specific monocyte subsets[48, 49, 85]. On the other hand, the circulating monocyte pool may simply not reflect the macrophage pool in the plaque. Indeed, a study by Robbins and colleagues has established that plaque macrophages in a murine atherosclerosis model mostly derive from proliferation of the existing macrophage pool rather than recruitment of new monocytes[87]. Furthermore, besides technical issues such as different gating strategies or isolation methods, monocyte

plasticity could also explain why the circulating monocytes do not per se mirror plaque macrophages. Indeed, experimental evidence indicates that plaque macrophages can switch phenotype over time in advanced atherosclerotic lesions[88, 89].

Neutrophils

As described in CAD patients, conflicting reports were published about the prognostic value of neutrophil counts. In ACS patients, the results are also not uniform, though more positive. Two studies were unable to establish an independent predictive value for neutrophil counts[83, 84]. However, in the majority of the studies assessing the prognostic value of neutrophils, their counts significantly predicted short term, i.e. <30 days, (average OR 5)[90-92] and long term, i.e. 3 years, (average HR 1.75)[74, 93, 94] secondary cardiovascular events and mortality (**Tables 3 and 4**). Although different studies have described that elevated neutrophil counts associate with worse outcome, it is surprising that there is no consistent evidence. Elevated neutrophil counts have been associated with endothelial disruption as a consequence of released reactive oxygen species and MPO[62]. In addition, neutrophils can induce vascular plugging, thereby extending infarct size[63]. The relative neutrophil count (as percentage of total WBC) might add prognostic value, but only few studies have examined their predictive power. However, the neutrophil to lymphocyte ratio described later in this review has been studied extensively.

Lymphocytes

There is limited evidence for a role of total lymphocytes in risk prediction of ACS patients. Total lymphocyte counts were not independently associated with follow-up events and mortality[74, 83, 84] except for one study, where low lymphocyte counts were predictive of mortality[95] (**Tables 3 and 4**). In addition, as for CAD patients, an increase of pro-atherogenic $CD4^{+}CD28^{null}$ T cells was associated with increased rate of recurrent CVD events[96] (**Table 4**). The information on the prognostic value of lymphocytes and specific lymphocyte subtypes is limited. Considering that both T and B cell subsets can have distinct pro- or anti-atherogenic or inflammatory characteristics[53, 97], the prognostic value of these specific subtypes could hold more value compared to the complete lymphocyte count. With advances made in multicolor flow cytometry the last decade[98], this research area deserves more attention in future biomarker studies.

Neutrophil to Lymphocyte Ratio

Although the number of studies describing neutrophil or lymphocyte counts alone are limited and inconclusive, the neutrophil to lymphocyte ratio (NLR) has been well studied during the past years (**Tables 3 and 4**). Similar to stable CAD patients, elevated NLR prognosticates adverse outcome in ACS patients as shown by several studies both at short term, i.e. <30 days[99-105], and long term, i.e. >6 months, of follow-up[74, 75, 83, 101, 103, 104, 106-108] with an average fourfold increased risk. Only in two studies, the NLR was not independently associated with MACE and CVD mortality[109, 110]. Apparently, the measurement combining neutrophil and lymphocyte counts adds significantly to the prognostic power of neutrophils or lymphocytes alone.

Table 3. Mortality risk predicted by total WBC and WBC differential in ACS patients categorized by follow-up time.

Study characteristics			WBC		Monocytes		Neutrophils		Lymphocytes		Neutrophil to Lymphocyte Ratio		Ref
Population	**FU**	**Size (n)**	**Risk [95% CI]**	***p***	**Risk [95% CI]**	***p***	**Risk [95% CI]**	***p***	**Risk [95% CI]**	***p***	**Risk [95% CI]**	***p***	
AMI	< 1	153213	2.3 [2.2 - 2.5]	***									3
AMI	< 1	751	2.3 [1.2 - 4.5]	*									66
AMI	< 1	1016	3.7 [1.6 - 8.7]	**									67
AMI	< 1	115273	2.7 [2.5 - 2.9]	NR									68
AMI	< 1	2863	1.6 [1.1 - 2.5]	**									76
STEMI	< 1	305					4.6 [1.5 - 14.4][a]	*					91
STEMI	< 1	404					2.9 [1.0 - 8.4]	*					92
STEMI + pPCI	< 1	304									1.1 [1.0 - 1.2]	n.s.	109
STEMI	< 1	522									3.8 [1.7 - 8.3]	**	100
ACS	< 1	2833									2.0 [1.2 - 3.6]	*	103
STEMI	< 1	636									2.4 [1.3 - 4.4]	n.s.	110
STEMI + PCI	< 1	538									15.8 [1.6 - 154]	*	104
ACS	1-6	2833									3.9 [3.2 - 8.1]	***	103
STEMI	> 6-36	470	2.5 [1.3 - 4.9]	NR	1.8 [1.0 - 3.4]	NR	2.2 [1.2 - 4.3]	NR	0.4 [0.2 - 1.0]	NR	4.2 [1.7 - 10.2]	**	74
AMI	> 6-36	1016	2.9 [1.2 - 7.1]	*									67
ACS + PCI	> 6-36	4329	1.1 [1.0 - 1.1][b]	***									70
pPCI	> 6-36	958	1.1 [1.0 - 1.2][b,d]	NR									71

AMI	> 6-36	447	NR	n.s.									81
NSTE ACS	> 6-36	1315	1.5 [1.1 - 2.0]	*									72
MI	> 6-36	2047					2.3 [1.8 - 2.8]	***					92
STEMI + pPCI	> 6-36	304									1.1 [1.1 - 1.2]	n.s.	109
STEMI + pPCI	> 6-36	210									2.7 [1.0 - 7.2]	*	106
STEMI + PCI	> 6-36	325									3.1 [1.1 - 8.6]	*	110
NSTEMI	> 36	619	1.0 [0.9 - 1.2]	n.s.	0.1 [0.0 - 1.1]	n.s.	1.0 [0.9 - 1.2]	n.s.	0.6 [0.2 - 1.7]	n.s.	1.1 [1.0 - 1.1]	**	83
STEMI	> 36	458	1.6 [1.1 - 2.3]	**									73
NSTE-ACS	> 36	476	2.0 [1.4 - 2.7]	***									73
AMI	> 36	144	1.3 [NR]	n.s.									78
STEMI + PCI	> 36	1377							2.4 [1.3 - 4.7][c]	***			95
STEMI + PCI	> 36	538									2.2 [1.0 - 4.8]	*	104

The risk of mortality in ACS patients is categorized by follow-up time. The risk can be relative risk, odds ratio or hazard rate. Unless otherwise stated, the indicated risk regards the risk of patients in the group with the highest cell count compared to patients with low cell counts. FU indicates follow-up time in months.
[a]Risk regarding cell percentage.
[b]Risk increase per 1 x 10e9 cells per liter of blood.
[c]Lowest tertile or quartile compared to highest.
[d]The presented risk score is not significant for both 1 and 2.6 years of follow-up.
The level of significance in multivariate analyses is indicated by * p<0.05; ** p<0.01; *** p<0.001; n.s. indicates not significant. NR indicates not reported.

Table 4. Cardiovascular events predicted by total WBC and WBC differential in ACS patients categorized by follow-up time.

Study characteristics			WBC		Monocytes		Neutrophils		Lymphocytes		Neutrophil to Lymphocyte Ratio		Ref
Population	**FU**	**Size (n)**	**Risk [95% CI]**	***p***	**Risk [95% CI]**	***p***	**Risk [95% CI]**	***p***	**Risk [95% CI]**	***p***	**Risk [95% CI]**	***p***	
AMI	< 1	1016	2.0 [1.1 - 3.6]	*									67
pPCI	< 1	80	1.3 [1.0 - 1.5][b]	*									69
ACS / nSTEMI	< 1	352	2.5 [1.4 - 5.7]	n.s.									80
AMI	< 1	177	1.0 [1.0 - 1.1]	n.s.									82
nSTE ACS	< 1	160					6.5 [1.6 - 27.2]	*					90
AMI + pPCI	< 1	440									1.3 [1.2 - 1.5]	*	99
STEMI + pPCI	< 1	304									1.1 [1.0 - 1.6]	n.s.	109
STEMI	< 1	682									1.2 [1.0 - 1.3]	***	101
STEMI	< 1	101									3.6 [1.2 - 10.7]	*	102
STEMI + pPCI	< 1	170									1.2 [1.0 - 1.3]	*	105
ACS	> 6 - 36[c]	2661	0.9 [0.9 - 1.0]	n.s.			1.0 [0.8 - 1.0]	n.s.	0.9 [0.8 - 1.0]	n.s.			84
STEMI +PCI	> 6 - 36	331	2.4 [1.4 - 4.3]	**							3.8 [2.2 - 6.7]	***	75
MI	> 6 - 36	107	NR	n.s.									78
MI	> 6 - 36	64	0.4 [0.2 - 2.1]	n.s.									79
STEMI	> 6 - 36	100			3.6 [1.2 - 10.8][d]	*							85
ACS	> 6 - 36	166							1.2 [1.1 - 1.3][a]	**			96
STEMI + pPCI	> 6 - 36	304									1.1 [1.1 - 1.2]	n.s.	109

STEMI + pPCI	> 6 - 36	326			3.8 [1.1 - 12.6]	*	107
STEMI	> 36	1287	1.3 [1.2 - 1.3]	***			94
STEMI	> 36	682			1.3 [1.1 - 1.3]	***	101

The risk of major adverse cardiovascular events (MACE) in ACS patients is ordered by follow-up time. The risk can be relative risk, odds ratio or hazard rate. Unless otherwise stated, the indicated risk regards patients in the group with the highest cell count compared to patients with low cell counts. FU indicates follow-up time in months.

[a]Risk increase regarding $CD28^{null}$ CD4 T cell percentage.

[b]Risk increase per 1 x 10e9 cells per liter of blood.

[c]The presented risk is for all cell types also not significant for in-hospital MACE.

[d]Total monocytes, $CD14^{++}CD16^{-}$ and $CD14^{++}CD16^{+}$, but not $CD14^{+}CD16^{++}$ monocytes, were predictive when measured at post MI day 2.

The level of significance in multivariate analyses is indicated by * $p<0.05$; ** $p<0.01$; *** $p<0.001$; n.s. indicates not significant. NR indicates not reported.

SUMMARY AND FUTURE PERSPECTIVES

Adequate risk assessment for patients with stable and unstable CAD is crucial to prevent recurrent events. Total circulating WBCs and WBC subtypes are strongly associated with risk of recurrent adverse events independently of many risk factors, both during hospitalization and up to 10 years of follow-up. Overall, elevated NLR appears to be most consistently associated with adverse outcomes in CAD and ACS patients. In comparison to the WBC count, four out of eight studies found that the prognostic value of NLR was superior[37, 38, 40, 83]. The remaining four studies showed comparable prognostic power for NLR and WBC count[31, 33, 74, 75]. Moreover, although limited, there is evidence that the NLR adds significantly to the prognosis assessed by existing prediction models such as the GRACE[83, 102, 103] and TIMI[107] risk scores. Despite the fact that it should be thoroughly established that NLR has added value in existing risk prediction models like the TIMI and GRACE risk score[111, 112], clinical implementation of NLR can be reached relatively quickly. The NLR is an inexpensive marker and can easily be calculated from the white blood cell differentiation that is routinely analyzed in every hospital. Of note, several factors may influence the prognostic value of NLR. For instance, the NLR is higher in women below 50 years of age compared to age matched man, while in postmenopausal women NLR is lower compared to age matched men[113]. Besides, ethnical differences may also influence the white blood cell profile. The NLR is lower in African-Americans as compared to other ethnicities, including Caucasian, Asian and Hispanic[114-116]. Therefore, a tailored cut-off value of the NLR for age, sex and ethnicity might lead to a more accurate risk prediction. Caution should be taken regarding the limitations of circulating cells as biomarker. Numbers of circulating inflammatory cells, or their subtypes, do not necessarily reflect the amount or phenotype of these cells in the plaque[48, 49, 85, 87]. Nevertheless, implementation of the NLR has a great potential to identify high risk CAD and ACS patients, thereby offering opportunities for intervention and prevention of recurrent cardiovascular events.

2

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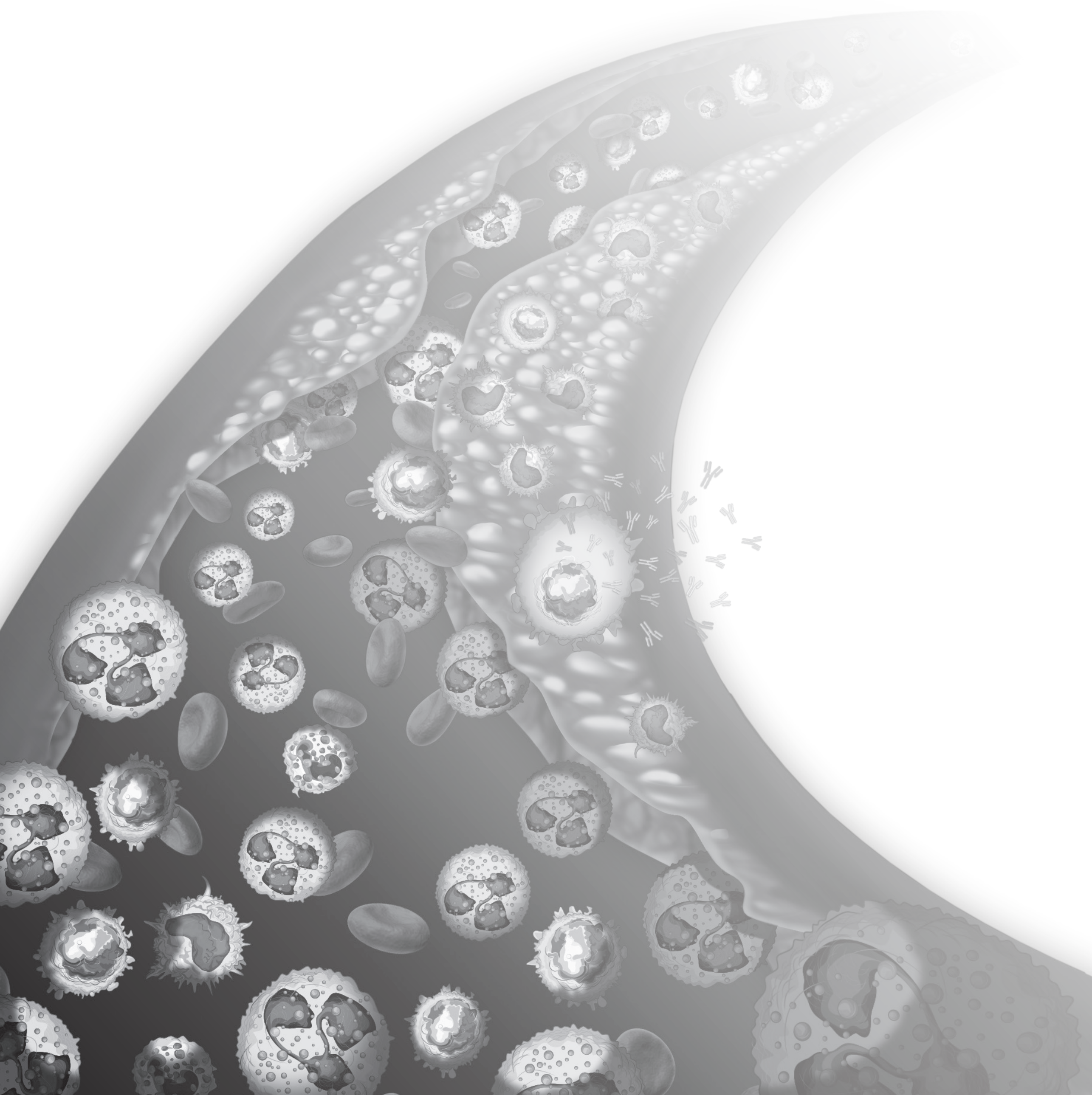

CHAPTER

3

Circulating $CD14^+CD16^-$ classical monocytes do not associate with a vulnerable plaque phenotype, and do not predict secondary events in severe atherosclerotic patients

Submitted

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ABSTRACT

Aim

Mouse studies have established distinct monocyte subtypes that participate in the process of atherosclerotic lesion formation. The pro-inflammatory Ly6C^{high} monocyte subtype actively contributes to murine plaque progression and destabilization. Also in humans, different peripheral monocyte subtypes have been identified, of which the CD14^{+}CD16^{-} classical monocyte is suggested to display similar pro-atherosclerotic properties as the murine Ly6C^{high} subtype. We aimed to investigate if circulating CD14^{+}CD16^{-} classical monocytes associate with characteristics of a vulnerable carotid atherosclerotic plaque and if they associate with the risk of secondary adverse manifestations of atherosclerotic disease.

Methods and results

We enrolled 175 carotid endarterectomy patients of the Athero-Express biobank in our study. Just prior to surgical procedure, blood was collected and peripheral blood mononuclear cells were isolated. Characterization of monocyte subsets was performed by flow cytometry. Plaque characteristics were semi-quantitatively scored for the presence of fat, collagen, intraplaque hemorrhage and calcification. Vessel density, smooth muscle cells and macrophages were assessed quantitatively on a continuous scale. All features of a vulnerable plaque phenotype, including low amounts of collagen and smooth muscle cells, and increased fat content, vessel density, intraplaque hemorrhage and plaque macrophages were not significantly associated with differential levels of peripheral classical CD14^{+}CD16^{-} monocytes or other monocyte subsets. Using Cox regression models to evaluate the prognostic value of circulating monocyte subtypes, we found that total counts of peripheral monocytes, as well as CD14^{+}CD16^{-} classical and other monocyte subtypes were not associated with the risk of secondary cardiovascular events during three-year follow-up.

Conclusion

Circulating classical CD14^{+}CD16^{-} monocytes do not associate with specific vulnerable plaque characteristics. In addition, they do not predict secondary adverse manifestations. This suggests that in patients with established carotid artery disease, the circulating monocytes do not reflect plaque characteristics and have no value in identifying patients at risk for future cardiovascular events.

INTRODUCTION

The link between monocytes and atherosclerosis is well established. Plaque macrophages primarily derive from infiltrating circulating monocytes in early atherosclerosis, while they may also arise from local macrophage proliferation during plaque progression[1]. Macrophages play a pivotal role in plaque initiation, development and progression by phagocytosis of (oxidized) LDL and the secretion of inflammatory cytokines[2-4]. In addition, dying foam cells form a major part of the necrotic core, which is known to further enhance the inflammatory response.

Experimental studies in mice show that circulating inflammatory Ly6C^{high} monocytes, the suggested equivalent of human classical CD14^{+}CD16^{-} monocytes[4, 5], contribute to the process of atherosclerotic lesion formation. Ly6C^{high} monocytes attach to the damaged endothelium, and upon arrest, they migrate through the endothelial layer into the plaque, primarily involving the chemokine receptor CCR2[6-8]. The Ly6C^{high} monocytes can subsequently differentiate into inflammatory M1 plaque macrophages, which are associated with a vulnerable plaque phenotype[6, 9-11]. Ly6C^{low} monocytes patrol the vascular endothelium, and upon plaque infiltration may be involved in scavenging of lipids and dead cells[9, 12]. The association between Ly6C^{low} monocytes and atherosclerotic plaque development remains inconclusive. On the one hand, low levels of Ly6C^{low} monocytes were associated with increased atherosclerotic lesion size[13, 14], but showed no effect on atherosclerosis development on the other hand[15].

Since classical CD14^{+}CD16^{-} monocytes contribute to plaque progression and vulnerability they may hold value in predicting patients at risk for CVD. However, existing data regarding the prognostic value of circulating monocytes remain inconclusive. Some studies indicate that monocytes have no prognostic value[16-19], whereas other studies suggest that high monocyte counts associate with increased risk for recurrent CVD events or mortality[20-23]. This may partly be explained by the fact that different monocyte subsets exist with distinct plaque modifying properties[3, 4, 24, 25]. Indeed, high levels of classical and intermediate monocytes have been associated with increased risk for adverse manifestations in CVD patients[26-28]. Moreover, classical monocytes are linked to the presence and severity of atherosclerosis[29].

Thus, although it is evident that monocyte subsets play a divergent role in CVD, the question whether circulating monocyte subsets in humans do relate to plaque characteristics has to the best of our knowledge not been comprehensively addressed before. Moreover, prospective cohort studies examining the prognostic value of monocyte subsets in CVD patients are limited. We took advantage of our unique biobank enclosing elaborate information of both plaque composition and occurrence of secondary adverse manifestations during three-years follow-up. We investigated whether classical monocytes associate with atherosclerotic plaque characteristics and if they associate with the risk of secondary CVD events during three-years follow-up.

MATERIALS AND METHODS

Patient selection

The current study comprises 175 patients in a subset from the Athero-Express biobank[30]. All patients undergoing carotid endarterectomy between 2009 and 2011 were included based on availability of peripheral blood mononuclear cells (PBMCs). In addition to the standard procedure, including an extensive patient questionnaire and detailed histological plaque characterization, PBMCs were isolated from blood that was drawn preoperatively. Isolated PBMCs were stored in liquid nitrogen until further analyses were performed. Patient follow-up was executed for three years or until the occurrence of a secondary cardiovascular event (cardiovascular death, stroke, myocardial infarction, coronary intervention, peripheral intervention (including amputation)). All events were validated using health records kept by general practitioners. The study protocol conforms to the Declaration of Helsinki and has been approved by the Institution's ethics committee on research on humans. All patients provided written informed consent.

PBMC isolation

Twenty mL blood was collected in Li-Heparin tubes, and a complete blood count profile was determined by a general hematology cell counter (Cell Dyn 1800 Abbott, Minesota, USA). PBMCs were isolated by Ficoll gradient fractionation and stored in liquid nitrogen, as described previously[31], until further analyses were performed.

Flow cytometry

After gentle thawing from liquid nitrogen, the PBMCs were washed with RPMI 1640 ((61870010, Gibco Carlsbad, CA, USA) supplemented with GlutaMax (room temperature (RT)) containing 25nM HEPES, 1% penicillin/streptomycin and 2% Fetal Bovine Serum (FBS) (10270-106, Gibco, Carlsbad, CA, USA)). Cells were kept on ice during the whole procedure, unless stated otherwise. To obtain single cell suspensions, PBMCs were gently filtered over a 40µm cell strainer (542040, Greiner bio-one), washed with RPMI again and centrifuged at 350*g* for 5 min at 4°C. Subsequently, the cells were washed with cold PBS supplemented with 2% FBS and 20mM EDTA, centrifuged at 350*g* for 5 min at 4°C and resuspended in cold PBS with 1% BSA. Then, the cells were incubated with fluorescent antibodies **(Supplemental table 1)** for 30 min at RT in the dark, washed with cold PBS and centrifuged at 350*g* for 5 min at 4°C. To be able to exclude dead cells, the cells were incubated with fixable viability, eFluor506 (eBioscience) for 30 min at RT in the dark. Then, cells were washed with cold PBS with 1% BSA and measured on the flow cytometer (Gallios, Beckman Coulter, Fullerton, CA, USA). Gating analyses of the flow cytometry data were performed using Kaluza 1.3 software. We selected viable $CD11B^{+}CD115^{+}$ monocytes, and based on the combination of forward - sideward scatter and CD14 and CD16 expression, we identified classical ($CD14^{+}CD16^{-}$), intermediate ($CD14^{+}CD16^{+}$) and non-classical ($CD14^{-}CD16^{+}$) monocytes **(Supplemental figure 1)**. In addition, we measured the surface expression of CCR2, CXC3CR1 and CD11B and CD31 at the different monocyte subsets.

Atherosclerotic plaque characterization

We performed a comprehensive analysis of the atherosclerotic plaque composition, as described previously[30, 32, 33]. This analysis includes a semi-quantitative assessment of fat deposition, collagen deposition, intraplaque hemorrhage and calcification. In addition, vessel density, smooth muscle cells (SMC's), and plaque macrophages were quantitatively determined on a continuous scale. In short, the relative lipid core size was estimated visually as a percentage of total plaque area with the use of H&E and picrosirius red stains, and divided into (1) ≤10% and (2) >10% of the total plaque area. Collagen deposition (picrosirius red) was divided into (1) no or minor staining along part of the luminal border of the plaque; (2) moderate or heavy staining along the entire luminal border. Intraplaque hemorrhage (H&E and fibrin staining) was defined as the composite of plaque bleeding at the luminal side of the plaque as a result of plaque disruption and intraplaque hemorrhage, and scored as (1) absent or (2) present. Calcification (hematoxylin and eosin, H&E) was scored as (1) no or minor staining along part of the luminal border of the plaque or a few scattered spots within the lesion; (2) moderate or heavy staining along the entire luminal border or evident parts within the lesion. For quantitative assessments, plaque vessel density (CD34) was defined as the average number of vessels of 3 hotspots within every single plaque. SMC's (α-actin) and macrophages (CD68) were stained in three representative fields and quantified using computerized analyses (AnalySiS version 3.2, Soft Imaging GmbH, Munster, Germany) as percentage of plaque area[30, 32-34].

Statistical analyses

Normally distributed continuous variables were indicated as mean ± SD and compared by Student's *t* tests or a one-way ANOVA. Non-normal distributed data were presented as median [interquartile range] and compared by Mann-Whitney *U* tests or Kruskal-Wallis tests. Categorical variables were indicated as percentage and compared by Chi-squared or Fisher's exact tests. Univariable Cox proportional hazard models were used to study the association between monocyte subsets and the occurrence of adverse cardiovascular events over time. To obtain a normal distribution, we performed a log transformation on the values of smooth muscle cells and plaque macrophages. To analyze the correlation between peripheral monocytes and vessel density, smooth muscle cells or plaque macrophages, linear regression models were used. Data management and statistical analyses were performed with RStudio[35] and the R software package[36] (version 3.2.0., Vienna, Austria). *P*-values <0.05 were considered as significant. GraphPad Prism 7.02 software (La Jolla, CA, USA) was used to produce the graphs.

RESULTS

Patient characteristics

The current subcohort of the Athero-Express biobank comprised 175 patients with severe carotid atherosclerosis, undergoing carotid endarterectomy. During three years of follow-up, 55 of the 175 patients (31%) experienced a secondary adverse cardiovascular event. The median age of the patients was 71 years and 63.4% was male. Other risk factors, clinical manifestations, medication use and laboratory parameters are depicted in **table 1** for all patients, and also stratified by occurrence of secondary adverse CVD events. Since the clinical manifestations (asymptomatic and symptomatic) were comparable among patients regarding circulating monocyte subset counts (**Table 2**), and also between patients with and without secondary adverse events, we included all patients undergoing carotid endarterectomy in the current analyses. In addition, because the circulating monocyte subtypes did not differ between males and females (**Table 2**), we chose to evaluate their association with plaque characteristics and secondary CVD events in males and females together.

Circulating monocyte subsets

In the current study, patients have median monocyte values of 10.8% [interquartile range (IQR) 9.3, 12.3]. Absolute monocytes counts were approximately 800 cells/µL [IQR 600, 1000]. On average, classical $CD14^{+}CD16^{-}$ monocytes represented 89.2%, intermediate $CD14^{+}CD16^{+}$ monocytes 4.8%, and nonclassical $CD14^{-}CD16^{+}$ monocytes 5.9% of the total monocyte pool (**Supplemental figure 2**), which corroborates previous studies[24, 25].

To explore which patient characteristics associated with the number of circulating monocytes, monocyte counts were presented for the presence or absence of different patient characteristics (**Table 2**). The classical monocyte counts negatively correlated with HDL levels (p=0.03; table 2), and positively correlated with hsCRP levels (p=0.02). In addition, classical monocyte counts were higher in patients taking calcium antagonists (p=0.04). Peripheral nonclassical monocytes were lower in current smokers than nonsmokers (p=0.003), higher in patients on beta blocker medication (p=0.008), and increased upon increasing BMI (p=0.02). No statistically significant associations with patient characteristics were identified for circulating intermediate monocyte counts (**Table 2**).

Circulating classical monocytes do not associate with plaque characteristics

To address the question if classical monocytes associate with a more vulnerable plaque phenotype, we studied the number of monocytes in relation to atherosclerotic plaque characteristics. However, we observed no differences in the numbers of classical $CD14^{+}CD16^{-}$ monocytes, nor intermediate $CD14^{+}CD16^{+}$ or nonclassical $CD14^{-}CD16^{+}$ monocytes in relation to fat deposition, collagen deposition, intraplaque hemorrhage or calcification (**Figure 1**). In addition, we found no association between circulating monocytes and high plaque vessel density (**Figure 2**) or decreased numbers of smooth muscle cells (**Figure 3**), which have been linked to a more vulnerable plaque phenotype.

Table 1. Patients characteristics are depicted for all patients and stratified by occurrence of secondary adverse CVD events during follow-up.

	Overall (n=175)	No events (n=120)	Events (n=55)	*p*
Risk factors				
Age (years)	71.0 [63.0, 78.0]	71.0 [63.0, 78.0]	73.0 [63.5, 79.0]	0.50
Sex (% male)	111 (63.4)	74 (61.7)	37 (67.3)	0.59
BMI	26.0 [23.6, 29.1]	26.1 [23.5, 29.3]	25.7 [23.6, 28.6]	0.74
Current smoker (%)	62 (35.6)	42 (35.3)	20 (36.4)	1
Contralateral stenosis (%)	75 (48.4)	47 (44.8)	28 (56.0)	0.25
Diabetes mellitus (%)	45 (25.7)	32 (26.7)	13 (23.6)	0.81
Hypertension (%)	152 (86.9)	102 (85.0)	50 (90.9)	0.41
Hypercholesterolemia (%)	107 (71.8)	74 (70.5)	33 (75.0)	0.72
CAD history (%)	63 (36.0)	42 (35.0)	21 (38.2)	0.81
PAOD history (%)	32 (18.3)	16 (13.3)	16 (29.1)	**0.02**
Clinical manifestations				0.38
Asymptomatic (%)	27 (15.4)	15 (12.5)	12 (21.8)	
Amaurosis fugax (%)	29 (16.6)	19 (15.8)	10 (18.2)	
Stroke (%)	42 (24.0)	31 (25.8)	11 (20.0)	
TIA (%)	77 (44.0)	55 (45.8)	22 (40.0)	
Medication				
Statins (%)	142 (81.1)	96 (80.0)	46 (83.6)	0.72
Diuretics (%)	79 (45.1)	52 (43.3)	27 (49.1)	0.58
Beta blockers (%)	85 (48.6)	56 (46.7)	29 (52.7)	0.56
Calcium antagonists (%)	43 (24.6)	26 (21.7)	17 (30.9)	0.26
ACE-inhibitors (%)	58 (33.1)	37 (30.8)	21 (38.2)	0.43
Angiotensin-2 antagonists (%)	46 (26.4)	30 (25.2)	16 (29.1)	0.72
Anticoagulants (%)	11 (6.3)	5 (4.2)	6 (10.9)	0.10
Aspirin (%)	142 (81.6)	100 (84.0)	42 (76.4)	0.32
Antiplatelets (%)	153 (87.9)	106 (89.1)	47 (85.5)	0.67
Laboratory parameters				
Cholesterol (mmol/L)	4.0 [3.3, 4.8]	4.0 [3.3, 4.8]	4.0 [3.3, 4.7]	0.67
HDL (mmol/L)	1.0 [0.9, 1.2]	1.0 [0.9, 1.3]	1.0 [0.9, 1.2]	0.14
LDL (mmol/L)	2.1 [1.6, 2.8]	2.0 [1.6, 2.7]	2.2 [1.8, 2.9]	0.16
hsCRP (µg/mL)	1.7 [0.7, 3.6]	1.7 [0.7, 3.1]	2.0 [0.7, 4.8]	0.49
GFR MDRD (mL/min)	69.1 ± 18.4	70.1 ± 17.6	66.9 ± 20.1	0.30

Values are presented as mean ± SD for normal distributions, number of patients (frequency in percentage) for categorical variables, and median [interquartile range] for non-normal distributions. *P*-values are calculated using Students' *t* tests, Chi-squared tests, and Mann-Whitney *U* tests, respectively. BMI indicates body-mass index; Contralateral stenosis, 50-100% stenosis of the contralateral carotid artery; CAD history, history of Coronary Artery Disease; PAOD, peripheral artery occlusive disease; Amaurosis fugax, temporary loss of vision; ACE-inhibitors, angiotensin-converting-enzyme inhibitor; HDL, high-density lipoprotein; LDL, low-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; GFR MDRD, Glomerular filtration rate according to 'Modification of Diet in Renal Disease' formula.

Table 2. The monocyte subset counts are depicted in relation to the presence or absence of patient characteristics.

	Classical monocytes (cells/μL)		**Intermediate monocytes (cells/μL)**		**Nonclassical monocytes (cells/μL)**	
Categorial variables	**Risk factor present**		**Risk factor present**		**Risk factor present**	
Risk factors	**Yes**	**No**	**yes**	**no**	**yes**	**no**
Male sex	688 [497, 837]	656 [537, 770]	33 [18, 45]	31 [19, 50]	42 [25, 61]	38 [27, 58]
Current smoker	708 [554, 929]	644 [481, 810]	26 [18, 42]	33 [18, 49]	**35 [19, 46]**	**43 [28, 65]** **
Contralateral stenosis	663 [491, 828]	656 [481, 802]	28 [19, 46]	32 [17, 47]	41 [26, 56]	39 [24, 63]
Diabetes mellitus	722 [602, 1030]	656 [494, 799]	35 [24, 55]	30 [17, 46]	45 [32, 62]	39 [25, 60]
Hypertension	669 [505, 821]	631 [552, 848]	31 [17, 47]	35 [25, 47]	42 [25, 61]	32 [26, 45]
Hypercholesterolemia	672 [537, 825]	647 [475, 808]	32 [17, 45]	37 [22, 50]	43 [27, 60]	37 [21, 63]
CAD history	698 [557, 820]	656 [473, 832]	36 [17, 50]	28 [19, 44]	47 [27, 61]	37 [25, 60]
PAOD history	702 [554, 810]	659 [505, 830]	34 [17, 57]	32 [19, 47]	53 [28, 61]	39 [25, 60]
Medication use	**Yes**	**No**	**Yes**	**No**	**Yes**	**No**
Statins	679 [537, 839]	625 [434, 765]	33 [19, 48]	29 [15, 42]	42 [26, 62]	31 [23, 44]
Diuretics	663 [472, 772]	669 [543, 931]	29 [18, 44]	33 [19, 50]	40 [26, 60]	42 [24, 61]
Beta blockers	657 [503, 826]	700 [536, 826]	31 [18, 46]	32 [18, 48]	**46 [31, 63]**	**36 [22, 51]** **
Calcium antagonists	733 [605, 940]	**644 [471, 812]** *	36 [16, 68]	31 [19, 44]	42 [34, 63]	40 [25, 60]
ACE inhibitors	663 [475, 760]	670 [520, 836]	33 [22, 50]	31 [17, 46]	41 [25, 64]	39 [25, 60]
Angiotensin-2 antagonists	719 [530, 883]	649 [508, 820]	30 [16, 47]	32 [18, 48]	42 [31, 60]	39 [24, 61]
Anticoagulants	755 [640, 839]	663 [494, 822]	24 [19, 37]	32 [18, 48]	45 [40, 70]	39 [25, 60]
Aspirins	659 [487, 805]	756 [556, 867]	32 [17, 48]	31 [19, 42]	39 [25, 60]	45 [29, 78]
Antiplatelets	666 [497, 825]	684 [589, 832]	32 [18, 47]	28 [19, 59]	39 [25, 60]	56 [37, 86]

Clinical manifestations			
asymptomatic	677 [472, 909]	30 [21, 62]	47 [27, 60]
Amaurosis fugax	625 [463, 774]	31 [21, 39]	39 [24, 64]
stroke	657 [530, 919]	34 [19, 51]	43 [31, 67]
TIA	672 [549, 799]	30 [14, 44]	40 [23, 57]
Continuous variables			
Risk factors			
Age (per 1 year increase)	-2.16 [-6.47, 2.16]	0.16 [-0.26, 0.58]	0.36 [-0.1, 0.81]
BMI (per unit increase)	2.25 [-6.74, 11.24]	0.21 [-0.67, 1.09]	**1.12 [0.17, 2.06]** *
Laboratory parameters			
Cholesterol (per mmol/L increase)	-38.28 [-78.35, 1.79]	1.01 [-3.15, 5.17]	-3.98 [-8.42, 0.47]
HDL (per mmol/L increase)	**-167.82 [-320.22, -15.42]** *	1.25 [-14.64, 17.15]	-15.07 [-32.03, 1.89]
LDL (per mmol/L increase)	-24.01 [-72.61, 24.59]	3.99 [-1.17, 9.15]	-1.67 [-7.23, 3.9]
hsCRP (per µg/mL increase)	**0.65 [0.09, 1.2]** *	0.05 [-0.01, 0.1]	0.03 [-0.03, 0.09]
GFR MDRD (per mL/min increase)	2.06 [-0.32, 4.44]	-0.1 [-0.33, 0.13]	-0.04 [-0.29, 0.22]

Values are presented as median [interquartile range] for categorical variables, and beta [95% confidence interval] for continuous variables. BMI indicates body-mass index; CAD history, history of Coronary Artery Disease; PAOD, peripheral artery occlusive disease; Amaurosis fugax, temporary loss of vision; ACE-inhibitors, angiotensin-converting-enzyme inhibitor; Contralateral stenosis, 50-100% stenosis of the contralateral carotid artery; HDL, high-density lipoprotein; LDL, low-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; GFR MDRD, Glomerular filtration rate according to 'Modification of Diet in Renal Disease' formula. *P*-values were calculated using a one-way ANOVA or Kruskal-Wallis test for categorical variables where appropriate, and linear regression models for continuous variables. * indicates $p<0.05$ and ** $p<0.01$.

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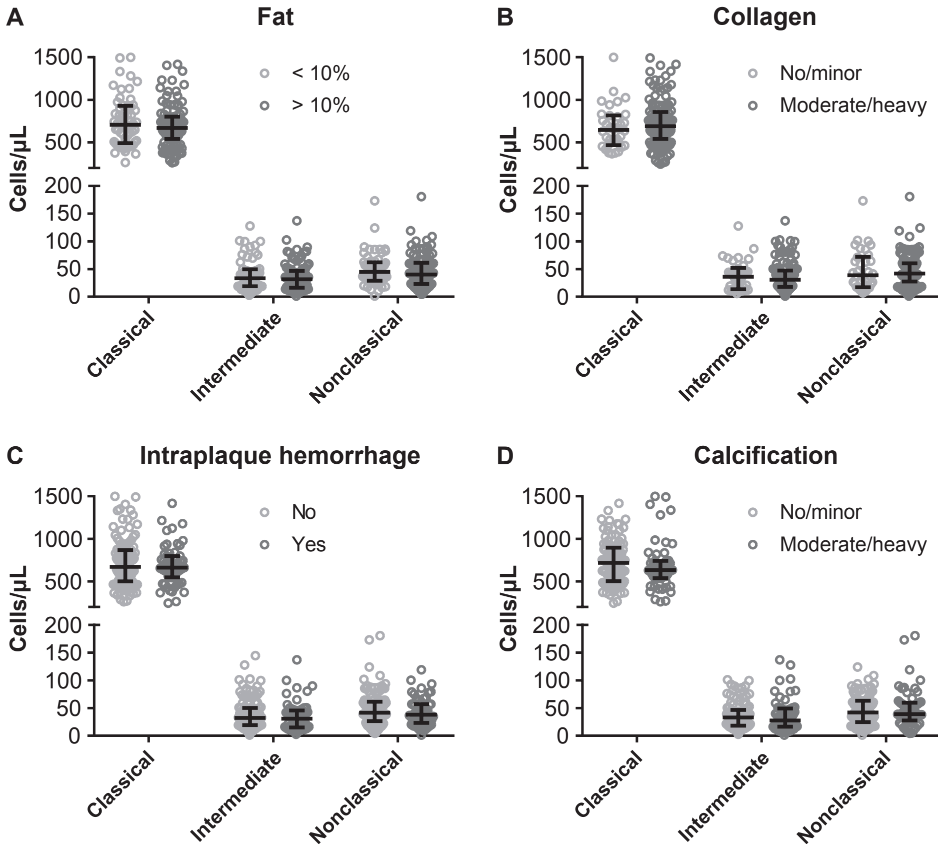

Figure 1. Classical monocyte subsets are not associated with vulnerable plaque characteristics. Absolute cell numbers of classical, intermediate and nonclassical monocytes are not associated with fat deposition (A) presence of collagen (B), intraplaque hemorrhage (C) and calcification (D). For each monocyte subtype, median and interquartile range is indicated. Statistical significance was considered as $p<0.05$, calculated with the Mann-Whitney *U* test (n=166).

Since plaque macrophages are thought to derive –at least partly– from circulating monocytes, we studied the association between circulating monocytes and plaque macrophages. Absolute counts of total monocytes or classical monocytes were not associated with macrophage plaque content (R^2=0.01, p=0.24) and (R^2=0.00, p=0.46), respectively (**Figure 4A-B**). On the other hand, statistically significant, though weak negative correlations were observed for absolute counts of intermediate (R^2=0.04, p=0.02) and nonclassical (R^2=0.06, p<0.01) monocytes with plaque macrophages (**Figure 4C-D**).
Because monocyte migration, activation and plaque infiltration has been described to be crucially dependent on the presence of surface receptors like CCR2, CD11B and CXCR3 in mouse studies[6-8], we also analyzed the association between monocyte surface expression of these receptors and the number of plaque macrophages. However, we found no

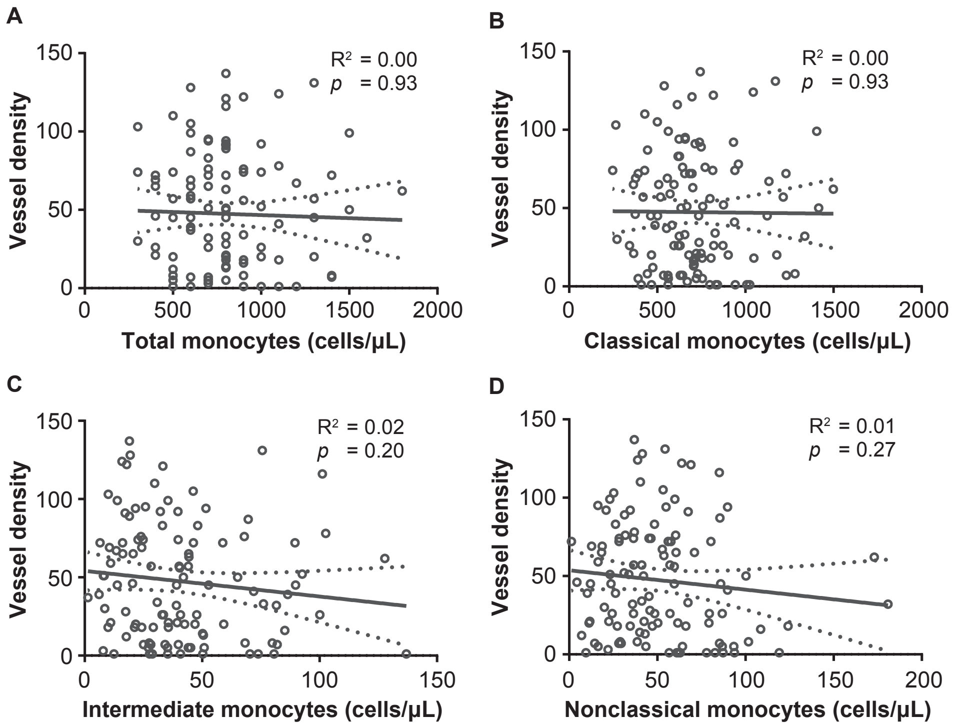

Figure 2. Classical monocyte subsets are not associated with intraplaque vessel density. Total (A), classical (B), intermediate (C), and nonclassical monocytes (D) show no significant association with intraplaque vessel density. Solid line indicates the slope and dashed lines indicate the 95% confidence interval of the slope. Statistical significance was considered as $p<0.05$, calculated using linear regression analyses (n=118).

3

association between monocyte surface expression of CCR2, CD11B, and CXCR3 and plaque macrophages (**Supplemental table 2**).

Circulating monocytes do not associate with secondary cardiovascular endpoints

We then investigated whether the sheer number of circulating monocyte subsets differed between patients who experienced a secondary cardiovascular event during follow-up as compared to patients who did not. Classical monocyte numbers were comparable among patients with secondary events as compared to patients without (median 670 cells/µL [IQR 475, 805] vs. 668 [IQR 536, 864], p=0.59) as were the numbers of intermediate (28 [IQR 19, 46] vs. 33 [IQR 17, 47], p=0.62) and nonclassical (39 [IQR 25, 60] vs. 40 [IQR 25, 61], p=0.74) monocytes (**Figure 5**).

To investigate if monocyte subsets associated with the risk of secondary adverse manifestations over time, we performed univariable Cox proportional hazard regression analyses. Circulating monocytes were not associated with the risk of secondary events during follow-up in univariable Cox regression analyses (**Supplemental table 3**).

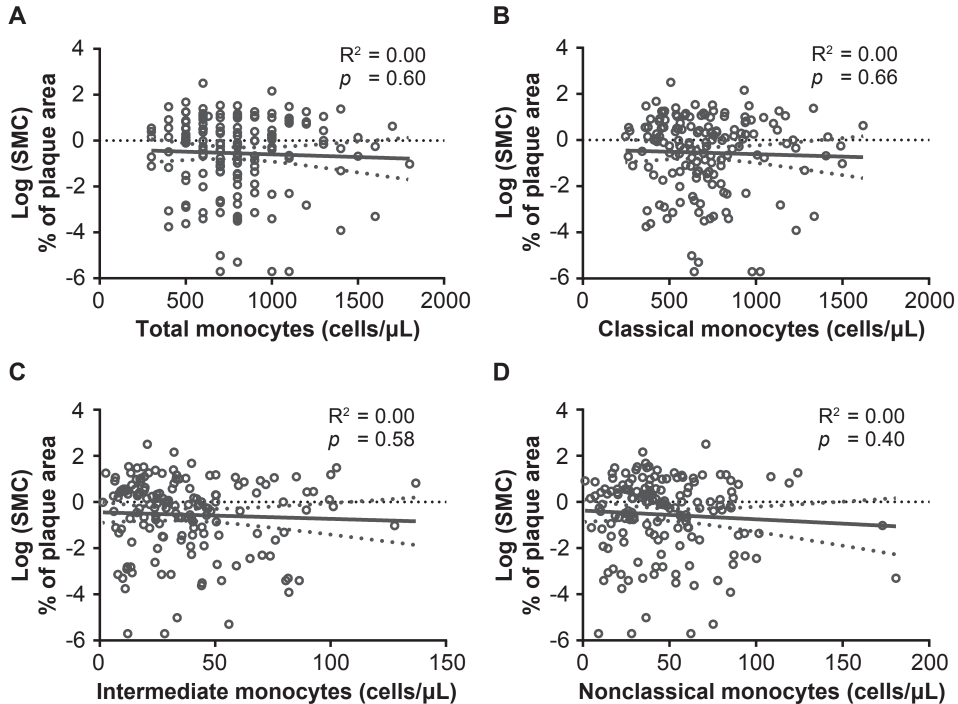

Figure 3. Classical monocyte subsets are not associated with the amount of smooth muscle cells in atherosclerotic plaques. Total (A), classical (B), intermediate (C), and nonclassical monocytes (D) reveal no significant association with relative area of smooth muscle cells in the plaque. Solid line indicates the slope and dashed lines indicate the 95% confidence interval of the slope. Statistical significance was considered as $p<0.05$, calculated using linear regression analyses (n=166).

DISCUSSION

In the current study, we investigated the role of monocyte subsets in association to the plaque phenotype, and in relation to recurrent adverse CVD events in a patient cohort with advanced atherosclerotic disease. We observed no association between classical $CD14^{+}CD16^{-}$ monocytes and plaque characteristics. In addition, we found that monocyte subsets could not prognosticate secondary CVD events during three-year follow-up.
First, we addressed the question if circulating classical monocytes were related to atherosclerotic plaque phenotype. In contrast to their role in experimental mice studies, in the current study in patients with an advanced stage of atherosclerotic disease, classical monocytes were not associated with characteristics of a vulnerable plaque phenotype. In agreement with our study, a recent study using optical coherence tomography also showed no association between classical monocytes and vulnerable plaque characteristics, including thickness of the fibrous cap, arc of lipid core and plaque calcification in UAP and STEMI patients[37]. However, absolute numbers of intermediate monocytes were inversely

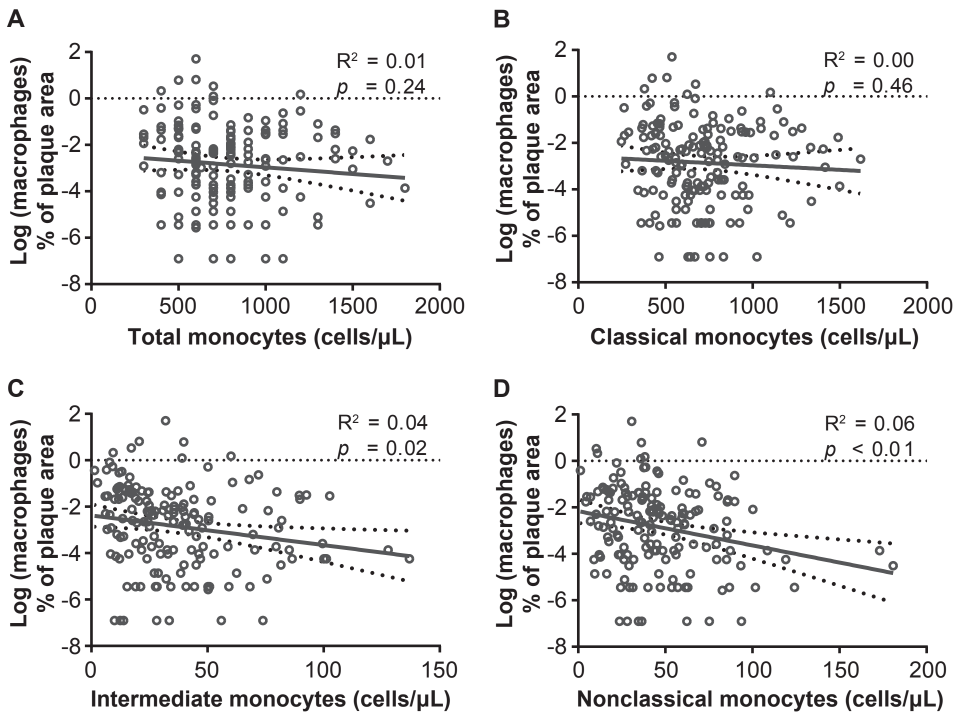

Figure 4. Circulating intermediate and nonclassical monocytes weakly associate with the number of plaque macrophages. The relation between peripheral blood monocyte counts and relative area of plaque $CD68^+$ staining is shown. No correlation was observed between total monocyte count and plaque macrophages (A). Within the monocyte subsets, classical monocytes were not associated (B), whereas intermediate and nonclassical monocytes displayed a weak inverse correlation with plaque macrophages (C-D). Solid line indicates the slope and dashed lines indicate the 95% confidence interval of the slope. Statistical significance was considered as $p<0.05$, calculated using linear regression analyses (n=166).

correlated with thickness of the fibrous cap and positively correlated with arc of lipid core[37]. In another study in patients with carotid and systemic atherosclerosis, researchers used ultrasonography to determine plaque neovascularization. They observed that patients with grade 1 or grade 2 neovascularization had higher levels of classical monocytes (464 or 482 cells/μL, respectively) as compared to patients without intraplaque neovascularization (406 cells/μL)[29]. However, contrasting results were obtained in a study where asymptomatic patient with carotid plaque neovascularization had lower levels of classical monocytes (255 cells/μL) than patients without (310 cells/μL). The discrepancy between these studies and our current study might be explained by the difference in study populations, and the different techniques used for intraplaque vessels. Thus, the exact association between classical monocytes and plaque neovascularization remains to be further investigated[38].

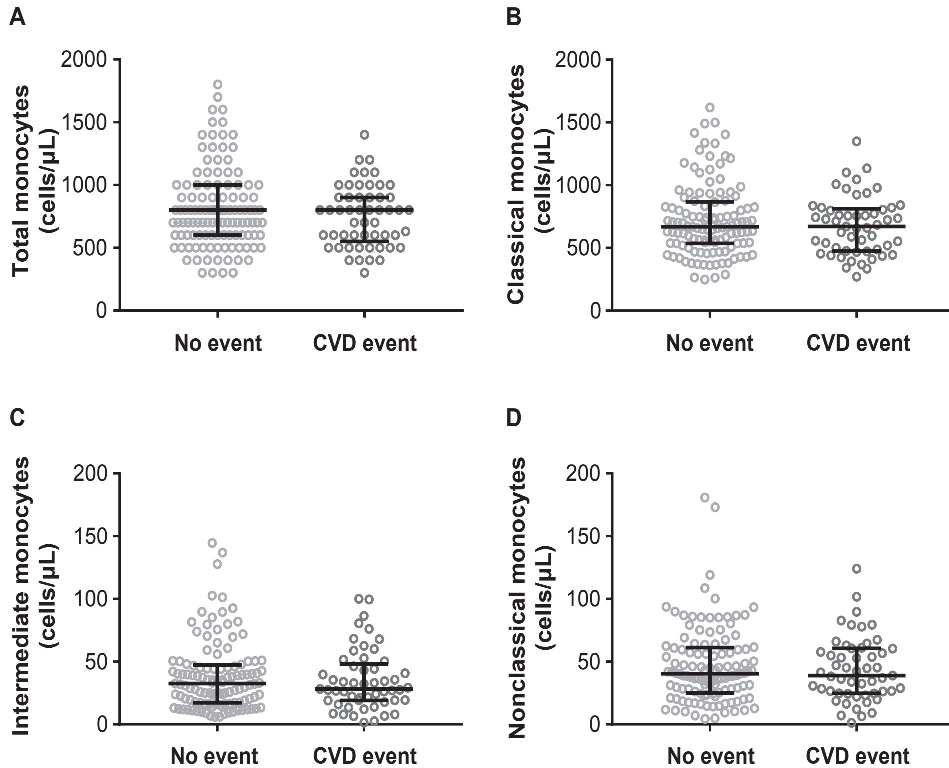

Figure 5. Circulating monocyte subsets do not associate with secondary CVD events. Absolute counts of total monocytes (A) and monocyte subtypes (B-D) are depicted for patients without and with secondary adverse CVD manifestations. Median and interquartile range are indicated. Statistical significance was considered as $p<0.05$, calculated with the Mann-Whitney *U* test (n=175).

In addition, we questioned if monocyte subsets reflect the presence of plaque macrophages. We observed no association between circulating classical $CD14^+CD16^-$ monocytes and plaque macrophages, and a statistically significant, but weak correlation between peripheral intermediate $CD14^+CD16^+$ and nonclassical $CD14^+CD16^-$ monocytes and plaque macrophages, which might indicate that in advanced atherosclerosis the peripheral monocytes only marginally contribute to the intraplaque macrophage pool. This observation may be explained by a combination of factors. First, plaque macrophages encompass different subsets as well, including inflammatory M1 macrophages, immune regulatory M2 macrophages and foam cells. For example, classical $CD14^+CD16^-$ monocytes might correlate better with M1 macrophages than with total plaque macrophages. Secondly, it is well appreciated that the vulnerable shoulder region of the plaque is most densely infiltrated with immune cells. In our study, we assessed macrophage accumulation in the entire plaque as we were not able to clearly distinguish the shoulder area. Third, murine studies have shown that plaque macrophages display signs of plasticity[39, 40] and

local proliferation[1]. Strikingly, local proliferation accounted for approximately 90% of the plaque macrophages in advanced murine plaques[1]. Thus, although monocyte influx is a crucial process in atherogenesis (as displayed by the fact that atherosclerosis is diminished in absence of chemokine receptors that facilitate monocyte trafficking[6, 7, 10, 11, 41, 42]), monocyte recruitment may be less important in more advanced atherosclerotic plaques[43-46]. In line, our patient population presents with advanced atherosclerotic plaques, where monocyte influx might be less pronounced.
We observed no prognostic value for total monocytes, nor for monocyte subsets for the identification of high risk patients. In patients with coronary artery disease (CAD), different studies observed diverse associations regarding the prognostic value of total monocytes, i.e. no risk[16-19] or increased risk[20-23] of CVD events and mortality. Cross-sectional studies have indicated that mainly intermediate monocytes are associated with CVD and plaque progression[24]. However, because of the cross-sectional design of these studies, no straightforward conclusions can be drawn about the prognostic value for the occurrence of adverse cardiovascular events. Cohort studies assessing the prognostic value of specific monocyte subsets are limited, but suggest classical and intermediate monocytes to be predictive of CVD events during follow-up[26-28]. In the current study, we found no differences in monocyte subsets between patients with and without recurrent adverse events. This discrepancy might be explained by the fact that patients in the current study are older, receive pharmaceutical treatment and have more severe atherosclerosis than random older Swedish subjects[28] or patients undergoing elective angiography[26]. In addition, differences in clinical manifestations (i.e. patients undergoing elective angiography[26], STEMI patients[27], or random older Swedish subjects[28]), and follow-up time (2 years in the STEMI patients, up to 9 years in random Swedish subjects), may contribute to the inconsistency between the studies.

Future perspectives

As we investigated the correlation between classical $CD14^{+}CD16^{-}$ monocytes and plaque vulnerability in a cross-sectional manner, it would be interesting to see whether monocyte numbers can predict plaque composition over time. A marker of plaque progression could for example be a useful indicator for intervention in asymptomatic patients. In addition, since monocyte influx is crucially involved in developing plaques, the association between peripheral monocyte subsets and plaque phenotype merits further investigation in developing plaques, rather than advanced atherosclerotic plaques. Recently, technetium-99m labeled PBMCs were tracked *in vivo,* in CVD patients and healthy controls. Interestingly, PBMCs accumulation could be visualized in atherosclerotic plaques[47]. This approach might be used to study monocyte trafficking in CVD patients in order to gain mechanistic insights of monocyte plaque infiltration, to support the identification of vulnerable plaques, or to validate therapeutic interventions that target inflammatory cells in CVD. Regarding the prognostic value of monocyte subsets, it is important that the findings from the current study will be replicated in large standardized cohort studies of CVD patients. Meanwhile, easily available risk markers, such as the NLR[19, 48-51], may be

considered for short term implementation, in order to enhance risk prediction and to prevent hospitalization, secondary events and mortality.
In conclusion, classical monocytes do not associate with a vulnerable plaque phenotype. In addition, we show that circulating monocyte subsets have no prognostic value for secondary CVD manifestations in a population of severe atherosclerotic patients. These results suggest that circulating monocytes do not reflect plaque phenotype and have only limited value in identifying patients at risk for future cardiovascular events.

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Conflict of interest
None declared.

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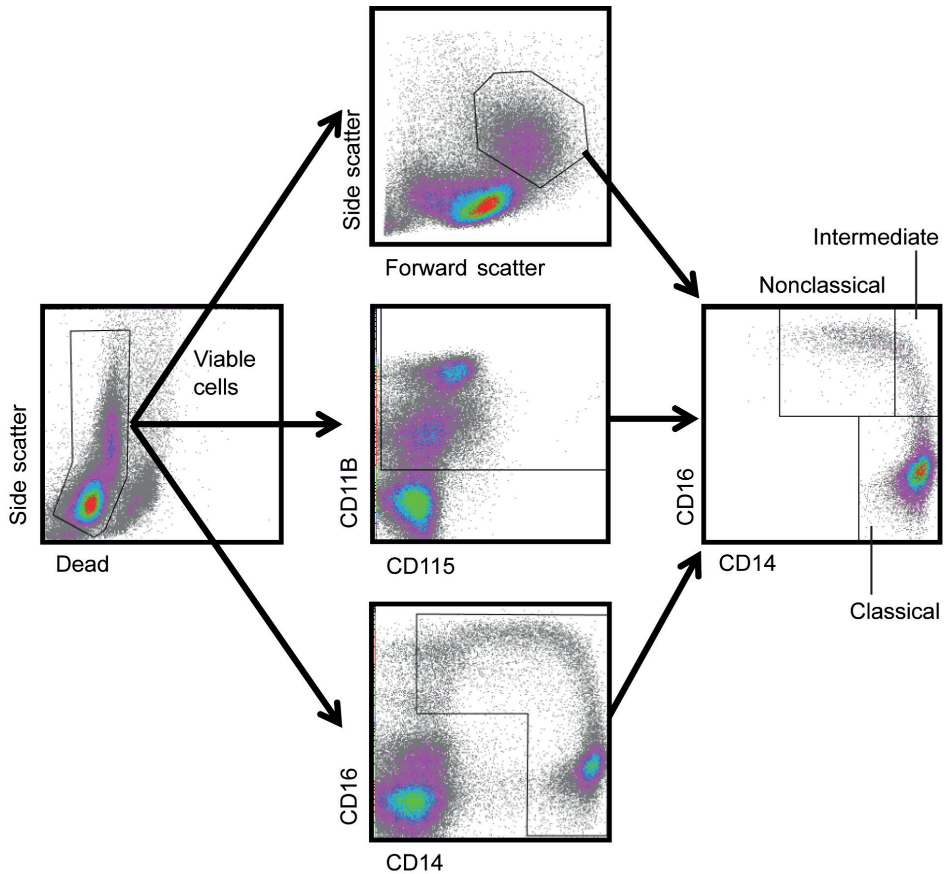
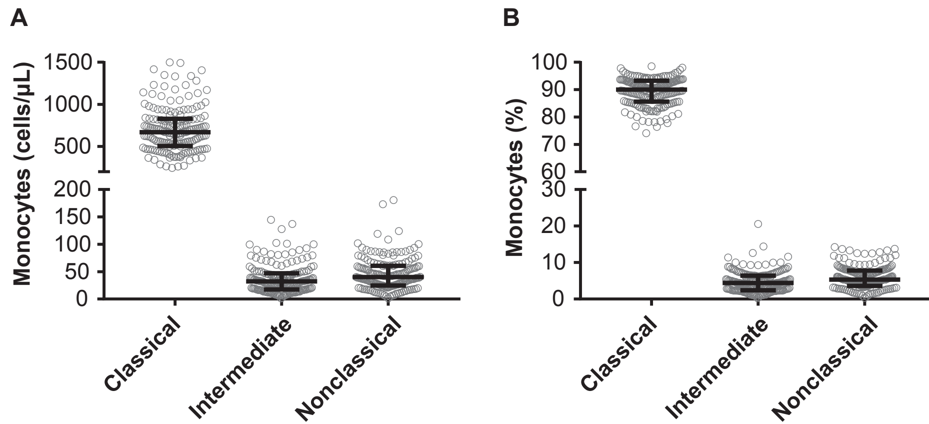
SUPPLEMENTAL DATA



Supplemental figure 1. Gating strategy of monocyte subsets.After doublet exclusion (not shown), viable cells were selected. Next, monocytes were distinguished based on size and granularity, positive expression of CD115 and CD11B. Based on surface expression of CD14 and CD16, we selected $CD14^{+}CD16^{-}$ classical, $CD14^{+}CD16^{+}$ intermediate and $CD14^{+}CD16^{++}$ non-classical monocytes.



Supplemental figure 2. Circulating monocyte subset counts and percentages. Absolute counts (A) and the percentages of $CD14^{+}CD16^{-}$ classical, $CD14^{+}CD16^{+}$ intermediate and $CD14^{+}CD16^{++}$ non-classical monocytes (B) are depicted for the complete patient cohort (n=175).

Supplemental table 1. Antibodies used for flow cytometry.

Marker	Fluorochrome	Clone	Ordering information		Amount (µL)
CD31	FITC	5.6E	Beckman Coulter	IM1431U	10.0
CCR2	RPE	58607	RnD	FAB151P	10.0
CD16	RPE/Cy5	3G8	Beckman Coulter	A07767	5.0
CD14	RPE/Cy7	Rmo52	Beckman Coulter	PN A22331	5.0
CX3CR1	APC	2A9-1	Biolegend	341604	2.5
CD115	Alexa 700	61708	RnD	FAB329N	5.0
CD11B	APC/Alexa 750	Bear1	Beckman Coulter	PN A97052	5.0
CXCR4	Bright Vision 421	12G5	BD Biosciences	562448	5.0

CD indicates cluster of differentiation; FITC, Fluorescein isothiocyanate; RPE, R-phycoerythrin; Cy, Cyanine; APC, allophycocyanin.

Supplemental table 2. The relation between monocyte counts and surface expression with the log transformed plaque macrophage area as outcome is shown.

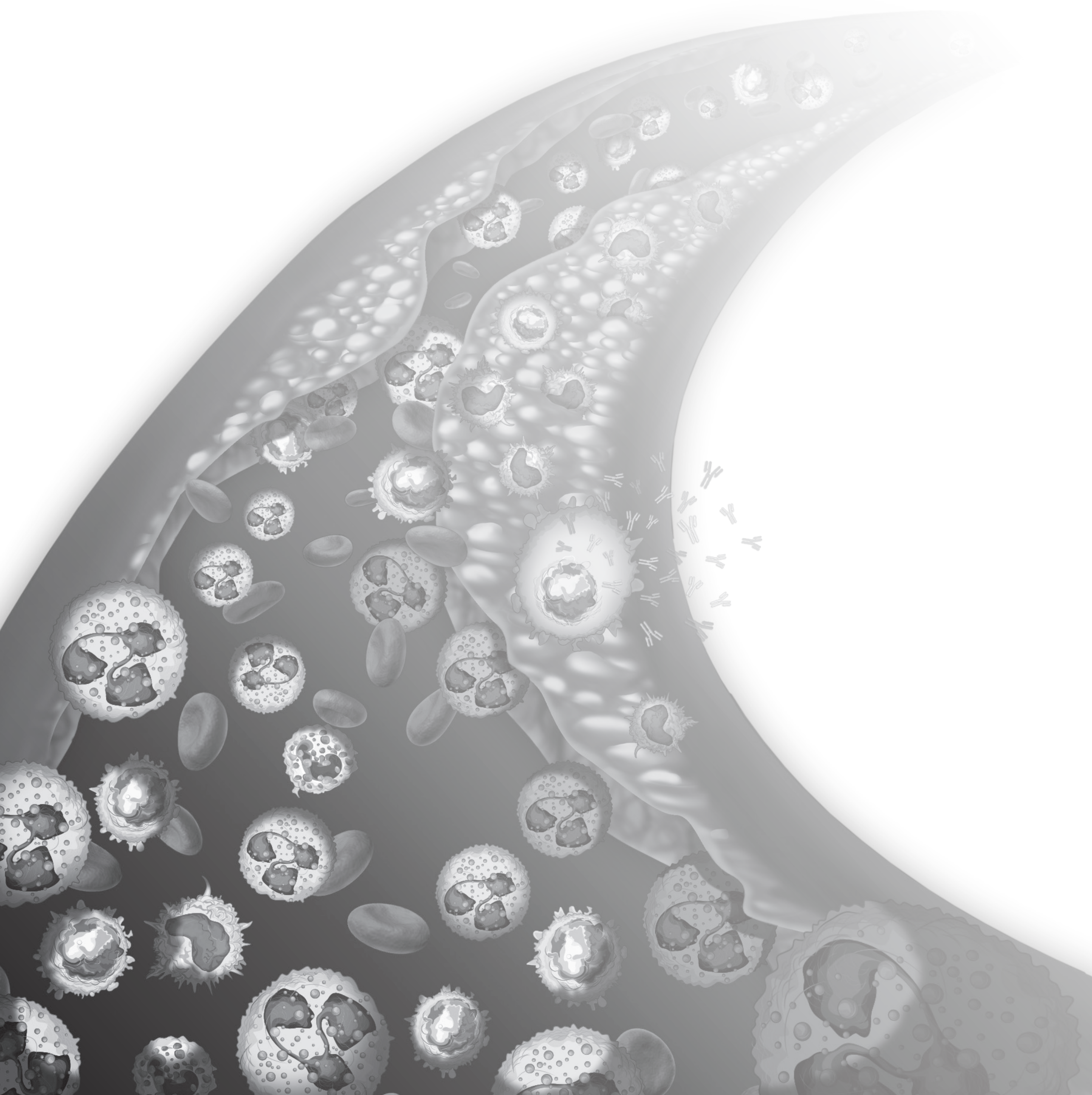
	Log plaque macrophage area change (Beta ± SE)	**R^2**	***p***
Total monocytes	-0.01 ± 0.01	0.01	0.24
Classical monocytes	-0.00 ± 0.01	0.00	0.46
Intermediate monocytes	-0.13 ± 0.05	0.04	**0.02**
Nonclassical monocytes	-0.15 ± 0.05	0.06	**<0.01**
CCR2 (total monocytes)	-0.00 ± 0.04	0.00	0.99
CCR2 (classical monocytes)	-0.01 ± 0.04	0.00	0.90
CCR2 (intermediate monocytes)	-0.03 ± 0.03	0.01	0.35
CCR2 (nonclassical monocytes)	-0.22 ± 0.19	0.01	0.25
CX3CR1 (total monocytes)	-0.03 ± 0.06	0.00	0.67
CX3CR1 (classical monocytes)	-0.03 ± 0.07	0.00	0.64
CX3CR1 (intermediate monocytes)	0.02 ± 0.03	0.00	0.46
CX3CR1 (nonclassical monocytes)	0.01 ± 0.03	0.00	0.67
CD11B (total monocytes)	0.00 ± 0.01	0.00	0.70
CD11B (classical monocytes)	0.00 ± 0.01	0.00	0.86
CD11B (intermediate monocytes)	0.01 ± 0.01	0.00	0.41
CD11B (nonclassical monocytes)	-0.01 ± 0.02	0.00	0.51

Beta indicates the slope or the direction of the relation between monocyte numbers (per 10 cells/µL) or per MFI increase of CCR2, CX3CR1 and CD11B surface expression of total monocytes or monocyte subsets. R^2 indicates how much variance in plaque macrophage content is explained by the different monocyte subsets. For example, the variance within the number of nonclassical monocytes explains 6% of the variance in plaque macrophage area. Statistical significance was considered as $p<0.05$, calculated using linear regression analyses (n=166). SE indicates standard error.

Supplemental table 3. No significant association was observed between monocyte subsets and risk of secondary CVD events during three-year follow-up.

	HR	**95% CI**	***p***
Total monocytes	0.99	[0.99 - 1.01]	0.31
Classical monocytes	0.99	[0.98 - 1.01]	0.32
Intermediate monocytes	0.97	[0.87 - 1.08]	0.58
Nonclassical monocytes	0.97	[0.88 - 1.08]	0.59

Hazard rates (indicated per increase of 10 cells/µL) and *p*-values were calculated using Cox proportional hazard models (n=175).



CHAPTER

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High levels of (un)switched memory B cells associate with better outcome in patients with advanced atherosclerotic disease

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ABSTRACT

Background

Atherosclerosis is an inflammatory lipid disorder and the main underlying pathology of acute ischemic events. Despite a vast amount of data from murine atherosclerosis models, evidence of B cell involvement in human atherosclerotic disease is limited. We therefore investigated the association of circulating B cell subtypes with the occurrence of secondary cardiovascular events in advanced atherosclerotic disease.

Methods and results

This cohort study consists of 168 patients who were included in the Athero-Express biobank between 2009-2011. Before surgery, peripheral blood mononuclear cells (PBMCs) were isolated and stored in liquid nitrogen. After gentle thawing of the PBMCs, different B cell subtypes including naïve, (un)switched memory and $CD27^{+}CD43^{+}$ B1-like B cells, were analyzed by flow cytometry. Univariable and multivariable Cox proportional hazard models were used to analyze associations between B cell subtypes, circulating antibodies and secondary cardiovascular manifestations during the three-year follow-up period. Mean age was 70.1 ± 9.6 years, males represented 62.8% of the population and 54 patients had secondary manifestations during follow-up. High numbers of unswitched memory cells were protective against secondary outcome (hazard ratio (HR) 0.30 [95% CI 0.13 - 0.69], $p<0.01$). Similar results were obtained for the switched memory cells that also showed to be protective against secondary outcome (HR 0.33 [95% CI 0.14 - 0.77], $p=0.01$).

Conclusion

A high number of (un)switched memory B cells is associated with better outcome following carotid artery endarterectomy. These findings suggest a potential role for B cell subsets in prediction and prevention of secondary cardiovascular events in patients with atherosclerosis.

CLINICAL PERSPECTIVE

What is new?

- We show an extensive flow cytometry profiling of B cells in severe cardiovascular patients and associate this profile to risk for future events.
- We show for the first time that both unswitched and switched memory B cells associate with better outcome in patients with existing cardiovascular disease.
- OxLDL IgG autoantibody titers strongly correlate to memory B cell numbers, but oxLDL autoantibodies were not predictive for future events.

What are the clinical implications?

These findings indicate that memory B cells might have value in prediction and prevention of adverse secondary cardiovascular manifestations.

INTRODUCTION

Although the prevention of cardiovascular disease (CVD) has improved in the past decades, it remains one of the major causes of death worldwide[1]. Its main underlying pathology, atherosclerosis, is an inflammatory lipid disorder and the major cause of acute cardiovascular syndromes[2-4]. Many inflammatory cell types, including monocytes, macrophages, mast cells, neutrophils, T and B cells, have been implicated in the initiation, progression and destabilization of atherosclerosis[5]. Increased insights into how these inflammatory cells are involved in CVD may lead to the identification of novel biomarkers or therapeutic targets for primary or secondary manifestations of CVD.

The risk of cardiovascular events is particularly high in patients with earlier CVD manifestations[6, 7]. For example, the 3-year cumulative incidence of major adverse cardiovascular events in patients undergoing carotid endarterectomy was 13%[8]. Increased inflammation is an important risk factor for recurrent CVD events[2]. For example, different studies showed that high white blood cell (WBC) counts are associated with the recurrence of CVD events and mortality[9-13]. These studies, however, mainly consider total white blood cell count or total lymphocyte counts and do not provide additional information regarding cell specific subtypes.

Besides their well-known role in humoral immunity through antibody production, B cells are important for T cell activation and cytokine production in maintaining immune homeostasis[14]. The total peripheral B-cell pool in humans mainly consists of naïve B cells, $CD43^+$ B1-like cells[15-17], unswitched and switched memory cells (mainly expressing Immunoglobulin (Ig)M[18], or IgG and IgA antibodies respectively[18]), regulatory B cells and plasma cells. As such, B cells are important in immune homeostasis, but have also been shown to be detrimental in autoimmune diseases like common variable immunodeficiency and systemic lupus erythematosus[19, 20]. Interestingly, patients with these B-cell-driven autoimmune diseases are also at high risk for CVD[19, 20].

Evidence for an important role of B lymphocytes in human CVD is limited. In patients with acute myocardial infarction (MI), high levels of the B-cell specific cytokines chemokine (C-C motif) ligand 7 and B-cell activating factor predict increased risk of death and recurrent MI[21]. In addition, hypertensive patients with high percentages of $CD40^+$ B cells were at lower risk for stroke[22], while high numbers of $CD86^+$ B cells showed higher risk for stroke[22]. A larger body of evidence is derived from elaborate mouse studies which identified a subset-specific role for B lymphocytes in atherosclerosis[23-25]. To date, it is generally accepted that (auto)antibody-producing B1 B cells are atheroprotective[26, 27] and this protective effect depends on IgM secretion[28], while conventional B2 B cells are generally considered proatherogenic[29]. This proatherogenic phenotype in mice has mainly been attributed to the production of pathogenic IgG antibodies against oxidized low-density lipoprotein (oxLDL) and the immune effector function of these B cells[27, 28, 30-33]. In human atherosclerosis, however, there is conflicting evidence about the role of auto-antibodies directed against oxLDL. On the one hand, oxLDL antibodies have been associated with the presence[34] and progression[35-37] of atherosclerosis and the risk for MI. On the other hand, anti-oxLDL autoantibodies have also been associated with lower oxLDL levels[38] and decreased carotid atherosclerosis as well[39]. In addition, vaccination with a pneumococcal vaccine, mimicking oxLDL epitopes, was shown to attenuate atherosclerotic lesion formation in mice[40] and, according to a recent meta-analysis, also prevents CVD in adults[41].
Taken together, accumulating evidence points to an important role of B cells in CVD, but human evidence is limited. In order to establish if a specific B-cell profile is associated with secondary cardiovascular events, we measured circulating B-cell subtypes, including naïve, $CD43^+$ B1-like, unswitched and switched memory B cells, as well as oxLDL antibodies, in severe atherosclerotic patients derived from the Athero-Express biobank. B-cell subtypes were identified by flow cytometry and associated with the occurrence of secondary cardiovascular manifestations during follow-up after carotid endarterectomy.

MATERIALS AND METHODS

Study population

This study includes a sub cohort of 168 patients from the Athero-Express biobank, a cohort study of patients undergoing carotid endarterectomy that were included between 2009 and 2011[42]. In addition to the standard procedure, including an extensive patient questionnaire and detailed histological plaque characterization, peripheral blood mononuclear cells (PBMCs) were isolated from blood that was drawn preoperatively. Isolated PBMCs were stored in liquid nitrogen until further analyses were performed. Patient follow-up was 3 years or until the occurrence of secondary cardiovascular events (cardiovascular death, stroke, myocardial infarction, coronary intervention, peripheral intervention (including amputation) or a combination). All events were validated using health records kept by general practitioners. All patients provided written informed consent. The study protocol conforms to the Declaration of Helsinki and has been approved by the Institution's ethics committee on research on humans.

Blood collection and PBMC isolation

Twenty milliliters of blood were collected in Li-Heparin blood tubes. A complete blood count profile was determined by a general hematology cell counter (Cell Dyn 1800; Abbott Laboratories, Abbott Park, IL). Directly after collection, the platelet rich plasma fraction was isolated by centrifugation for 10 minutes at 150*g* at room temperature without brake. The blood volume was restored to its original volume with PBS. Subsequently, blood was gently layered on a Ficoll (17-1440-03; GE Healthcare, Chalfont St. Giles, UK) loaded Leucosep tube (227 290, Greiner bio-one, Alphen aan den Rijn, The Netherlands) and centrifuged at 1000*g* for 15 minutes at room temperature without brake. PBMCs were carefully isolated from the interphase. To remove any residual Ficoll, PBMCs were washed with cold PBS, centrifuged at 330*g* for 10 minutes at 4°C with brake and resuspended in 1 mL of sterile, serum-free cell freezing medium with DMSO (C6295; Sigma-Aldrich, St. Louis, MO, USA). PBMCs were slowly frozen overnight at -80°C using a Nalgene freezing container and stored in liquid nitrogen until further analyses were performed.

Flow Cytometry

PBMCs were gently thawed and washed with RPMI 1640 ((61870010; Gibco Carlsbad, CA, USA) supplemented with GlutaMax, 25nmol/L HEPES, 1% penicillin/streptomycin and 2% FBS (10270-106; Gibco, Carlsbad, CA, USA)). Cells were kept on ice during the whole procedure, unless stated otherwise. To obtain single cell suspensions, PBMCs were gently filtered over a 40-µm cell strainer (542040; Greiner bio-one), washed with RPMI again and centrifuged at 350*g* for 5 minutes at 4°C. Subsequently, the cells were resuspended in cold PBS (supplemented with 2% FBS and 20mmol/L EDTA) centrifuged at 350*g* for 5 minutes at 4°C and resuspended in cold PBS with 1% BSA. Subsequently, the cells were incubated with antibodies (**Table 1**) for 30 minutes at room temperature in the dark, washed with PBS (4°C) and centrifuged at 350*g* for 5 minutes at 4°C. Next, cells were incubated for 30 minutes with fixable viability dye eFluor-506 (eBioscience, San Diego, CA, USA), washed, centrifuged and measured on the flow cytometer (Gallios; Beckman Coulter, Fullerton, CA, USA). Analysis of the flow cytometry data was performed using Kaluza 1.3 software. We selected viable $CD19^{+}CD3^{-}$ lymphocytes, excluded plasmablasts ($CD24^{-}CD38^{+}$; **Figure 1**), and gated $CD43^{+}CD27^{+}$ cells, which are suggested to resemble B1 B cells[15]. Next, we selected unswitched memory cells ($CD27^{+}CD43^{-}IgD^{+}$) and switched memory cells ($CD27^{+}CD43^{-}IgD^{-}$). From the $CD27^{-}IgD^{+}$ B cells, we selected the naïve $CD24^{+}CD38^{+}$ B cells (**Figure 1**). Absolute B-cell numbers were calculated from the ratio measured by flow cytometry multiplied by the absolute number of lymphocytes obtained from the hematology cell counter.

Anti-oxLDL antibody measurements

Serum levels of IgM and IgG-α-oxLDL antibodies were measured as described previously[40, 43]. In short, serum samples were diluted 500 times for IgM and 2000 times for IgG-α-oxLDL antibody measurements. Antibody titers were determined by chemiluminescent enzyme immunoassays and values are presented as relative light units per 100 ms.

Table 1. Antibody characteristics.

Marker	Fluorochrome	Clone	µL
IgD	AF488	IA6-2	2.5
CD24	PE	ML-5	2.5
CD19	PE-Cy5	HIB19	2.5
CD38	PE-Cy7	HIT2	2.5
CD43	APC	10G7	2.5
IgM	APC-Cy7	MHM-88	5
CD27	PB	O323	1
CD3	BV510	OKT3	1

Panel of antibodies used. AF indicates alexa fluor, PE, (R-)phycoerythrin; APC, allophycocyanin; PB, pacific blue; BV, brilliant violet. All antibodies are from Biolegend San Diego, CA, USA.

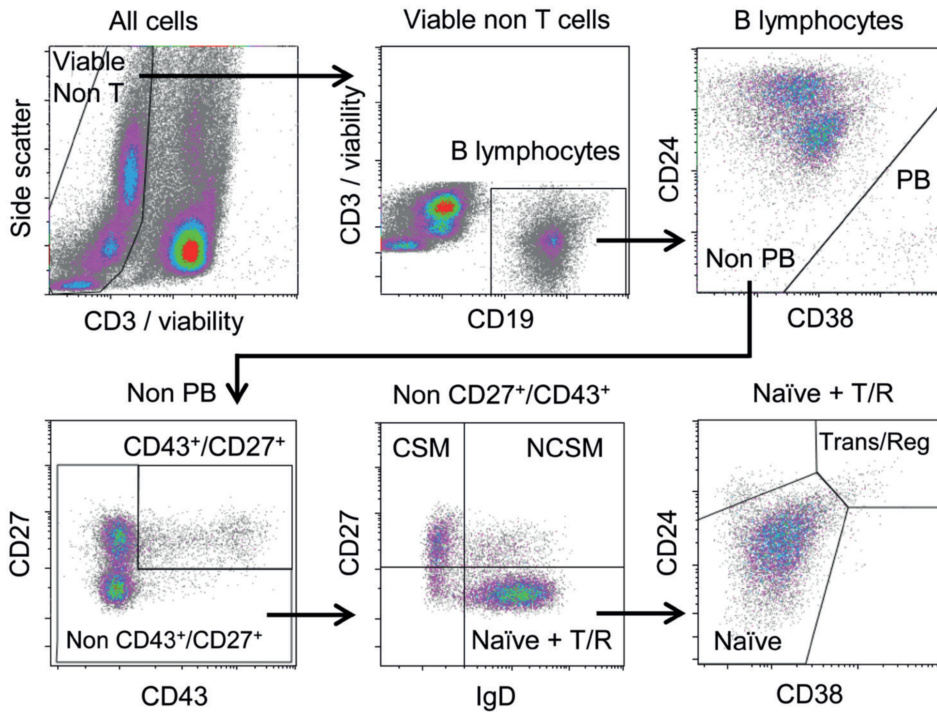

Figure 1. Gating strategy for the selection of different B-cell subtypes from a representative sample. First, dead cells and $CD3^+$ T cells were excluded. Next, from the viable non-T cells, the $CD19^+$ B cells were identified. Then, the $CD24^{low}CD38^+$ plasmablasts (PB) were excluded and from the non-PB, the $CD43^+CD27^+$ cells were selected. Next, based on surface expression of CD27 and IgD class switched (CSM) and non-class switched memory cells (NCSM) were identified. From the IgD^+CD27^- cells, the $CD38^+CD24^+$ transitional and regulatory (Trans/Reg) could be distinguished from the naïve B cells. An overview of the antibody characteristics is provided in **table 1**. Ig indicates immunoglobulin.

Statistical analyses

Normally distributed continuous variables are indicated as means ± SD and compared by Student's *t* tests or a one-way ANOVA. Non-normal distributed data are presented as medians [interquartile ranges; IQRs] and compared by Kruskal-Wallis tests. Categorical variables are indicated as percentages and compared by Chi-squared or Fisher's exact tests. As confounders, we selected variables that associate with CVD risk but also influence B cell numbers and have been established as confounders in literature, including age, sex, smoking, history of coronary artery disease and glomerular filtration rate[22, 44-47]. We also tested for a sex interaction between the association of B cells and cardiovascular end points.

Univariable and multivariable Cox proportional hazard models were used to study the association of B cell subtypes and anti-oxLDL antibodies with occurrence of secondary cardiovascular events over time. Next, to visualize this association, subjects were divided into tertiles according to the absolute numbers of B cell subtypes and plotted against the occurrence of secondary cardiovascular events over time. Data management and statistical analyses were performed with RStudio[48] and the R software package[49] (version 3.2.0.; R Foundation for Statistical Computing, Vienna, Austria). *P* <0.05 were considered as significant.

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RESULTS

Clinical characteristics

Baseline characteristics are shown in **table 2**, stratified into patients with and without secondary cardiovascular events. Of the included 168 patients 54 patients experienced secondary cardiovascular events during follow-up, which included cardiovascular death (n=11), stroke (n=13), coronary events (n=11) peripheral intervention (n=35). Risk factors for the presence of cardiovascular disease such as age, sex, smoking, and kidney function (glomerular filtration rate), did not differ between patients with or without secondary events (**Table 2**).

Total B cells and (un)switched memory B cells are higher in patients without secondary cardiovascular events

We measured different B cell subtypes using flow cytometry. Total B cell numbers (approximately 200 cells/µL) and B cell subset numbers corroborate with earlier observations[50] (**Table 3**). Next, we investigated whether baseline levels of B cells differed between patients who developed secondary cardiovascular events during follow-up after endarterectomy (cases) compared to those without a secondary event (controls) (**Table 3**). Total numbers of $CD19^+$ B cells (167 [interquartile range (IQR) 115, 236] vs 209 [IQR 121, 415] cells/µL, *p*=0.04), unswitched memory cells (8 [IQR 4, 12] vs 12 [IQR 6, 25] cells/µL, *p*<0.01) and switched memory cells (21 [IQR 13, 34] vs 27 [IQR 15, 54] cells/µL, *p*=0.02, were lower in cases than in controls. Although numbers of naïve B

Table 2. Baseline characteristics of the study cohort stratified by occurrence of secondary cardiovascular events.

	Overall (n=168)	No events (n=114)	Events (n=54)	*p*
Age (years)	70.1 ± 9.6	69.8 ± 9.3	70.9 ± 10.3	0.50
Sex (% male)	108 (62.8%)	72 (61.5%)	36 (65.5%)	0.74
Current smoker	61 (35.7%)	41 (35.3%)	20 (36.4%)	1
BMI	26.7 ± 4.6	26.7 ± 4.7	26.7 ± 4.4	1
Contralateral stenosis	72 (47.7%)	45 (44.6%)	27 (54.0%)	0.36
Diabetes	41 (23.8%)	28 (23.9%)	13 (23.6%)	1
Hypercholesterolemia	106 (71.6%)	74 (70.5%)	32 (74.4%)	0.78
Hypertension	149 (86.6%)	100 (85.5%)	49 (89.1%)	0.68
CAD history	62 (36.0%)	41 (35.0%)	21 (38.2%)	0.82
Clinical manifestations				0.26
Asymptomatic	25 (14.5%)	13 (11.1%)	12 (21.8%)	
Ocular	29 (16.9%)	19 (16.2%)	10 (18.2%)	
Stroke	42 (24.4%)	31 (26.5%)	11 (20.0%)	
TIA	76 (44.2%)	54 (46.2%)	22 (40.0%)	
Medication				
Statins	138 (80.2%)	92 (78.6%)	46 (83.6%)	0.57
Beta blockers	82 (47.7%)	53 (45.3%)	29 (52.7%)	0.46
Anticoagulants	11 (6.4%)	5 (4.3%)	6 (10.9%)	0.11
Laboratory parameters				
Total cholesterol (mmol/L)	4.0 [3.3, 4.8]	4.0 [3.4, 4.7]	4.0 [3.3, 4.8]	0.75
Triglycerides (mmol/L)	1.5 [1.1, 2.0]	1.4 [1.1, 1.9]	1.5 [1.1, 2.0]	0.54
HDL cholesterol (mmol/L)	1.0 [0.9, 1.3]	1.0 [0.9, 1.2]	1.0 [0.9, 1.3]	0.15
LDL cholesterol (mmol/L)	2.1 [1.6, 2.8]	2.2 [1.8, 2.9]	2.1 [1.6, 2.8]	0.20
hsCRP plasma (µg/mL)	16.0 ± 78.4	17.2 ± 90.7	13.3 ± 37.1	0.78
GFR MDRD (mL/min)	69.5 ± 18.3	70.5 ± 17.4	67.3 ± 20.2	0.31
WBC count (10e6 cells/mL)	7.2 [5.5, 8.6]	7.0 [5.5, 8.9]	7.4 [5.6, 8.2]	0.99
Lymphocytes (10e6 cells/mL)	1.5 [1.2, 2.1]	1.6 [1.2, 2.2]	1.4 [1.1, 1.9]	0.09
Monocytes (10e6 cells/mL)	0.8 [0.6, 0.9]	0.8 [0.6, 1.0]	0.8 [0.6, 0.9]	0.59
Granulocytes (10e6 cells/mL)	4.6 [3.4, 6.1]	4.4 [3.3, 6.1]	5.1 [3.5, 6.0]	0.34

The demographic characteristics of the study cohort are given for the whole population and also stratified by occurrence of secondary cardiovascular events. The values are presented as mean ± SD for normal distributions, number of patients (frequency in percentage) for categorical variables, and median [interquartile range] for non-normal distributions. *P*-values are calculated using Student's *t* tests, Chi-squared or Fisher's exact tests, and Kruskal-Wallis tests, respectively. BMI indicates body-mass index; CAD history, history of Coronary Artery Disease; Contralateral stenosis, 50-100% stenosis of the contralateral carotid artery; HDL, high-density lipoprotein; LDL, low-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; GFR MDRD, Glomerular filtration rate according to 'Modification of Diet in Renal Disease' formula;

cells or $CD43^{+}CD27^{+}$ B cells also tended to be higher, these differences were not statistically significant between groups. Baseline characteristics of patients stratified by B lymphocyte tertiles are shown in **table 4**.

Associations of B cell subtypes with univariate risk factors

We then investigated whether the numbers of B cells or B-cell subtypes were associated with cardiovascular risk factors. As expected, total B-cell numbers decreased with age and were higher in women and in current smokers. Specific associations of each B cell subtype with the cardiovascular risk factors are depicted in **table 5**. We selected age, sex, smoking, history of coronary artery disease and glomerular filtration rate as potential confounders, based on their established prognostic value in CVD.

Cox regression analysis of B cells with secondary cardiovascular events

Because of the known B cell related immunological differences between sexes we tested sex interaction between the association of B cells and cardiovascular endpoints, but there was no statistically significant interaction. Univariable and multivariable Cox proportional hazard regression models were used to calculate the hazard ratios (HRs) for each B-cell subset (**Table 6**). Patients were then categorized into tertiles according to the absolute number of B cells and B-cell subsets. Interestingly, patients with high numbers of unswitched memory B cells were at lower risk of experiencing a secondary cardiovascular event during follow-up as compared to patients with low numbers (HR 0.30 [95% CI 0.13 - 0.69], $p<0.01$; **Figure 2**). Likewise, patients in the highest tertile of switched memory cell numbers were at lower risk than patients in the lowest tertile (HR 0.33 [95% CI 0.14 - 0.77], $p=0.01$). When we combined both the numbers of switched and unswitched memory cells, patients in the highest tertile of total memory cells were still at lower risk (HR 0.35 [95% CI 0.15 - 0.83], $p=0.02$) compared to patients in the lowest tertile of total memory cells, however the combination did not further decrease the risk for recurrent CVD events as the hazard ratio was comparable.

Table 3. Baseline numbers of total B cells and B cell subtypes stratified by occurrence of secondary cardiovascular events.

B cell (sub)type (cells/µL)	Surface markers	Overall (n=168)	No events (n=114)	Events (n=54)	*p*
B lymphocytes	$CD19^{+}CD3^{-}$	193 [121, 323]	209 [122, 415]	167 [115, 236]	**0.04**
B1 like	$CD27^{+}CD43^{+}$	8 [3, 15]	9 [4, 15]	7 [3, 13]	0.39
Unswitched	$CD27^{+}CD43^{-}IgD^{+}$	10 [5, 21]	12 [6, 25]	8 [4, 12]	**<0.01**
Switched	$CD27^{+}CD43^{-}IgD^{-}$	26 [15, 47]	27 [16, 54]	21 [13, 34]	**0.02**
Naïve	$CD27^{-}CD43^{-}IgD^{+}$	102 [58, 184]	106 [62, 213]	89 [56, 141]	0.17

The numbers of B cells are presented for the whole cohort and also stratified by occurrence of secondary cardiovascular events during follow-up. The absolute numbers of B cells and B-cell subtypes with their surface markers are indicated as median [interquartile range]. *P*-values were calculated using the Kruskal-Wallis test for non-parametric distributions.

Table 4. Baseline characteristics of the study cohort stratified by tertiles of total B lymphocytes.

	Low (n=56)	**Intermediate (n=56)**	**High (n=56)**	***p***
B lymphocytes (cells/µL)	**96 [67, 121]**	**193 [168, 214]**	**430 [326, 506]**	
Age (years)	72.2 ± 10.5	69.9 ± 9.2	68.0 ± 8.9	0.07
Sex (% male)	36 (64.3%)	40 (71.4%)	30 (53.6%)	0.14
Current smoker	14 (25.5%)	21 (37.5%)	26 (46.4)	0.07
BMI	27.4 ± 4.4	25.4 ± 4.0	27.3 ± 5.2	**0.03**
Contralateral stenosis	31 (59.6%)	18 (39.1%)	20 (40.8%)	0.07
Hypertension	49 (87.5%)	47 (83.9%)	49 (87.5%)	0.82
Diabetes	11 (19.6%)	11 (19.6%)	17 (30.4%)	0.30
Hypercholesterolemia	38 (77.6%)	27 (56.2%)	38 (79.2%)	**0.02**
CAD history	25 (44.6%)	17 (30.4%)	18 (32.1%)	0.23
Clinical manifestations				0.58
asymptomatic	7 (12.5%)	9 (16.1%)	8 (14.3%)	
ocular	12 (21.4%)	11 (19.6%)	6 (10.7%)	
stroke	10 (17.9%)	14 (25.0%)	17 (30. 4%)	
TIA	27 (48.2%)	22 (39.3%)	25 (44.6%)	
Medication				
Statins	48 (85.7%)	42 (75.0%)	46 (82.1%)	0.34
Beta blockers	30 (53.6%)	24 (42.9%)	25 (44.6%)	0.48
Anticoagulants	2 (3.6%)	7 (12.5%)	2 (3.6%)	0.11
Laboratory parameters				
Cholesterol (mmol/L)	3.8 [3.2, 4.6]	4.1 [3.3, 4.9]	3.9 [3.4, 4.4]	0.71
Triglycerides (mmol/L)	1.5 [1.2, 2.1]	1.4 [1.0, 1.9]	1.6 [1.2, 2.0]	0.30
HDL cholesterol (mmol/L)	1.0 [0.9, 1.2]	1.0 [0.9, 1.3]	1.0 [0.9, 1.2]	0.72
LDL cholesterol (mmol/L)	2.1 [1.6, 2.5]	2.1 [1.6, 3.1]	2.1 [1.6, 2.7]	0.60
hsCRP plasma (µg/mL)	5.0 (15.7)	9.7 (29.6)	33.8 (130.8)	0.14
GFR MDRD (mL/min)	66.0 (17.7)	74.8 (19.0)	68.8 (17.5)	**0.04**

The baseline characteristics are depicted for patients with low, intermediate and high numbers of total B lymphocytes. The values are presented as mean ± SD for normal distributions, number of patients (frequency in percentage) for categorical variables, and median [interquartile range] for non-normal distributions. *P*-values are calculated using a one-way ANOVA, Chi-square or Fisher's exact tests, and Kruskal-Wallis tests, respectively. BMI indicates body-mass index; CAD history, history of coronary artery disease; contralateral stenosis, 50-100% stenosis of the contralateral carotid artery; GFR MDRD, glomerular filtration rate according to 'Modification of Diet in Renal Disease' formula; HDL, high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; TIA, transient ischemic attack.

Table 5. Univariable associations of the total B cells and B cell subtypes with classical cardiovascular risk factors.

	B lymphocytes (cells/µL)		Naïve (cells/µL)		Unswitched memory (cells/µL)	
	beta [95% CI]	***p***	**beta [95% CI]**	***p***	**beta [95% CI]**	***p***
Age (years)	-3.14 [-6.35, 0.07]	0.05	-0.49 [-2.83, 1.85]	0.68	-0.5 [-0.78, -0.22]	**<0.01**
Sex (male)	-74.19 [-137.92, -10.47]	**0.02**	-55.49 [-101.4, -9.58]	**0.02**	-1.74 [-7.49, 4.02]	0.55
Current smoker	72.06 [7.83, 136.3]	**0.03**	1.83 [-45.21, 48.87]	0.94	9.94 [4.34, 15.53]	**<0.01**
BMI	1.43 [-5.36, 8.22]	0.68	1.63 [-3.26, 6.52]	0.51	0.23 [-0.37, 0.83]	0.45
Contralateral stenosis	-24.9 [-85.71, 35.91]	0.42	-10.17 [-57.28, 36.93]	0.67	-5.22 [-11.3, 0.85]	0.09
Diabetes	65.49 [-7.81, 138.79]	0.08	53.35 [0.61, 106.09]	0.05	0.89 [-5.7, 7.47]	0.79
Hypercholesterolemia	21.71 [-52.45, 95.88]	0.56	2.27 [-50.23, 54.78]	0.93	3.16 [-3.76, 10.07]	0.37
Hypertension	-42.66 [-133.3, 47.98]	0.35	-25.09 [-90.52, 40.35]	0.45	-0.89 [-8.98, 7.2]	0.83
CAD history	-53 [-117.68, 11.69]	0.11	-22.71 [-69.61, 24.19]	0.34	-5.19 [-10.94, 0.56]	0.08
Total cholesterol (mmol/L)	-10.87 [-42.01, 20.27]	0.49	-14.67 [-37.07, 7.73]	0.2	1.06 [-1.64, 3.77]	0.44
Triglycerides (mmol/L)	5.68 [-31.34, 42.7]	0.76	-4.56 [-31.29, 22.17]	0.74	4.22 [1.07, 7.37]	**0.01**
HDL cholesterol (mmol/L)	-3.97 [-122.77, 114.83]	0.95	-26.37 [-112.05, 59.31]	0.54	-1.18 [-11.49, 9.13]	0.82
LDL cholesterol (mmol/L)	-17.96 [-56.83, 20.91]	0.36	-14.09 [-42.21, 14.02]	0.32	-1.79 [-4.71, 1.13]	0.23
hsCRP plasma (µg/mL)	0.04 [-0.39, 0.47]	0.86	0.03 [-0.28, 0.34]	0.85	0 [-0.04, 0.03]	0.91
GFR MDRD (mL/min)	0.59 [-1.2, 2.38]	0.52	-0.09 [-1.39, 1.2]	0.89	0.08 [-0.07, 0.23]	0.31

	Switched memory (cells/µL)		$CD43^+CD27^+$ (cells/µL)	
	beta [95% CI]	***p***	**beta [95% CI]**	***p***
Age (years)	-1.13 [-1.79, -0.46]	**<0.01**	-0.4 [-0.61, -0.19]	**<0.01**
Sex (male)	-5.35 [-18.98, 8.28]	0.44	-0.4 [-4.78, 3.97]	0.86
Current smoker	39.49 [27.14, 51.84]	**<0.01**	3.39 [-0.98, 7.77]	0.13
BMI	-0.35 [-1.78, 1.07]	0.63	0 [-0.45, 0.46]	0.98
Contralateral stenosis	-3.14 [-12.63, 6.34]	0.51	-1.35 [-6.02, 3.32]	0.57
Diabetes	3 [-12.6, 18.6]	0.70	1.84 [-3.15, 6.83]	0.47
Hypercholesterolemia	10.24 [-6.22, 26.69]	0.22	3.09 [-2.05, 8.24]	0.24
Hypertension	-13.23 [-32.3, 5.83]	0.17	2.64 [-3.49, 8.77]	0.40
CAD history	-12.09 [-25.72, 1.53]	0.08	-3.06 [-7.44, 1.32]	0.17
Total cholesterol (mmol/L)	1.69 [-4.83, 8.22]	0.61	1.16 [-0.91, 3.23]	0.27
Triglycerides (mmol/L)	5.79 [-1.91, 13.5]	0.14	2.14 [-0.3, 4.58]	0.09
HDL cholesterol (mmol/L)	14.07 [-10.71, 38.85]	0.26	-2.26 [-10.16, 5.64]	0.57
LDL cholesterol (mmol/L)	-1.97 [-10.12, 6.19]	0.63	0.2 [-2.2, 2.6]	0.87
hsCRP plasma (µg/mL)	0.01 [-0.08, 0.09]	0.87	0 [-0.02, 0.02]	0.93
GFR MDRD (mL/min)	0.33 [-0.05, 0.7]	0.09	0.02 [-0.08, 0.11]	0.73

The associations between risk factors and B cell numbers are presented as beta [95% confidence intervals]. For categorical variables, beta is the difference in B cell numbers (cells/µL) between groups. For continuous variables, beta is the linear regression coefficient relating a 1-unit increase in the variable to the associated change in B-cell numbers. BMI indicates body-mass index; Contralateral stenosis, 50-100% stenosis of the contralateral carotid artery; CAD history, history of Coronary Artery Disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; GFR MDRD, Glomerular filtration rate according to 'Modification of Diet in Renal Disease' formula. "*p" is the p*-value of the linear or logistic regression model for association.

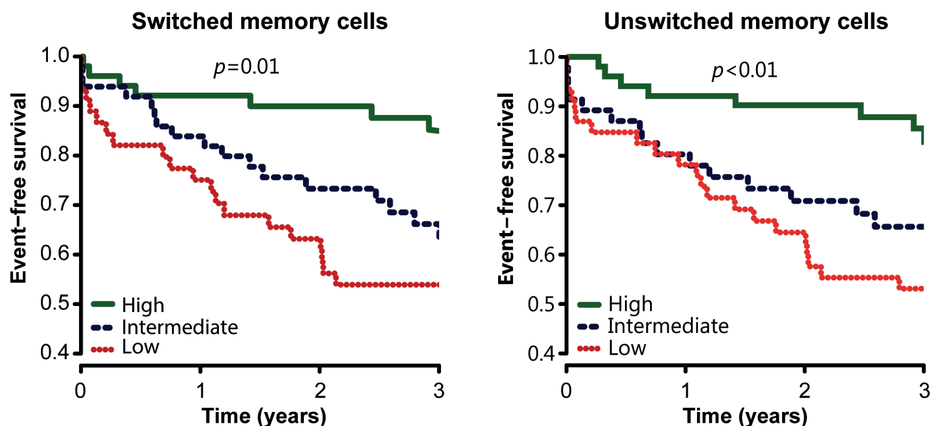

Figure 2. High numbers of (un)switched memory cells associate with decreased risk of secondary adverse cardiovascular events. Cox proportional hazard models are shown by tertiles of (un)switched memory cells. The model is adjusted for age, sex, smoking, history of coronary artery disease and glomerular filtration rate. The indicated *p*-value is derived from comparison of highest to lowest tertile.

Comparable results were obtained when we excluded secondary surgical interventions from the composite endpoint (25 endpoints left). In multivariable Cox proportional hazard regression models, patients in the highest tertile were at lower risk of events, now only consisting of cardiovascular death, stroke, or MI, as compared to patients in the lowest tertile (HR 0.35 [95% CI 0.15 - 0.82], p=0.01) of unswitched memory cells and (HR 0.32 [95% CI 0.13 - 0.75], p=0.01) of switched memory cells.

Surface IgM expression is higher in patients with high numbers of unswitched memory cells

IgM has been shown to be atheroprotective in mouse atherosclerosis models. To assess if the protective effect of the (un)switched memory B cells is reflected by high expression of IgM we measured surface expression of IgM. Indeed, in patients with high numbers of unswitched memory cells, the surface expression of IgM was also higher as compared to patients with low numbers of unswitched memory B cells (median fluorescence intensity 62.5 [IQR 51.2, 70.0] versus 53.4 [IQR 40.4, 65.1], p=0.03), suggesting that these patients not only possessed higher numbers of unswitched memory B cells, but that these cells also produce more IgM antibodies.

Serum levels of anti-oxLDL autoantibodies

To further investigate the antibody production by these (un)switched memory B cells we determined antibody titers of antibodies directed against oxLDL. Although there was no correlation between IgM-α-oxLDL antibodies and memory B cells, levels of IgG-α-oxLDL antibodies positively correlated with class switched memory cells (spearman's rho = 0.28, p<0.01) (**Figure 3**). IgM- and IgG-α-oxLDL titers were not significantly increased in patients

Table 6. Cox proportional hazard models of total B lymphocytes and B cell subtypes.

				Univariable		Multivariable	
	Patients (Events)	**Cells/µL**		**HR [95% CI]**	***p***	**HR [95% CI]**	***p***
Total B lymphocytes			continuous	0.98 [0.96, 1.00]	**0.01**	0.98 [0.96, 1.00]	**0.01**
	56 (21)	40-141	low	1 (Ref)		1 (Ref)	
	56 (21)	142-257	intermediate	0.90 [0.49, 1.66]	0.74	0.92 [0.48, 1.75]	0.79
	56 (12)	258-1351	high	0.5 [0.25, 1.01]	0.05	0.47 [0.22, 1.00]	0.05
Naïve			continuous	0.98 [0.95, 1.00]	0.05	0.98 [0.95, 1.00]	0.06
	56 (22)	2-71	low	1 (Ref)		1 (Ref)	
	56 (19)	72-141	intermediate	0.80 [0.43, 1.48]	0.48	0.82 [0.42, 1.59]	0.56
	56 (13)	141-759	high	0.53 [0.27, 1.06]	0.07	0.59 [0.29, 1.20]	0.15
Unswitched memory			continuous	0.75 [0.59, 0.95]	**0.02**	0.63 [0.46, 0.86]	**<0.01**
	56 (23)	1-6	low	1 (Ref)		1 (Ref)	
	56 (21)	7-15	intermediate	0.94 [0.52, 1.7]	0.84	0.89 [0.47, 1.67]	0.72
	56 (10)	16-126	high	0.37 [0.18, 0.79]	**<0.01**	0.30 [0.13, 0.69]	**<0.01**
Class switched memory			continuous	0.86 [0.77, 0.97]	**0.02**	0.80 [0.69, 0.93]	**<0.01**
	56 (22)	1-17	low	1 (Ref)		1 (Ref)	
	56 (20)	18-39	intermediate	0.87 [0.47, 1.59]	0.65	0.74 [0.39, 1.42]	0.37
	56 (12)	40-398	high	0.48 [0.24, 0.96]	**0.04**	0.33 [0.14, 0.77]	**0.01**
All memory			continuous	0.89 [0.82, 0.97]	**<0.01**	0.84 [0.75, 0.93]	**<0.01**
	56 (21)	2-26	low	1 (Ref)		1 (Ref)	
	56 (22)	27-60	intermediate	1.05 [0.58, 1.92]	0.86	0.88 [0.46, 1.69]	0.70
	56 (11)	61-447	high	0.47 [0.23, 0.97]	**0.04**	0.35 [0.15, 0.83]	**0.02**
$CD43^+/CD27^+$			continuous	0.96 [0.77, 1.18]	0.67	0.84 [0.61, 1.16]	0.29
	56 (20)	1-5	low	1 (Ref)		1 (Ref)	
	56 (19)	6-12	intermediate	0.86 [0.46, 1.62]	0.65	0.88 [0.43, 1.80]	0.72
	56 (15)	13-116	high	0.68 [0.35, 1.33]	0.26	0.71 [0.33, 1.50]	0.36

Cox proportional hazard models are presented for different B-cell (subtype) numbers. In the continuous model, the hazard rate [95% confidence interval (CI)] and *p*-values are indicated per increase of 10 cells/µL. In the other models, the intermediate or high tertile is compared to the low tertile in a univariable model or multivariable model adjusted for age, sex, smoking, history of coronary artery disease and glomerular filtration rate.

with a secondary CVD event compared to those without (18247 [IQR 8809, 35064] vs. 16982 [IQR 8251, 33146] RLU/100ms, p=0.67 for IgG-α-oxLDL, and 20328 [IQR 11706, 29514] vs. 22111 [IQR 13041, 29698] RLU/100ms, p=0.72 for IgM-α-oxLDL). When assessing IgG-α-oxLDL antibodies in a Cox regression model no association with the risk for secondary CVD events was found (HR 1.01 [95% CI 0.98 - 1.03], p=0.67; data not shown).

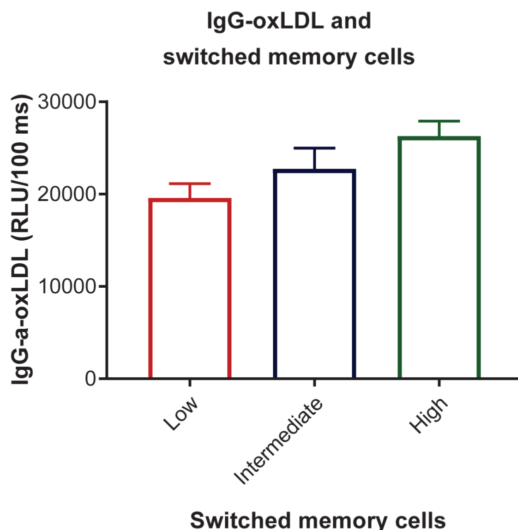

Figure 3. Serum IgG autoantibody titers directed against oxLDL correlate with the numbers of circulating class switched memory cells. Patients are divided in tertiles by numbers of class switched memory cells and corresponding IgG-α-oxLDL titers are shown ($p<0.01$). Data are presented as mean and error bars indicate standard error of the mean (SEM). Ig indicates immunoglobulin; oxLDL, oxidized low-density lipoprotein.

DISCUSSION

We investigated the association of circulating B cell subsets with the occurrence of secondary cardiovascular events in severe carotid atherosclerotic patients undergoing carotid endarterectomy. We found that high levels of (un)switched memory cells were independently associated with the freedom of recurrent cardiovascular events, suggesting that patients with high numbers of (un)switched B cells are protected against secondary cardiovascular manifestations.

Patients with auto-immune diseases, such as SLE and CVID, have an increased risk of developing CVD. Production of (auto)antibodies by B lymphocytes is a hallmark of auto-immune disease and auto-antibody formation is also evident in atherosclerotic patients. SLE patients have reduced levels of both switched and unswitched memory cells[19], and decreased levels of unswitched memory cells are associated with increased levels of SLE auto-antibodies[19]. Furthermore, in patients with common variable immunodeficiency (CVID) levels of switched memory cells are lower compared to controls[20]. As such, low levels of switched and unswitched memory B cells are associated with an unfavorable inflammatory status and, as we show here for the first time, with secondary cardiovascular events in patients with severe atherosclerosis.

Similar to our observations regarding the memory B cells, it has previously been shown that patients with high $CD40^{+}$ B cell percentages exhibit a lower stroke risk[22]. Accordingly,

we also observed a lower rate of cerebrovascular events in patients with high memory cell numbers (0.6%) compared to patients with low numbers (3.6%). However, due to the low prevalence of cerebrovascular events within our population we were not sufficiently powered to confirm these findings statistically.
The role of IgM antibodies in cardiovascular disease has gained much attention in the last decades. In mouse models, it was shown that IgM secreted by B1 B cells was responsible for their atheroprotective effect[28]. In contrast to their role in mouse studies[28, 33], $CD43^+$ B cells, suggested to resemble B1 cells in mice[15], did not associate with protection against secondary events in our study. Contrasting results have been reported for the association of serum IgM levels with primary cardiovascular events[51-53]. In our study, patients with high numbers of unswitched memory cells expressed more IgM on their cell surface, suggesting an increased production and release of IgM antibodies. However, it remains to be elucidated if the increase of surface IgM expression translates to higher circulating IgM antibody levels and to which antigens this IgM is directed. To investigate if the protective effect of memory B cells involves autoantibodies directed against oxLDL, we measured oxLDL specific titers of IgM and IgG. IgM-α-oxLDL antibody titers were not different between patients with and without a follow-up event, nor were they associated with the number of unswitched memory B cells. The number of class switched memory cells did show significant correlations with serum IgG-α-oxLDL antibody titers, indicating that the switched memory cells might indeed be responsible for the production of IgG-α-oxLDL antibodies. However, IgG-α-oxLDL titers were not associated with the occurrence of secondary CVD events, suggesting that oxLDL antibodies are not responsible for the protective effect of memory B cells in our population of severe atherosclerotic patients. These data are in line with other researchers which also show no association with secondary CVD events[54, 55], but do not corroborate with studies showing oxLDL antibodies to be associated with occurrence and severity of CVD[34-37]. Apart from their pivotal role in antibody generation, B cells can also regulate T cell responses and produce cytokines, suggesting a more intricate role in cardiovascular disease[14, 56].
In conclusion, we show that high numbers of unswitched and switched memory B cells are associated with lower risk of secondary cardiovascular events in a population with severe cardiovascular disease. These findings are based on a relative small patient cohort and should be established in larger patient cohorts. However, our findings do suggest a potential role for B cell subsets in prediction and prevention of secondary cardiovascular events in patients with atherosclerosis.

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Disclosures

None.

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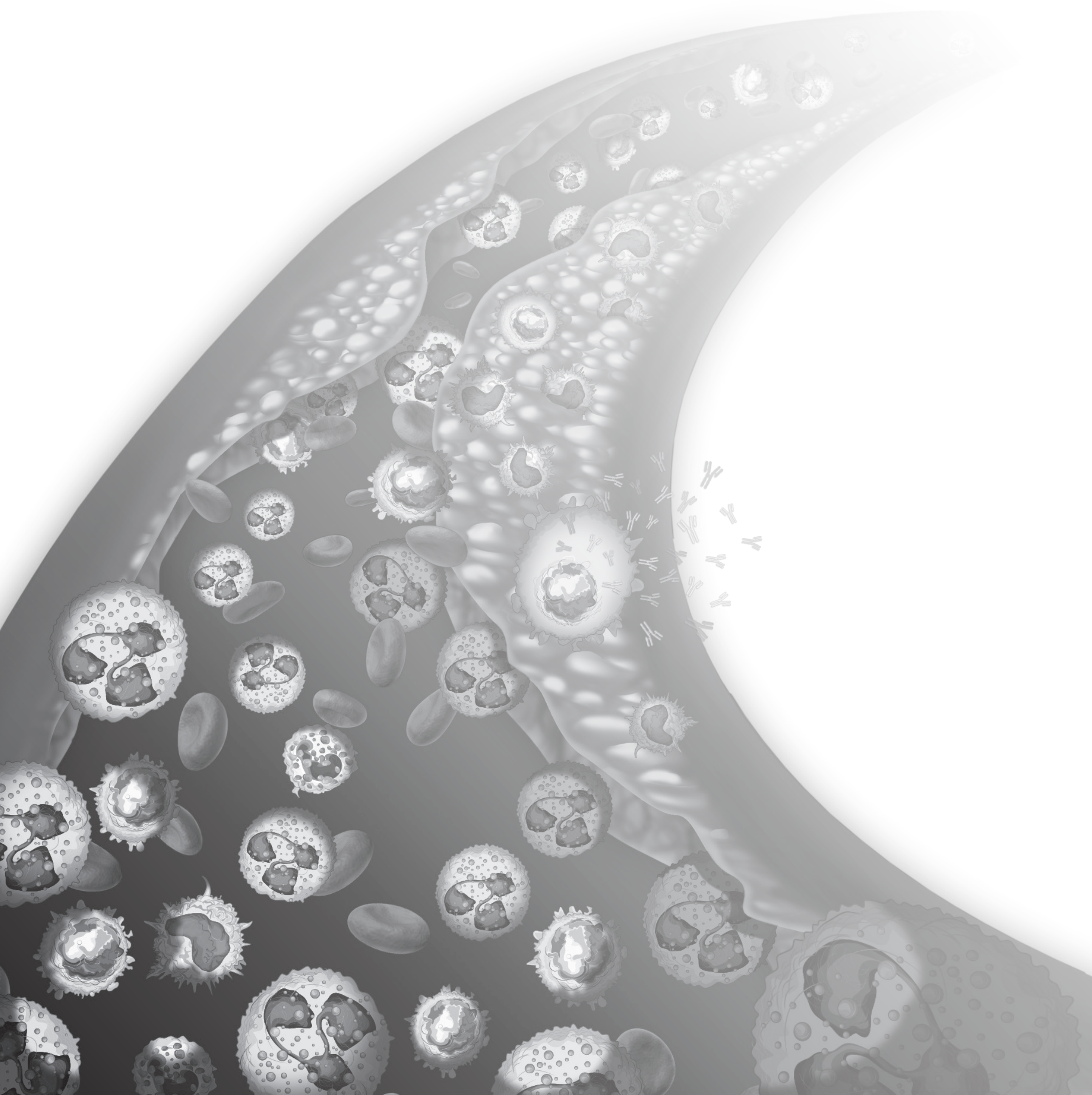

CHAPTER

5

Serum IgG4 levels prognosticate secondary cardiovascular events in females with severe atherosclerosis

In preparation

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ABSTRACT

Background

Atherosclerotic cardiovascular disease (CVD) results from high lipid levels and chronic inflammation. The mechanisms underlying the acute manifestations of CVD, like plaque rupture or erosion, are not completely understood. Nonetheless, clear differences between men and women have been observed in the pathology and clinical presentation of CVD. Antibodies have been investigated for a long time in CVD, especially anti-oxidized low-density lipoprotein (LDL) (auto)antibodies have gained a lot of attention. More recently, Immunoglobulin G4 (IgG4) related inflammation has been suggested to increase the risk of atherosclerosis. However, longitudinal studies examining the prognostic value of serum IgG4 antibodies are lacking. We therefore investigated the association between IgG4 levels and the occurrence of cardiovascular events in men and women with severe atherosclerotic disease, undergoing carotid endarterectomy (CEA). In addition, we investigated if the presence of $IgG4^+$ deposits was associated with a vulnerable plaque phenotype.

Methods and results

We measured total serum IgG and IgG4 with a luminex assay in 632 CEA patients from the Athero-Express biobank. We performed sex stratified Cox regression analyses, adjusted for age and smoking status, to investigate the association between IgG4 and adverse CVD manifestations during three-year follow-up. The risk for secondary adverse events was approximately four times higher in females with a high IgG4/IgG ratio as compared to females with a low IgG4/IgG ratio (hazard rate HR 4.38 [95% confidence interval (CI) 1.23 - 15.57], *p*= 0.02). We observed no differences in risk for future CVD events in males between the lowest and highest tertile of IgG4/IgG (HR 1.06 [95% CI 0.53 - 2.13], *p*=0.87). Moreover, we found that IgG4 positive deposits were enriched in advanced plaques from female, but not in male carotid autopsy sections.

Conclusion

We show that serum IgG4 levels are associated with the risk for secondary cardiovascular events in females, but not in males, undergoing CEA, suggesting that serum IgG4 may prove a valuable biomarker for secondary cardiovascular manifestations specifically in women.

INTRODUCTION

Atherosclerosis is the most prevalent underlying pathology of cardiovascular disease (CVD), leading to approximately 17.9 million deaths annually, which is mainly attributed to ischemic heart disease and stroke[1]. The expected growing burden of CVD is a major public health concern. Moreover, differences between male and female pathology and clinical manifestations are much more appreciated nowadays and it is important to characterize these differences in order to better understand and treat cardiovascular disease in both men and women[2-4].
Atherosclerosis is characterized by chronic inflammation in the context of high lipid levels[5-7]. Different (auto)antibodies have been implicated in atherosclerosis. A recent systematic review concluded that high levels of anti-cardiolipin, IgG-oxLDL, unspecified anti-cyclic citrullinated protein, and anti-human Heat Shock Protein 60 IgA autoantibodies are associated with increased risk of cardiovascular outcome. On the other hand, a protective effect of anti-phosphorylcholine IgM autoantibodies was observed for cardiovascular outcome[8].
IgG4 is the least abundant (<5%) antibody subclass within the IgG type of antibodies. In contrast to the other IgG subclasses, the IgG4 subclass is characterized by Fab-arm exchange, which results in the formation of heterobivalent antibodies. These heterobivalent antibodies can bind two different antigens, but are often unable to form immune complexes by crosslinking. IgG4-related disease (IgG4-RD), is a systemic fibro inflammatory disease and every organ in the body can be affected. IgG4-RD is characterized by the presence of tumour like lesions, $IgG4^+$ plasma cell infiltrates and storiform fibrosis[9]. During the past years, the association between IgG4 and aortic atherosclerosis became a target for investigation. For example, in a small study investigating the IgG4 response in patients with chronic infectious aortitis, it was shown that $IgG4^+$ plasma cell infiltration was increased in the presence of aortic atherosclerosis[10]. In addition, a positive association between aortic wall area, a surrogate marker for vascular remodelling and atherosclerosis, and IgG4 levels has been observed in patients with periaortitis[11] and in patients undergoing cardiac computed tomography[12]. Moreover, serum IgG4 levels were elevated in patients with coronary artery disease (CAD) as compared to non-CAD controls (median 39.3 mg/dL, [IQR 21.5, 66.5] vs. 27.0 mg/dL [IQR 14.9, 45.1], $p<0.01$)[13].
Although these findings point to an important role for IgG4 antibodies in cardiovascular disease, to the best of our knowledge, their prognostic value has not been described in literature yet. In this study we investigated if circulating IgG4 antibodies can prognosticate adverse cardiovascular events during follow-up in severe atherosclerotic patients from the Athero-Express biobank. In addition, we investigated if the presence of $IgG4^+$ deposits was associated with a vulnerable plaque phenotype in carotid arteries from the Maastricht Pathology Tissue Collection.

MATERIALS AND METHODS

Study populations

Athero-Express biobank

Serum IgG4 was measured in a sub cohort from the Athero-Express biobank, including 632 patients undergoing carotid endarterectomy between 2002 and 2013. Inclusion criteria were presence of serum and follow-up information, and we aimed for equal distribution of male and female subjects. Through random selection 319 male and 313 female patients were selected. Details about inclusion and analysis of patients from the prospective cohort study Athero-Express have been described before[14]. Patients were followed during three years or until the occurrence of major adverse cardiovascular events (MACE), consisting of stroke, myocardial infarction or cardiovascular death. The secondary endpoint, termed as composite event, included coronary interventions (percutaneous coronary interventions and coronary artery bypass grafting) and peripheral interventions (percutaneous transluminal angioplasty, surgical endarterectomy and amputations) in addition to the MACE endpoints. All events were validated using health records kept by general practitioners or by hospital information systems. All patients provided written informed consent. The study protocol conforms to the Declaration of Helsinki and has been approved by the Institution's ethics committee on research on humans.

Maastricht Pathology Tissue Collection

59 carotid autopsy sections from the Maastricht Pathology Tissue Collection (MPTC) were stained for IgG4 and CD138 after informed consent, in agreement with the 'Code for Proper Secondary Use of Human Tissue in the Netherlands' and after approval by the medical and ethical committee of Maastricht University Medical Centre, Maastricht, The Netherlands.

Luminex assay immunoglobulins

Serum levels of IgG4 and total IgG were determined simultaneously using a Bioplex bead based human isotyping assay (BioRad, Hercules, CA, USA). Briefly, serum was diluted 40,000 times and incubated for 1 hour with beads pre-coated with isotype specific antibodies at room temperature under continuous shaking. After washing, biotinylated detection antibodies were added to the assay and left to incubate for 30 minutes at room temperature under continuous shaking. Subsequently, another washing step was performed. Then, Phycoerythrin (PE)-conjugated streptavidin was added and left to incubate for 10 minutes at room temperature under continuous shaking. Once more, a washing step was performed. Next, the beads were resuspended in assay buffer. Subsequent data acquisition and analyses were performed with the Bio-Plex-200 flow cytometry analyzer (BioRad, Hercules, CA, USA) complemented with Bio-Plex Manager 6.1.1 software.

IgG4 and CD138 staining in plaque

Carotid autopsy material from the Maastricht Pathology Tissue Collection (MPTC) was collected, fixed in paraformaldehyde, embedded in paraffin and sections were staged by histological analyses of adjacent HE stained sections according to Virmani *et al.*[15], (IT indicates intimal thickening (n=22); PIT, pathological intimal thickening (n=12); TkFCA, thick fibrous cap atheroma (n=16) and IPH, lesions with thrombus or intraplaque hemorrhage (n=9)). In some cases, different plaque stages from a single plaque of one individual patient were collected and used for analysis. Antigen retrieval was performed by boiling in EDTA buffer (1mM EDTA in distilled water, pH 8.0) for 20 minutes. Unspecific binding sites were blocked for 15 minutes using Ultra Vision block buffer (Thermo Fisher). First, slides were incubated with mouse α human CD138 (MCA681 clone, Bio-Connect, Huissen, The Netherlands) for 1 hour at room temperature. After gentle washing in PBS, slides were incubated with secondary goat α mouse alkaline phosphatase conjugated antibodies for 30 minutes at room temperature (Immunologic, Duiven, The Netherlands). Antibody binding was visualized using liquid permanent red (DAKO, Santa Clara, USA). Subsequently, slides were further processed to stain for IgG4. After boiling the sections again for 10 min in EDTA buffer, sections were incubated overnight at 4°C with mouse α human IgG4 (Invitrogen, Waltham, MA USA) followed by incubation with alkaline phosphatase conjugated goat α mouse antibodies. Signal was detected using vector blue (Vector laboratories, Burlingame, CA USA) and sections were embedded in ClearVue Mountant (ThermoFisher Scientific, Waltham, MA USA).

Statistical analyses

Normally distributed continuous variables were indicated as mean ± SD and compared by Student's *t* tests or a one-way ANOVA. Non-normal distributed data were presented as median [interquartile range] and compared by Mann-Whitney *U* or Kruskal-Wallis tests. Categorical variables were indicated as percentages and compared by Chi-squared or Fisher's exact tests. Cardiovascular risk factors were compared across tertiles of serum IgG4 due to their skewed distribution.

Univariable and adjusted Cox proportional hazard models were used to study the association of absolute and relative serum IgG4 levels with occurrence of secondary cardiovascular events over time. The proportional hazard assumption was tested using statistical tests and visual inspection of Schoenfeld residuals. Because of the established role of sex in immunology and cardiology, we also tested for a sex interaction between the association of serum IgG4 levels and cardiovascular endpoints. Covariates for multivariable Cox regression analyses were based on literature and previous knowledge, including age and smoking. To visualize this association, subjects were divided into tertiles according to the absolute numbers of serum IgG4 levels and plotted against the survival of secondary cardiovascular events over time. In addition, the dose responsive association between IgG4 levels and the hazard ratio was evaluated. Data management and statistical analyses were performed with RStudio[16] and the R software package[17] (version 3.2.0., Vienna, Austria). *P*-values <0.05 were considered as significant.

RESULTS

Patients from the Athero-Express biobank

The current study included 632 patients from the Athero-Express biobank. In 618 patients, IgG4 was measured successfully. Of these 618 patients, 50% (n=309) was female. Secondary adverse events during three-year follow-up, consisting of cardiovascular death, stroke and coronary events, occurred more often in males 15.2% (n=47), than in females 7.4% (n=23). Overall baseline characteristics are depicted in **supplemental table 1**. In addition, baseline characteristics for males and females, stratified by the occurrence of adverse cardiovascular manifestations during follow-up are shown in **table 1**.

Serum IgG and IgG4 levels at baseline

Circulating median [interquartile range (IQR)] IgG levels were 8.7 mg/L [IQR 6.63, 12.25], IgG4 levels were 19.6 mg/dL [IQR 7.9, 42.9] and the IgG4 to IgG ratio was 2.3% [IQR 1.0, 4.2]. These levels are comparable to those reported in earlier studies[13, 18, 19]. Total IgG levels were lower in males than females (7.8 mg/L [IQR 6.1, 11.5] vs. 9.5 [IQR 7.5, 13.1], p<0.01). However, males tended to have higher absolute IgG4 levels than females (21.9 mg/dL [IQR 9.9, 43.6] vs. 18.3 mg/dL [IQR 6.9, 41.4], p=0.05), which was also reflected in relative IgG4 levels (2.6 % [IQR 1.5, 4.7] in males vs. 1.8 % [IQR 0.8, 3.7] in females, p<0.01). Patients in the highest tertile of relative IgG4 levels were likely to be older than patients in the lowest tertile (70.0 ± 9.5 years vs. 67.4 ± 8.7 years, p=0.01). In addition, patients in the highest tertile of IgG4 (% of IgG) tended to have a history of peripheral artery occlusive disease more often than patients in the lowest tertile (25.6% vs. 16.3%, p=0.06; **Table 2**). Next, we investigated if serum levels of IgG, IgG4 and the IgG4/IgG ratio were different between patients with or without three-year follow-up events. Total IgG levels were comparable between men experiencing follow-events (**Table 3**). Serum IgG4 levels were also not significantly different in males without a follow-up event as compared to males without a follow-up event (20.5 mg/dL [IQR 10.0, 43.6] vs 27.8 mg/dL [IQR 10.9, 45.3], p=0.42; **Table 3**). Likewise, the IgG4/IgG ratio was comparable between men with and without events (3.5 % [IQR 1.6, 4.8] vs. 2.5 % [IQR 1.5, 4.6], p=0.29; **Table 3**). While total IgG levels were comparable in females with and without follow-up events, IgG4 levels tended to be higher in females experiencing secondary CVD events during follow-up as compared to females who did not experience secondary CVD events (32.2 mg/dL [IQR 12.1, 55.9] vs. 17.6 mg/dL [IQR 6.7, 40.1], p=0.07; **Table 3**). Moreover, the IgG4/IgG ratio was significantly higher in females with as compared to females without events (3.2% [IQR 1.6, 5.0] vs. 1.7% [IQR 0.8, 3.6], p=0.03; **Table 3**).

Cox regression analyses for three-year follow-up

Because of the observed differences in IgG4 levels between males and females (**Table 3**), we decided to evaluate the association between IgG4 levels and secondary CVD events in a sex stratified manner. To determine if IgG4 specifically prognosticates secondary cardiovascular events or merely reflects total IgG antibody levels, total serum IgG levels were also evaluated for their association with secondary CVD manifestations.

Table 1. Patient characteristics stratified by sex and the occurrence of major adverse CVD events during follow-up.

	Males			Females		
	No Events (n = 262)	**Events (n=47)**	***p***	**No Events (n = 277)**	**Events (n=23)**	***p***
Age (years)	67.5 ± 8.2	73.4 ± 8.3	<0.01	68.4 ± 10.0	71.7 ± 11.1	0.13
Current smoker	93 (35.6%)	21 (44.7%)	0.31	107 (38.1%)	9 (40.9%)	0.97
BMI	26.3 ± 3.6	26.8 ± 3.6	0.33	26.5 ± 4.8	26.3 ± 4.8	0.92
Contralateral stenosis	113 (45.2%)	25 (56.8%)	0.21	99 (39.0%)	13 (61.9%)	0.07
Diabetes mellitus	47 (17.9%)	12 (25.5%)	0.31	62 (21.7%)	6 (26.1%)	0.82
Hypercholesterolemia	163 (64.9%)	23 (54.8%)	0.27	183 (69.1%)	13 (68.4%)	1.00
Hypertension	223 (85.1%)	41 (87.2%)	0.88	241 (84.3%)	20 (87.0%)	0.97
CAD history	91 (34.7%)	18 (38.3%)	0.76	68 (23.9%)	7 (30.4%)	0.65
Stroke history	70 (26.7%)	16 (34.0%)	0.39	88 (30.8%)	8 (34.8%)	0.87
PAOD history	58 (22.1%)	16 (34.0%)	0.12	46 (16.1%)	5 (21.7%)	0.69
Clinical manifestations			0.13			0.44
Asymptomatic	56 (21.4%)	4 (8.5%)		38 (13.3%)	5 (21.7%)	
TIA	116 (44.3%)	25 (53.2%)		120 (42.1%)	6 (26.1%)	
Stroke	52 (19.8%)	13 (27.7%)		77 (27.0%)	7 (30.4%)	
Ocular	38 (14.5%)	5 (10.6%)		50 (17.5v)	5 (21.7%)	
Medication						
Statins	188 (71.8%)	33 (70.2%)	0.97	217 (76.1%)	20 (87.0%)	0.35
Beta-blockers	108 (41.2%)	25 (53.2%)	0.17	135 (47.4%)	10 (43.5%)	0.89
Anticoagulants	25 (9.5%)	9 (19.1%)	0.09	26 (9.1%)	2 (8.7%)	1.00*
Laboratory parameters						
Total cholesterol (mmol/L)	3.9 [3.2, 4.8]	3.5 [3.1, 3.9]	0.05	4.2 [3.5, 5.2]	3.9 [3.5, 4.9]	0.32
HDL cholesterol (mmol/L)	1.0 [0.8, 1.2]	0.9 [0.8, 1.0]	0.05	1.1 [0.9, 1.3]	0.9 [0.7, 1.1]	**0.01**
LDL cholesterol (mmol/L)	2.1 [1.5, 2.7]	2.0 [1.6, 2.3]	0.38	2.2 [1.6, 3.0]	2.4 [2.2, 2.7]	0.62
Triglycerides (mmol/L)	1.4 [0.9, 2.0]	1.4 [1.0, 1.9]	0.86	1.5 [1.1, 2.0]	1.5 [1.2, 1.6]	0.67
hsCRP (µg/mL)	7.6 ± 30.5	43.5 ± 203.9	0.01	11.1 ± 35.8	18.7 ± 42.8	0.41
GFR MDRD (mL/min)	72.4 ± 18.5	69.6 ± 18.3	0.35	71.4 ± 19.9	58.3 ± 23.6	**<0.01**

Values are presented as mean ± SD for normal distributions, number of patients (frequency in percentage) for categorical variables, and median [interquartile range] for non-normal distributions. *P*-values are calculated using Student's *t* tests, Chi-squared or Fisher's exact tests (*), and Mann-Whitney *U* tests, respectively. BMI indicates body-mass index; Contralateral stenosis, 50-100% stenosis of the contralateral carotid artery; CAD history, history of Coronary Artery Disease; PAOD history, history of peripheral artery occlusive disease; TIA, transient ischemic attack; Ocular, temporary loss of vision (amaurosis fugax); HDL, high-density lipoprotein; LDL, low-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; GFR MDRD, Glomerular filtration rate according to 'Modification of Diet in Renal Disease' formula.

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Table 2. Baseline characteristics stratified per tertile of relative serum IgG4 levels.

Tertiles of IgG4 (% of IgG)	Low n=203	Intermediate n=203	High n=203	*p*
Age (years)	67.4 ± 8.7	68.0 ± 9.5	70.0 ± 9.5	**0.01**
Sex (% male)	75 (36.9%)	115 (56.7%)	116 (57.1%)	**<0.01**
Current smoker	86 (42.6%)	70 (35.2%)	74 (36.6%)	0.27
BMI	26.4 ± 4.8	26.2 ± 3.6	26.5 ± 4.2	0.76
Contralateral stenosis	74 (39.8%)	88 (46.8%)	84 (44.7%)	0.37
Diabetes mellitus	36 (17.7%)	44 (21.7%)	44 (21.7%)	0.52
Hypercholesterolemia	131 (67.9%)	135 (72.6%)	112 (58.9%)	**0.02**
Hypertension	166 (81.8%)	178 (87.7%)	172 (84.7%)	0.25
CAD history	56 (27.6%)	62 (30.7%)	62 (30.5%)	0.74
Stroke history	56 (27.6%)	64 (31. 5%)	58 (28.6%)	0.66
PAOD history	33 (16.3%)	39 (19.3%)	52 (25.6%)	0.06
Clinical manifestations				0.23
Asymptomatic	30 (14.8%)	44 (21.7%)	28 (13.9%)	
TIA	94 (46.3%)	75 (36.9%)	94 (46.5%)	
Stroke	49 (24.1%)	47 (23.2%)	49 (24.3%)	
Ocular	30 (14.8%)	37 (18.2%)	31 (15.3%)	
Medication				
Statins	152 (74.9%)	155 (76.7%)	143 (70.4%)	0.33
Beta-blockers	90 (44.3%)	93 (46.0%)	92 (45.3%)	0.94
Anticoagulants	19 (9.4%)	18 (8.9%)	25 (12.3%)	0.47
Laboratory parameters				
Total cholesterol (mmol/L)	4.2 [3.4, 5.2]	4.0 [3.3, 4.9]	3.9 [3.3, 4.8]	0.52
HDL cholesterol (mmol/L)	1.1 [0.9, 1.3]	1.0 [0.8, 1.2]	1.0 [0.9, 1.3]	0.05
LDL cholesterol (mmol/L)	2.2 [1.4, 2.9]	2.1 [1.7, 2.8]	2.2 [1.6, 2.8]	0.94
Triglycerides (mmol/L)	1.5 [1.0, 2.0]	1.5 [1.1, 2.0]	1.3 [1.0, 1.9]	0.39
hsCRP (µg/mL)	9.7 ± 34.3	6.9 ± 25.0	20.7 ± 105.0	0.10
GFR MDRD (mL/min)	72.0 ± 18.3	69.7 ± 20.2	72.1 ± 19.8	0.39

Values are presented as mean ± SD for normal distributions, number of patients (frequency in percentage) for categorical variables, and median [interquartile range] for non-normal distributions. *P*-values are calculated using one-way ANOVA, Chi-squared tests, and Kruskal-Wallis tests, respectively. BMI indicates body-mass index; Contralateral stenosis, 50-100% stenosis of the contralateral carotid artery; CAD history, history of Coronary Artery Disease; PAOD history, history of peripheral artery occlusive disease; TIA, transient ischemic attack; Ocular, temporary loss of vision (amaurosis fugax); HDL, high-density lipoprotein; LDL, low-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; GFR MDRD, Glomerular filtration rate according to 'Modification of Diet in Renal Disease' formula.

Table 3. Serum IgG4 and total IgG levels for males and females without or with major CVD events during three-year follow-up.

	Males			Females		
	No Events (n = 262)	Events (n=47)	p	No Events (n = 286)	Events (n=23)	p
Total IgG (mg/L)	7.9 [6.0, 11.6]	7.8 [6.3, 10.7]	0.88	9.6 [7.5, 13.1]	9.00 [7.5, 10.7]	0.45
IgG4 (mg/dL)	20.5 [10.0, 43.6]	27.8 [10.9, 45.3]	0.42	17.6 [6.7, 40.1]	32.2 [12.1, 55.9]	0.07
IgG4 (% of IgG)	2.5 [1.5, 4.6]	3.5 [1.6, 4.8]	0.29	1.7 [0.8, 3.6]	3.2 [1.6, 5.0]	**0.03**

Because the values are not normally distributed, values are presented as median [Interquartile range]. *P*-value is derived from the Mann-Whitney *U* test.

Females in the highest tertile of absolute serum IgG4 levels had significantly higher risk for three-year follow-up events in both univariable (HR 4.51 [95% confidence interval (CI) 1.27 - 15.99], *p*=0.02) and age and smoking adjusted (HR 3.97 [95% CI 1.10 - 14.32], *p*=0.04) Cox regression models, as compared to females in the lowest tertile of serum IgG4. Moreover, relative serum IgG4 (% of total IgG) levels were associated with even higher risk for three-year adverse cardiovascular events in univariable (HR 4.80 [95% CI 1.37 - 16.86], *p*=0.01) and adjusted (HR 4.38 [95% CI 1.23 - 15.57], *p*=0.02) Cox proportional hazard models. Interestingly, in contrast to males (**Figure 1E** and **2E)**, females with low IgG4 levels (<20mg/dL or <2% of total IgG) appear to be better protected against secondary manifestations (**Figure 2F**) during the first 4 months after CEA procedure (in the first tertile; **Figure 1F**). Though, it has to be mentioned that continuous analyses of absolute and relative IgG4 showed no significant association with adverse CVD outcome, which is possibly due the relatively low event rate in the female population (**Table 4**). Compared to females in the lowest tertile of total IgG, females in the highest tertile of total IgG were also not at increased risk for future CVD events in univariable (HR 0.57 [95% CI 0.19 - 1.70], *p*=0.31) and age and smoking adjusted (HR 0.40 [95% CI 0.12 - 1.31], *p*=0.13) Cox regression analyses (**Figure 1C, 2C** and **table 4**).

Males in the highest tertile of total serum IgG levels had comparable risk for secondary CVD events as compared to males in the lowest tertile of serum IgG in univariable (HR 0.90 [95% CI 0.44 - 1.85], *p*=0.32) or in age and smoking adjusted Cox regression analyses (HR 1.05 [95% CI 1.21 - 1.31], *p*=0.55; **Figure 1B** and **table 4**). Likewise, males in the highest tertile of serum IgG4 levels showed no increased risk as compared to patients in the lowest tertile in univariable (HR 1.26 [95% CI 0.63 - 2.49], *p*=0.51) or in age and smoking adjusted Cox regression models (HR 1.08 [95% CI 0.54 - 2.16], *p*=0.82; **Figure 1E, 2E** and **table 4**). In addition, relative IgG4 levels were not associated with adverse events during one-year follow-up in univariable (HR 1.51 [95% CI 0.76 - 2.98], p=0.24) or in age and smoking adjusted Cox regression models (HR 1.06 [95% CI 0.53 - 2.13], *p*=0.87; **Figure 1H, 2H** and **table 4**).

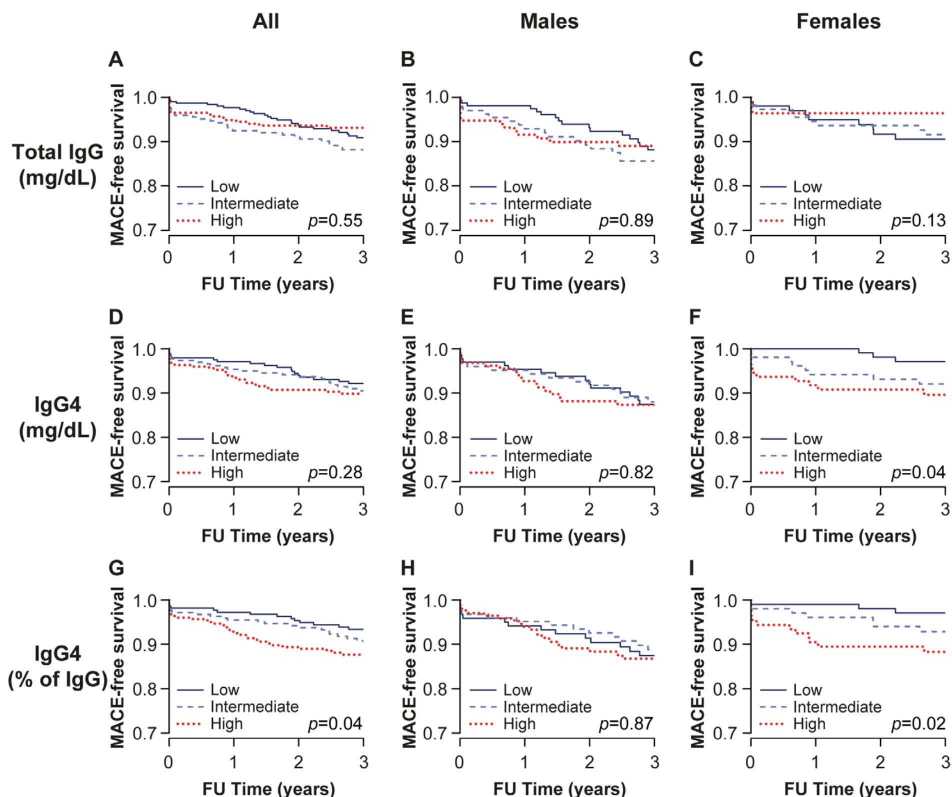

Figure 1. **Cox regression analyses show the association between total serum IgG or IgG4 levels and the occurrence of major adverse cardiovascular events during three-year follow-up.** For each analysis, patients were divided in equal tertiles with low, intermediate and high levels of serum IgG or IgG4. FU time indicates follow-up time. *P*-values are derived from age, sex (when applicable) and smoking status adjusted Cox regression models, comparing patients in the highest to the lowest tertile.

An important assumption for Cox regression analyses is that the risk for outcome for a given variable is stable over time (Cox proportional hazards assumption). Although statistical tests and graphical inspection of the Schoenfeld residuals indicated that the proportional hazards assumption for three-year follow-up was violated for total IgG levels in males (p=0.09) and females (p<0.01), we show the results in order to compare them with the hazard ratios for absolute and relative IgG4 levels, as for IgG4 the proportional hazards assumptions were satisfied (all p-values >0.20).

In addition, we explored whether comparable results were obtained when we included (surgical) vascular interventions to the cardiovascular end points (composite endpoints). However, statistical and graphical inspection of the Schoenfeld residuals indicated that the assumption of proportional hazard was violated in females for absolute (p=0.08) and relative (p=0.03) IgG4 levels. Therefore, no reliable results could be obtained from Cox regression analyses of absolute IgG4 levels or the IgG4/IgG ratio.

Table 4. Cox regression analyses for major adverse CVD events during three-year follow-up.

Males and females		**Events (nr)**	**Unadjusted HR [95% CI]**	***p***	**Adjusted HR [95% CI]**	***p***
Total IgG (mg/dL)	Continuous	70	0.73 [0.48 - 1.14]	0.17	0.80 [0.53 - 1.21]	0.29
	Low	25	REF		REF	
	Intermediate	27	1.10 [0.64 - 1.90]	0.72	1.41 [0.81 - 2.47]	0.22
	High	18	0.74 [0.40 - 1.35]	0.32	0.83 [0.44 - 1.55]	0.55
IgG4 (mg/dL)	Continuous	70	1.07 [0.87 - 1.31]	0.54	1.01 [0.80 - 1.27]	0.96
	Low	18	REF		REF	
	Intermediate	23	1.33 [0.72 - 2.46]	0.37	1.22 [0.65 - 2.29]	0.54
	High	29	1.75 [0.97 - 3.15]	0.06	1.39 [0.77 - 2.54]	0.28
IgG4 (% of IgG)	Continuous	70	1.19 [0.98 - 1.77]	0.08	1.05 [0.85 - 1.28	0.67
	Low	14	REF		REF	
	Intermediate	21	1.55 [0.79 - 3.06]	0.20	1.40 [0.70 - 2.77]	0.34
	High	35	2.75 [1.48 - 5.12]	**<0.01**	1.98 [1.05 - 3.75]	**0.04**
Males		**Events (nr)**	**Unadjusted HR [95% CI]**	***p***	**Adjusted HR [95% CI]**	***p***
Total IgG (mg/dL)	Continuous	47	0.81 [0.49 - 1.32]	0.39	0.91 [0.58 - 1.43]	0.67
	Low	16	REF		REF	
	Intermediate	17	1.12 [0.56 - 2.21]	0.75	1.33 [0.67 - 2.64]	0.42
	High	14	0.90 [0.44 - 1.85]	0.78	1.05 [1.21 - 4.02]	0.89
IgG4 (mg/dL)	Continuous	47	0.99 [0.75 - 1.32]	0.97	0.97 [0.71 - 1.33]	0.87
	Low	15	REF		REF	
	Intermediate	14	0.95 [0.46 - 1.97]	0.89	0.97 [0.46 - 2.05]	0.94
	High	18	1.26 [0.63 - 2.49]	0.51	1.08 [0.54 - 2.16]	0.82
IgG4 (% of IgG)	Continuous	47	1.09 [0.84 - 1.40]	0.53	0.97 [0.74 - 1.26]	0.82
	Low	14	REF		REF	
	Intermediate	13	0.92 [0.43 - 1.95]	0.82	0.87 [0.40 - 1.85]	0.71
	High	20	1.51 [0.76 - 2.98]	0.24	1.06 [0.53 - 2.13]	0.87
Females		**Events (nr)**	**Unadjusted HR [95% CI]**	***p***	**Adjusted HR [95% CI]**	***p***
Total IgG (mg/dL)	Continuous	23	0.74 [0.35 - 1.55]	0.42	0.62 [0.27 - 1.42]	0.26
	Low	9	REF		REF	
	Intermediate	9	1.00 [0.40 - 2.52]	1.00	0.90 [0.36 - 2.27]	0.82
	High	5	0.57 [0.19 - 1.70]	0.31	0.40 [0.12 - 1.31]	0.13
IgG4 (mg/dL)	Continuous	23	1.15 [0.83 - 1.58]	0.40	1.09 [0.77 - 1.54]	0.64
	Low	3	REF		REF	
	Intermediate	8	2.79 [0.74 - 10.51]	0.13	2.87 [0.76 - 10.87]	0.12
	High	12	4.51 [1.27 - 15.99]	**0.02**	3.97 [1.10 - 14.32]	**0.04**

Table 4. *Continued*

Females		**Events (nr)**	**Unadjusted HR [95% CI]**	***p***	**Adjusted HR [95% CI]**	***p***
IgG4 (% of IgG)	Continuous	23	1.27 [0.93 - 1.74]	0.14	1.21 [0.87 - 1.67]	0.26
	Low	3	REF		REF	
	Intermediate	7	2.41 [0.62 - 9.34]	0.20	2.49 [0.64 - 9.68]	0.18
	High	13	4.80 [1.37 - 16.86]	**0.01**	4.38 [1.23 - 15.57]	**0.02**

Cox proportional hazard ratios are given for the association between total serum IgG, absolute and relative serum IgG4 levels and major adverse cardiovascular events. Results are depicted for the total cohort, and stratified by sex. In addition to continuous hazard rates (per SD), the hazard rates per tertile are given for crude and adjusted Cox regression models. Adjusted models contain age, sex (when males and females were analyzed together) and smoking status as covariates. REF indicates reference.

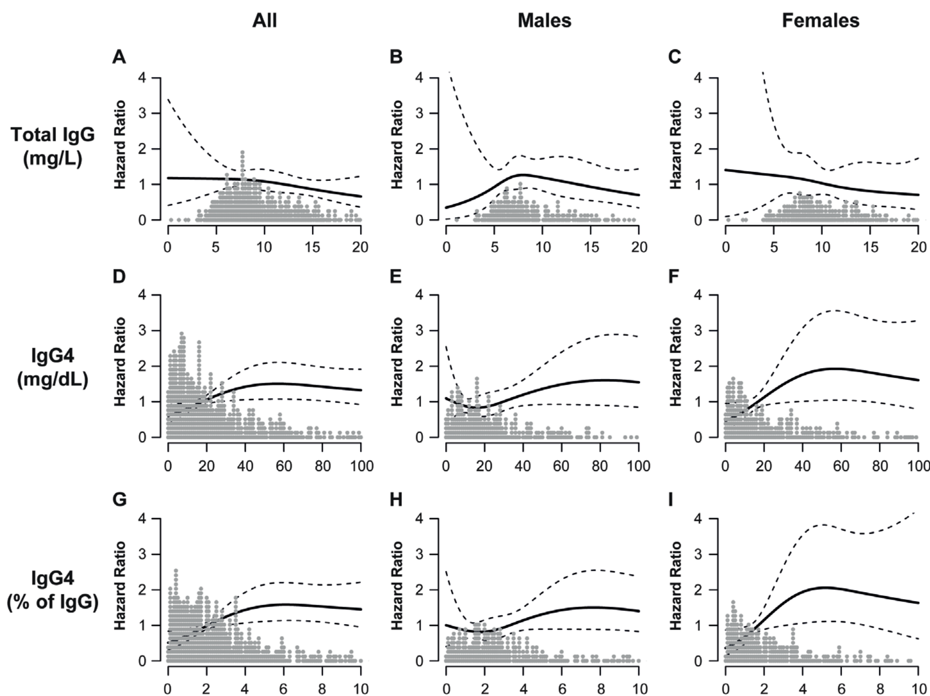

Figure 2. **The association between serum IgG (A-C) and absolute (D-F) or relative (G-I) IgG4 levels and the hazard ratio for major CVD events during three-year follow-up are depicted for all patients (left column), males (middle column) and females (right column).** The grey dots represent the individually measured values. The black lines represent the hazard ratio as a function of the serum IgG levels and the dotted lines show the 95% confidence intervals. Note that female patients with low serum levels of IgG4 (below 20mg/dL, F and below 2%, I) are protected against adverse CVD outcome.

Cox regression analyses for one-year follow-up

Because the risk association between total IgG levels and secondary CVD events changed over time, we also investigated the association between serum IgG levels and adverse outcome during one-year follow-up. Here, the Cox proportional hazard assumptions were satisfied (all *p*-values >0.2), indicating that the risk association between IgG or IgG4 with CVD outcome was stable over time. In line with the three-year follow-up analyses, we observed that females, but not males, with high IgG4 levels compared to females with low serum IgG4 levels were at increased risk for major CVD endpoints, including CVD death, stroke and MI (**Supplemental table 2**). Similar results were observed for composite end points (including (surgical) vascular interventions; **Supplemental table 3**). Because of the higher event rate, confidence intervals were smaller. We observed that males, but not females, in the highest tertile of total IgG levels were at increased risk for major CVD events during one-year follow-up (**Supplemental table 2**). A similar trend for the prognostic value of IgG was observed in males, but not in females, when we included surgical interventions as endpoints (**Supplemental table 3**).

Patients from the Maastricht Pathology Tissue Collection

Since serum IgG4 levels significantly associated with adverse CVD manifestations, we wondered if IgG4 positive plasma cells in atherosclerotic plaques also associated with advanced plaque phenotypes. Although plaques from Athero-Express patients were obtained and stored after carotid endarterectomy, this material mainly consists of intimal plaque with some media, but no adventitial tissue. As plasma cells are predominantly found in the adventitial part of the affected vessel[20], we evaluated carotid autopsy material from the Maastricht Pathology Tissue Collection for the presence of CD138 and IgG4 (Figure 3B and C). In total, 59 carotid autopsy sections were evaluated. The mean age was 77.2 ± 13.5 for females and 72.4 ± 9.6 for males. 25 (42.4%) of the sections were obtained from females. Within the male specimens, sections were classified as intimal thickening (IT, n=12), pathological intimal thickening (PIT, n=8), thick fibrous cap atheroma (TkFCA, n=10) and intraplaque hemorrhage (IPH, n=6) (**Figure 4B**). Within female specimens, 12 IT, 4 PIT, 6 TkFCA and 3 IPH sections were included (**Figure 4C**). CD138 cells were present in 34 plaque sections (57.6%) and IgG4 positive deposits were present in 44 plaque sections (74.6%) (**Figure 3D-I** and **4D-F**).

IgG4 positive deposits are associated with advanced plaque stage in females, but not in males

Although CD138 positive plasma cells positively associated with unstable plaque phenotype, the numbers of $CD138^{+}IgG4^{+}$ double positive plasma cells were very low (<2 plaque sections with double positive cells per plaque stage), and no reliable data analyses could be performed. However, we did find that IgG4 deposits tended to be more frequently present in advanced plaques (TkFCA, 81.2%) as compared to earlier plaque stages (IT, 68.2% and PIT, 75%) or hemorrhaged plaques (IPH, 77.8, *p*=0.83; **Figure 3D-I** and **4D**).

Because IgG4 serum levels were specifically associated with the occurrence of adverse cardiovascular events in females, we also performed sex stratification in plaque analyses. In males, the overall number of IgG4 deposits was higher than in females, but more equally distributed over different plaque stages (**Figure 3E** and **H; 4E** and **H**). In contrast, in females, IgG4 deposits were more frequently found in stable advanced plaques (TkFCA,

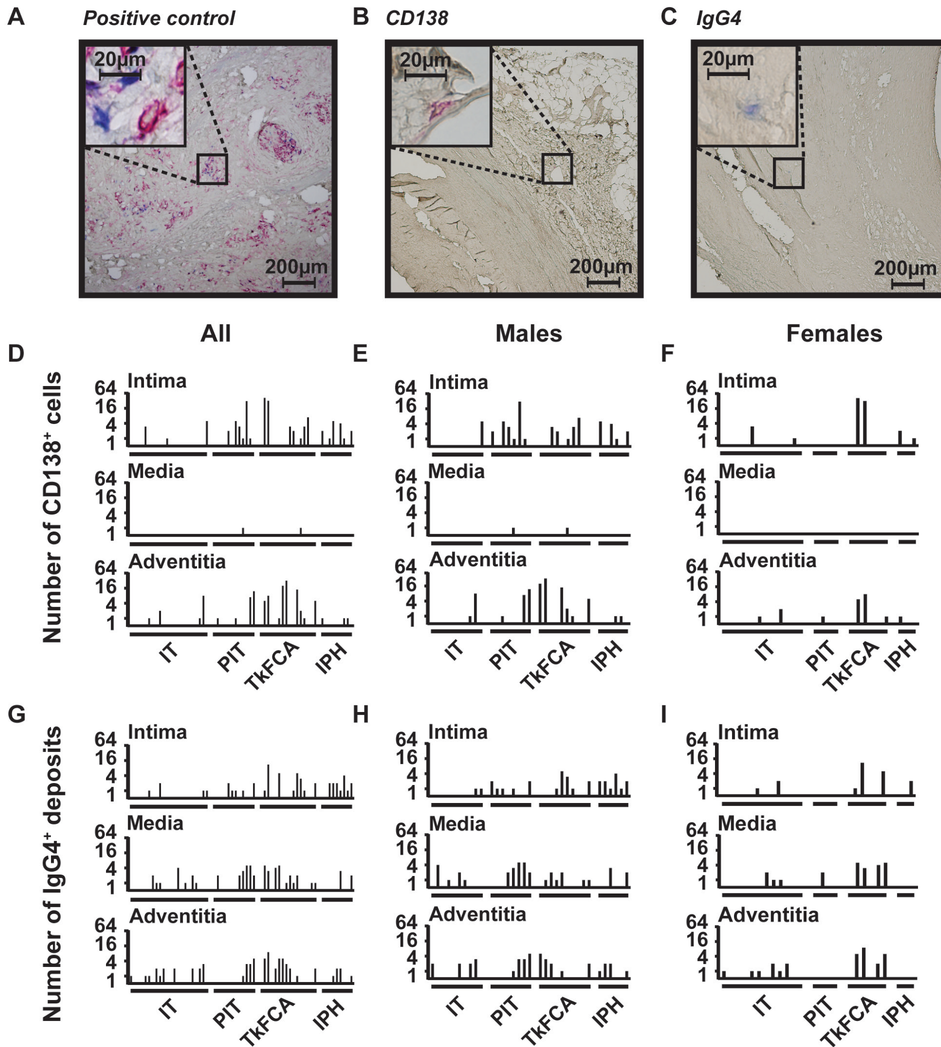

Figure 3. Staining of CD138 positive cells and IgG4 positive deposits in carotid vessels in different plaque stages. Top panel (A-C) shows a staining in positive control tissue (biopsy from orbital IgG4 related disease) (A) and a representative image of a CD138 positive cell (B) and diffuse cytoplasmic staining of an IgG4 positive deposit (C). Results are depicted for the whole population (D and G), for males (E and H) and females (F and I). While IgG4 positive deposits are generally more present in male vessels (H), they were specifically increased in female vessels with thick fibrous cap atheroma (I). Each bar represents an individual section, and no bar indicates no CD138 positive cells or IgG4 positive deposits present.

66.7%) than early plaques (IT, 58.3% and PIT, 25%), or hemorrhaged plaques (IPH, 33.3%; **Figure 3F** and **I; 4F and I),** although this difference was not statistically significant (*p*=0.57). Moreover, the median [interquartile range] IgG4 deposits found in the affected vessels were also higher in advanced plaques (TkFCA, 8.5 [IQR 1.5, 14.0]) than in early plaques (IT, 1.0 [IQR 0.0, 2.0] or PIT 0.0 [IQR 0.0, 0.5]) or in hemorrhaged plaques (IPH 0.0 [IQR 0.0, 1.0]), although not statistically significant, *p*=0.19; (**Figure 4I**). However, IgG4 deposits were significantly increased in the adventitial part of advanced plaques (TkFCA, 3.5 [IQR 0.8, 4.8]) as compared to early plaques (IT, 0.5 [IQR 0.0, 1.0] or PIT 0.0 [IQR 0.0, 0.0]) or hemorrhaged plaques (IPH 0.0 [IQR 0.0, 0.0]), *p*=0.047; (**Figure 3I**).

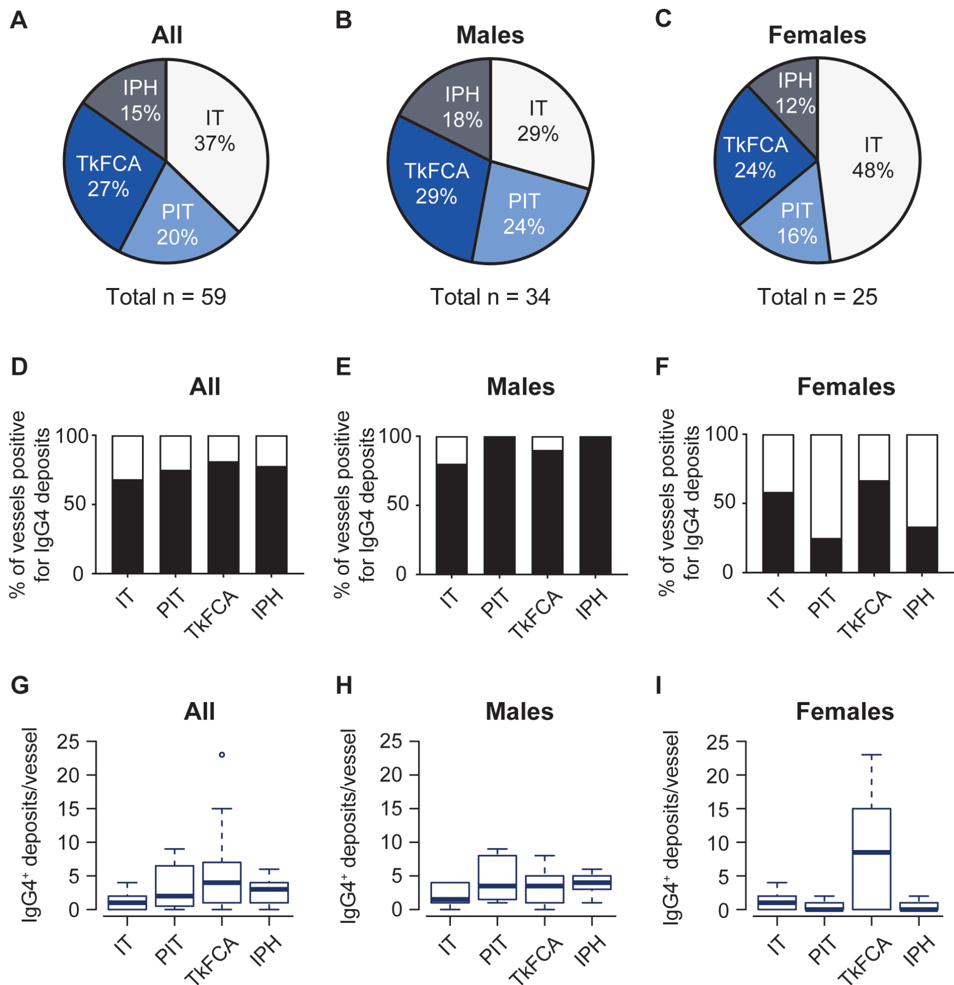

Figure 4. Overview of the plaque stages for all sections (A) and stratified for males (B) and females (C). The percentage of IgG4 positive vessels is shown in D-E. In addition, the number of IgG4 positive deposits per vessel is depicted for all sections (G) and separately in male sections (H) and female sections (I).

5

DISCUSSION

In this study we measured if circulating IgG4 antibodies could prognosticate adverse cardiovascular events during follow-up in severe atherosclerotic patients from the Athero-Express biobank. In addition, we investigated if $IgG4^+$ deposits were associated with a vulnerable plaque phenotype from the Maastricht Pathology Tissue Collection. We found that high serum IgG4 levels were associated with increased risk for cardiovascular events during follow-up in female, but not in male CEA patients. In addition, $IgG4^+$ deposits were more often present in vessels with thick fibrous cap atheroma compared to vessels with (pathological) intimal thickening in females, while in males more IgG4 deposits were present, but these were more equally distributed over the different plaque stages.
In previous work, it was shown that CAD patients had increased IgG4 serum levels compared to non-CAD controls[13]. In line, patients in the highest relative IgG4 (% of IgG) tertile were more likely to have a history of CAD and PAOD as compared to patients in the lowest tertile (30.5% vs. 27.6%, p=0.74 and 25.6% vs. 16.3%, p=0.06, respectively; **Table 2**). Moreover, in agreement with our study, sex stratified analyses in the aforementioned study showed that serum IgG4 predicted the presence of CAD more accurately in females than males (area under the receiver operating curve was 0.672 in women and 0.594 in men, no confidence intervals or p-values were reported)[13].
The underlying differences for the fact that IgG4 specifically prognosticates secondary cardiovascular events in females remain to be established. It is known that the inflammatory responses in males and females differ. Females are considered to be more efficient in clearing primary pathogenic infections[2]. On the other hand, females are also more prone to develop auto-immune diseases such as systemic sclerosis or rheumatoid arthritis[21]. Along this line, it has been postulated that the inflammatory response present in cardiovascular disease in women primarily involves the adaptive immune system, including the formation of autoantibodies, while the inflammatory response in men is more reflected by activation of the innate immune system[2].
We observed that circulating CRP levels were significantly higher in males with secondary CVD events as compared to males without events during three-year follow-up. In contrast, CRP levels were comparable between females with and without adverse manifestations during follow-up (**Table 1**). In line, CRP was significantly associated with the occurrence of CVD events in males (HR per SD 1.57 [95% CI 1.17 - 2.11], p<0.01), but not in females (HR per SD 0.96 [95% CI 0.57 - 1.64], p=0.89). We found no association between serum IgG4 and CRP levels (data not shown). These findings indicate that the mechanisms underlying secondary CVD events may differ between males and females undergoing CEA. Interestingly, albeit in a limited number of samples, we observed that specifically in females, the presence of IgG4 positive deposits was enriched in advanced plaques (thick fibrous cap atheroma), and not in early ((pathological) intimal thickening) or hemorrhaged plaques. Although hemorrhaged plaques have also been shown to predict for adverse outcome, IgG4 positive deposits were less abundant in these vessels. An explanation might be that hemorrhaged plaques are considered older and therefore might become

more calcified. Indeed, a recent study showed that serum IgG4 levels were higher in patients with non-calcified plaques than in patients with calcified plaques[22]. As IgG4 was mainly found in the adventitia, which was not available from the CEA patients in which we measured circulating IgG4 levels, we were not able to make a direct comparison between circulating IgG4 levels and IgG4 deposits in the vessel wall.

To speculate, IgG4 might be associated with plaque erosion, a phenomenon which has been observed more frequently in women than men presenting with acute myocardial infarction[15, 23, 24]. Plaque erosion is often observed on top of thick fibrous cap atheroma, which are characterized by high collagen and smooth muscle cell content and low inflammation[23-25]. Three arguments underlie this speculation, including the pro-fibrotic, anti-inflammatory and pro-thrombotic role of IgG4. First, the pro-fibrotic role of IgG4 is clear, since fibrosis is one of the main characteristics of IgG4-RD. In line, we observed an increase of IgG4 positive deposits in vessels with stable advanced plaques, which are characterized by a thick fibrous cap. Second, in IgG4-RD, IgG4 is rather considered a marker of inflammation than being inflammatory itself. In fact, IgG4 antibodies are suggested to be anti-inflammatory as they are hardly capable to form immune complexes because of Fab arm exchange[26]. The impaired ability to form immune complexes results in less activation of the immune system[9, 26]. Other arguments for the anti-inflammatory role of IgG4, include the fact that IgG4 production was found to be strongly dependent on type 2 helper T cells and driven by interleukin (IL)-4 and IL-13[27]. In parallel, IgG4-RD is characterized by increased numbers of regulatory T cells and related cytokines including IL-10 and TGF-β[28-30]. Of note, the anti-inflammatory TGF-β has also been linked to a more stable plaque phenotype[31-33]. Third, although information is limited, there is suggestive evidence that IgG4 autoantibodies play a pro-thrombotic role in thrombotic thrombocytopenic purpura, a disease characterized by general microvascular occlusion due to deposition of platelet thrombi[34, 35]. In this way, IgG4 antibodies might contribute to the formation of (micro)thrombi, thereby increasing the risk for clinical CVD manifestations such as MI. In addition, different IgG4-RD case reports have described the occurrence of CVD events like MI, stroke and venous thrombosis, also suggesting an association between IgG4 and thrombus formation[36-41]. Taken together, IgG4 is generally considered as a pro-fibrotic and anti-inflammatory antibody, and might exert pro-thrombotic properties. Therefore, IgG4 might be involved in plaque erosion which is more frequently seen in females with CVD[23-25].

In conclusion, we show that IgG4 serum levels, especially relative to total IgG, are significantly associated with the risk for cardiovascular events during follow-up in females, but not in males, with severe atherosclerosis. The role of IgG4 as prognostic marker for secondary CVD events in women with severe atherosclerosis is promising. In addition, the underlying mechanism of IgG4 in relation to secondary events deserves attention in future research.

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SUPPLEMENTAL DATA

Supplemental table 1. Baseline characteristics for the total population.

Risk factors	Overall (N = 618)
Age (years)	68.5 ± 9.3
Male gender	309 (50.0%)
Current smoker	230 (37.6%)
BMI	26.4 (4.2%)
Contralateral stenosis	250 (43.9%)
Diabetes mellitus	127 (20.6%)
Hypercholesterolemia	382 (66.2%)
Hypertension	525 (85.0%)
CAD history	184 (29.8%)
Stroke history	182 (29.4%)
PAOD history	125 (20.3%)
Clinical manifestations	
Asymptomatic	103 (16.7%)
TIA	267 (43.3%)
Stroke	149 (24.1%)
Ocular	98 (15.9%)
Medication	
Statins	458 (74.2%)
Beta-blockers	278 (45.1%)
Anticoagulants	62 (10.0%)
Laboratory parameters	
Total cholesterol (mmol/L)	4.0 [3.3, 4.9]
HDL cholesterol (mmol/L)	1.0 [0.8, 1.3]
LDL cholesterol (mmol/L)	2.2 [1.6, 2.8]
Triglycerides (mmol/L)	1.5 [1.0, 2.0]
hsCRP (µg/mL)	12.3 ± 64.6
GFR MDRD (mL/min)	71.2 ± 19.5

Values are presented as mean ± SD for normal distributions, number of patients (frequency in percentage) for categorical variables, and median [interquartile range] for non-normal distributions. BMI indicates body-mass index; Contralateral stenosis, 50-100% stenosis of the contralateral carotid artery; CAD history, history of Coronary Artery Disease; PAOD history, history of peripheral artery occlusive disease; TIA, transient ischemic attack; Ocular, temporary loss of vision (amaurosis fugax); HDL, high-density lipoprotein; LDL, low-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; GFR MDRD, Glomerular filtration rate according to 'Modification of Diet in Renal Disease' formula.

Supplemental table 2. Cox regression analyses for major adverse CVD events during one-year follow-up.

Males and females		**Events (nr)**	**Unadjusted HR [95% CI]**	***p***	**Adjusted HR [95% CI]**	***p***
Total IgG (mg/dL)	Continuous	39	1.00 [0.74 - 1.36]	0.99	1.03 [0.75 - 1.41]	0.87
	Low	7	REF		REF	
	Intermediate	18	2.65 [1.11 - 6.34]	**0.03**	3.29 [1.36 - 7.99]	**<0.01**
	High	14	2.06 [0.83 - 5.10]	0.12	2.18 [0.86 - 7.99]	0.11
IgG4 (mg/dL)	Continuous	39	1.09 [0.84 -1.42]	0.52	1.02 [0.76 - 1.39]	0.88
	Low	7	REF		REF	
	Intermediate	12	1.74 [0.68 - 4.41]	0.25	1.61 [0.63 - 4.14]	0.32
	High	20	2.98 [1.26 - 7.04]	**0.01**	2.29 [0.95 - 5.49]	0.06
IgG4 (% of IgG)	Continuous	39	1.13 [0.86 - 1.48]	0.39	0.99 [0.74 - 1.33]	0.95
	Low	6	REF		REF	
	Intermediate	11	1.86 [0.69 - 5.02]	0.22	1.75 [0.64 - 4.78]	0.28
	High	22	3.84 [1.56 - 9.46]	**<0.01**	2.82 [1.11 - 7.13]	**0.03**
Males		**Events (nr)**	**Unadjusted HR [95% CI]**	***p***	**Adjusted HR [95% CI]**	***p***
Total IgG (mg/dL)	Continuous	23	1.01 [0.68 - 1.49]	0.97	1.14 [0.79 - 1.64]	**0.49**
	Low	3	REF		REF	
	Intermediate	9	3.06 [0.83 - 11.3]	0.09	3.94 [1.06 - 14.65]	**0.04**
	High	11	3.88 [1.08 - 2.08]	**0.04**	4.93 [1.35 - 17.95]	**0.02**
IgG4 (mg/dL)	Continuous	23	0.94 [0.60 - 1.48]	0.80	0.89 [0.53 - 1.48]	0.64
	Low	6	REF		REF	
	Intermediate	6	1.01 [0.33 - 3.13]	0.99	1.05 [0.33 - 3.35]	0.93
	High	11	1.88 [0.70 - 5.09]	0.21	1.48 [0.54 - 4.05]	0.45
IgG4 (% of IgG)	Continuous	23	0.90 [0.57 - 1.42]	0.65	0.74 [0.45 - 1.22]	0.24
	Low	7	REF		REF	
	Intermediate	6	0.85 [0.29 - 2.53]	0.77	0.80 [0.27 - 2.39]	0.68
	High	10	1.45 [0.55 - 3.81]	0.45	0.91 [0.34 - 2.43]	0.84
Females		**Events (nr)**	**Unadjusted HR [95% CI]**	***p***	**Adjusted HR [95% CI]**	***p***
Total IgG (mg/dL)	Continuous	16	1.02 [0.65 -1.60]	0.94	0.96 [0.55 - 1.69]	0.89
	Low	5	REF		REF	
	Intermediate	6	1.20 [0.37 - 3.94]	0.76	1.11 [0.34 - 3.64]	0.87
	High	5	1.02 [0.30 - 3.52]	0.97	0.73 [0.19 -2.74]	0.64
IgG4 (mg/dL)	Continuous	16	1.25 [0.91 - 1.72]	0.18	1.20 [0.85 - 1.69]	0.31
	Low	0	REF		REF	
	Intermediate	6	NA [INFERIOR][$]	NA	NA [INFERIOR][$]	NA
	High	10	NA [INFERIOR][$]	NA	NA [INFERIOR][$]	NA

Supplemental table 2. *Continued*

Females		Events (nr)	Unadjusted HR [95% CI]	p	Adjusted HR [95% CI]	p
IgG4 (% of IgG)	Continuous	16	1.37 [0.99 - 1.89]	0.06	1.32 [0.94 - 1.86]	0.11
	Low	1	REF		REF	
	Intermediate	4	4.05 [0.45 - 36.22]	0.21	4.00 [0.44 - 35.95]	0.22
	High	11	11.69 [1.51 - 90.56]	**0.02**	10.35 [1.32 - 81.08]	**0.03**

Cox proportional hazard rates are given for the association between total serum IgG or IgG4 and the occurrence of major CVD events during one-year follow-up. In addition to continuous hazard rates (per SD), the hazard ratios per tertile are given for crude and adjusted Cox regression models. Adjusted models contain age, sex (when males and females are analyzed together) and smoking status as covariates.
$ Hazard ratios for tertiles of absolute IgG4 levels in females could not be calculated because the comparison to 0 events in the lowest group led to infinite confidence intervals. Nevertheless, a clear increase in the event rate is observed across serum IgG4 tertiles in females.

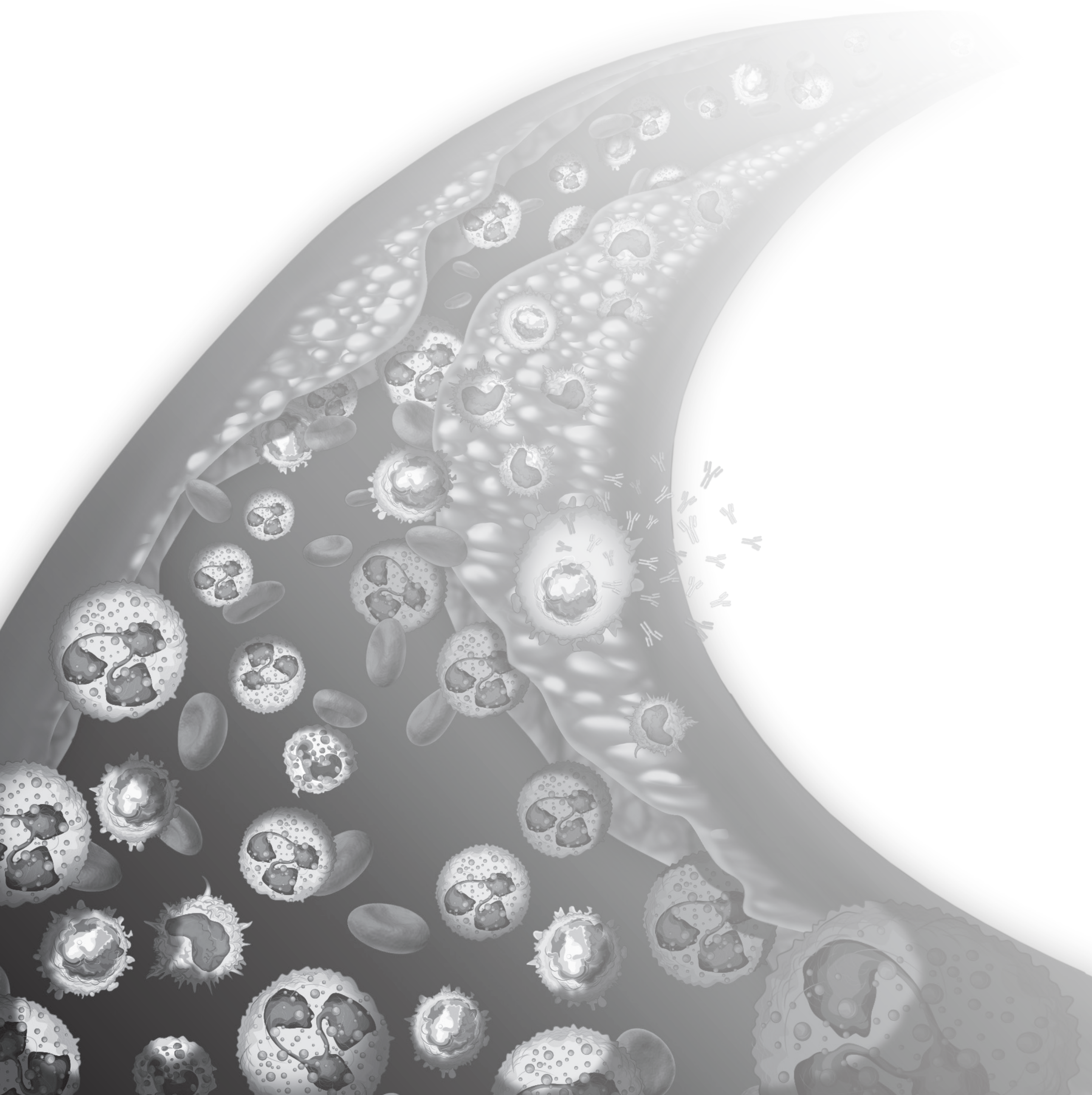
Supplemental table 3. Cox regression analyses for composite CVD end points during one-year follow-up.

Males and females		Events (nr)	Unadjusted HR [95% CI]	*p*	Adjusted HR [95% CI]	*p*
Total IgG (mg/dL)	Continuous	79	0.97 [0.76 - 1.24]	0.82	0.98 [0.77 - 1.25]	0.87
	Low	22	REF		REF	
	Intermediate	29	1.36 [0.78 - 2.37]	0.28	1.55 [0.88 - 2.72]	0.13
	High	28	1.32 [0.75 - 2.30]	0.33	1.37 [0.77 - 2.45]	0.28
IgG4 (mg/dL)	Continuous	79	1.02 [0.83 - 1.26]	0.85	0.99 [0.79 - 1.24]	0.92
	Low	19	REF		REF	
	Intermediate	27	1.45 [0.81 - 2.61]	0.22	1.37 [0.76 - 2.49]	0.30
	High	33	1.83 [1.04 - 3.22]	0.04	1.59 [0.89 - 2.84]	0.11
IgG4 (% of IgG)	Continuous	78	1.08 [0.88 - 1.32]	0.45	1.02 [0.82 - 1.26]	0.88
	Low	19	REF		REF	
	Intermediate	27	1.44 [0.80 - 2.59]	0.22	1.32 [0.72 - 2.40]	0.37
	High	32	1.78 [1.01 - 3.15]	0.05	1.51 [0.84 - 2.72]	0.17
Males		**Events (nr)**	**Unadjusted HR [95% CI]**	***p***	**Adjusted HR [95% CI]**	***p***
Total IgG (mg/dL)	Continuous	47	0.98 [0.72 -1.33]	0.87	1.01 [0.76 - 1.34]	0.95
	Low	11	REF		REF	
	Intermediate	17	1.57 [0.73 - 3.35]	0.24	1.76 [0.82 - 3.78]	0.15
	High	19	1.84 [0.88 - 3.87]	0.11	2.03 [0.96 - 4.28]	0.06
IgG4 (mg/dL)	Continuous	47	0.95 [0.69 - 1.29]	0.73	0.94 [0.68 - 1.31]	0.73
	Low	14	REF		REF	
	Intermediate	14	1.02 [0.48 -2.13]	0.97	1.05 [0.50 - 2.22]	0.90
	High	19	1.40 [0.70 - 2.80]	0.34	1.34 [0.67 - 2.69]	0.41

Supplemental table 3. *Continued*

Males		Events (nr)	Unadjusted HR [95% CI]	*p*	Adjusted HR [95% CI]	*p*
IgG4 (% of IgG)	Continuous	46	0.97 [0.73 - 1.30]	0.86	0.93 [0.69 - 1.26]	0.64
	Low	17	REF		REF	
	Intermediate	13	0.75 [0.36 - 1.54]	0.43	0.75 [0.36 - 1.54]	0.43
	High	16	0.94 [0.48 - 1.87]	0.87	0.83 [0.41 - 1.67]	0.60
Females		**Events (nr)**	**Unadjusted HR [95% CI]**	***p***	**Adjusted HR [95% CI]**	***p***
Total IgG (mg/dL)	Continuous	32	1.00 [0.71 - 1.41]	0.99	0.95 [0.62 - 1.45]	0.81
	Low	11	REF		REF	
	Intermediate	9	0.81 [0.34 - 1.96]	0.64	0.76 [0.31 - 1.84]	0.54
	High	12	1.11 [0.49 - 2.52]	0.80	0.87 [0.37 - 2.07]	0.75
IgG4 (mg/dL)	Continuous	32	1.10 [0.82 - 1.47]	0.53	1.08 [0.78 - 1.46]	0.69
	Low	4	REF		REF	
	Intermediate	13	3.37 [1.10 - 10.35]	**0.03**	3.44 [1.12 - 10.59]	**0.03**
	High	15	4.15 [1.38 - 12.51]	**0.01**	3.59 [1.17 - 11.05]	**0.03**
IgG4 (% of IgG)	Continuous	32	1.19 [0.89 - 1.58]	0.25	1.17 [0.87 - 1.58]	0.31
	Low	5	REF		REF	
	Intermediate	12	2.45 [0.86 - 6.94]	0.09	2.28 [0.79 - 6.61]	0.13
	High	15	3.25 [1.18 - 8.95]	**0.02**	3.04 [1.10 - 8.46]	**0.03**

Cox proportional hazard rates are given for the association between total serum IgG or IgG4 and the occurrence of composite CVD endpoints (including (surgical) vascular interventions) during one-year follow-up. In addition to continuous hazard rates (per SD), the hazard ratios per tertile are given for crude and adjusted Cox regression models. Adjusted models contain age, sex (when males and females are analyzed together) and smoking status as covariates.



CHAPTER

6

The agonistic CD200R antibody OX110 does not affect atherosclerotic plaque progression in LDL receptor knockout mice

In preparation

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ABSTRACT

Introduction

Atherosclerosis is a chronic inflammatory disease and the main underlying cause for CVD. Proper immune regulation is crucial to prevent atherosclerosis. While co-stimulatory molecules like CD40L, CD80 and CD86 display atherogenic properties, stimulation of co-inhibitory pathways via PD-1 and CTLA-4 have been associated with atheroprotective properties via reduction of inflammatory reactions. Likewise, the CD200 receptor (CD200R) pathway has been shown to be an important immune regulatory pathway, as disruption of CD200R signaling has been associated with the development of auto-immune diseases such as rheumatoid arthritis. Therefore, we hypothesized that stimulation of CD200R with OX110 reduces the inflammatory response and atherosclerotic plaque progression in low-density lipoprotein receptor knockout ($LDLR^{-/-}$) mice.

Materials and methods

Eight to ten weeks old $LDLR^{-/-}$ mice were placed on a high fat diet (T=0), received bilateral carotid collars (T=4 weeks), and were treated twice a week with intraperitoneal injections of 100 µg OX110, isotype or PBS control (from 7 till 10 weeks, n=13-14 per group). Primary outcome was plaque size, determined with hematoxylin and eosin (H&E) staining. Secondary outcomes included plaque stability and profiles of circulating inflammatory cells. To assess plaque stability, collagen was stained using picrosirius red and necrotic core size was determined on the H&E stained sections. Circulating myeloid cells, B lymphocytes and T lymphocytes profiles, as well as peritoneal cells, were measured using flow cytometry.

Results

We observed no difference in plaque progression between OX110 (45 ± 17 x 10e3 μm^2), isotype (36.8 ± 13 x 10e3 μm^2) or PBS (38 ± 22 x 10e3 μm^2) treated mice (p=ns). In addition, collagen intensity and necrotic core size was not statistically significantly different between OX110 and isotype treated mice. The percentage of M2-like $MHCII^+CD71^+CD301^+$ cells tended to be increased in the peritoneal cavity of OX110 treated mice (15.8 ± 9.7%) as compared to isotype (12.4 ± 9.1%) and PBS (6.0 ± 6.0%) treated mice (p=0.54 and p=0.01, respectively). Circulating numbers of white blood cells did not differ significantly between OX110 (median 5.9 x 10e9/L [interquartile range (IQR) 4.1, 8.2]) and isotype (5.8 x 10e9/L [IQR 4.1, 6.5]) or PBS (median 5.2 x 10e9/L [IQR 4.2, 6.5]) treated animals (p=ns). Likewise, circulating profiles of myeloid, B and T cells were comparable among the different groups.

Conclusion

Despite a tendency toward increased M2-like cells, CD200R stimulation with OX110 did not affect atherosclerotic plaque progression or stabilization. These findings indicate that inhibition of the inflammatory response via stimulation of CD200R has limited potential as therapeutic target for atherosclerotic disease.

INTRODUCTION

Although atherosclerosis has been described as an inflammatory lipid disorder[1-3], until recently, treatment of CVD remained highly dependent on downregulation of lipid levels. However, in the CANTOS trial, inhibition of IL-1β with canakinumab resulted in a 15% reduction of major adverse cardiovascular events in CAD patients that were already treated with statins[4]. These findings confirm the important pathological role of inflammation in atherosclerosis and underline the significance of proper immune regulation.
As demonstrated in the CANTOS trial, inhibition of excessive inflammation reduces the occurrence of adverse cardiovascular events[4]. Another way to dampen excessive inflammatory responses is via the inhibition of co-stimulatory molecules like CD40L and B7 members including CD28, B7.1 (CD80) and B7.2 (CD86)[5]. These co-stimulatory molecules have been shown to play an important role in atherosclerotic lesion formation and plaque destabilization. For example, administration of CTLA-4, which blocks interactions of CD80/CD86-CD28, inhibits atherosclerosis formation[6, 7]. Moreover, in human atherosclerotic plaques, CD80 and CD86 were co-expressed with $CD68^+$ macrophages and $CD3^+$ T cells and increased in vulnerable plaques as compared to stable plaques[8]. During homeostasis, inflammatory signals are well regulated via co-stimulatory molecules and co-inhibitory molecules. The CD200 receptor (CD200R) is a co-inhibitory molecule and upon binding of its ligand, CD200, CD200R has demonstrated anti-inflammatory properties, including suppression of T cell activity[9] and inhibition of mast cell activation[10]. CD200R is, among others, expressed by myeloid cells, T cells, and B cells[11]. Autoimmune diseases are characterized by chronic inflammation, and patients with autoimmune diseases have increased risk of cardiovascular disease. Interestingly, the presence and regulation of the CD200R is reduced in patients with rheumatoid arthritis[12,13]. In addition, CD200-deficiency accelerated the onset of experimental auto-immune encephalomyelitis and disruption of the CD200-CD200R axis resulted in collagen induced arthritis[14]. As such, CD200R has been suggested as a therapeutic target for autoimmune diseases.
Although atherosclerosis is recognized as a chronic inflammatory disease, and CD200-CD200R interaction has been shown to be crucial for immune homeostasis, the role of CD200R in atherosclerosis remains unexplored. Therefore, we investigated if stimulation of CD200R with an agonistic antibody, OX110, dampens the inflammatory response and inhibits plaque progression in LDL receptor knockout ($LDLR^{-/-}$) mice.

MATERIALS AND METHODS

Animals

Experiments were conducted according to the Guide for the Care and Use of Laboratory Animals and approved by the central animal experimental committee in the Netherlands. Male $LDLR^{-/-}$ mice of the background strain C57BL/6 were originally obtained from the Jackson laboratory (002207) and subsequently bred and kept at the animal laboratory of

the UMC Utrecht. All animals were genotyped prior to the experimental procedure. Mice were housed in filter top cages, normal light/dark cycle, with food and water ad libitum. Welfare-related assessments included daily visual inspections and mice were regularly weighed (**Supplemental figure 1**).

Atherosclerosis model

Eight to ten weeks old male LDLR$^{-/-}$ mice (weight 25.2 ± 1.6 grams) were placed on a western type diet containing 15% cocoa butter and 0.25% cholesterol (823233, Special Diets Services/Tecnilab, Someren, the Netherlands). After four weeks of western type diet feeding, bilateral collars were placed around the carotid arteries as described earlier[15]. Prior to placement of the carotid collars, mice were anaesthetized with dexdomitor (0.5 mg/kg), midazolam (5 mg/kg) and analgesized with fentanyl (0.05 mg/kg) through a single intraperitoneal (i.p.) injection. After surgery, anesthetics were antagonized through a single subcutaneous (s.c.) injection with antipamezole (5 mg/kg) and flumazenil (1 mg/kg). Analgesics were applied by s.c. injection with buprenorphine (0.1 mg/kg) directly after surgery and one day later. Four out of 45 animals died as a consequence of the disease model within one week after the surgical procedure (**Supplemental figure 2**). After seven weeks (T=49), mice were treated i.p. with PBS (n=13), 100µg of isotype (IgG_{2a} anti βGAL, clone GL117; n=14) or 100µg of OX110 (a kind gift of Louis Boon, Utrecht; n=14) twice a week, seven injections in total. The injected dose was based on previous experiments[16]. Blood cholesterol was frequently measured (**Supplemental figure 1**) using enzymatic colorimetric procedures in order to match the mice on cholesterol levels and to determine if OX110 treatment affects cholesterol levels. To reduce possible bias, the mice in the different treatment groups were matched on weight and cholesterol levels, and the executing researcher was blinded during treatment and analyses. In addition, experiments were replicated four times (n=9-12 per experiment, n=3-4 per treatment). The number of mice was derived from a sample size calculation with an effect size of 25% reduction in plaque size, alpha of 0.05, standard deviation of 22%, power of 80% and 15% mortality due to the procedure. In addition, the number was increased with 15% to correct for non-normal distributed measurements. After ten weeks (T=70), euthanasia was applied using an overdose of pentobarbital (i.p.). Upon termination, carotid arteries were collected for histochemical analyses to determine the primary outcome: plaque size. Secondary outcomes included plaque composition, and blood and intraperitoneal cells were collected for flow cytometry.

Histochemistry

To determine plaque size and composition, bilateral carotid arteries were collected and fixated in 4% paraformaldehyde, frozen in Tissue-Tek OCT compound (25608-930, Sakura Finetek, Alphen aan den Rijn, The Netherlands) and serial 5 µm cryosections were obtained using the Micro HM 560 Cryostat (Thermo Fisher Scientific, Boston, MA, USA). To assess plaque size, we used hematoxylin and eosin staining. Briefly, slides were fixed for 10 minutes in 4% formalin, rinsed with demi water for 2 minutes and stained with

hematoxylin solution for 5 minutes. After rinsing with tap water for 10 minutes, the slides were stained with eosin solution for 1 minute, rinsed with demi water and embedded in ClearVue Mountant (Thermo Fisher). Plaque burden was determined as absolute plaque size (in 10e3 μm^2) and stenosis (plaque area as percentage of intimal area) using cellSens software (Olympus Imaging, Center Valley, PA, USA).
Necrotic core size was scored on the HE stained plaques. We defined necrotic core as acellular areas that were often found close to the intima, but not to the lumen. Necrotic core size was calculated as absolute area (x 10e3 μm^2) and relative area (% of total plaque area).
The amount of collagen in the carotid plaques was assessed using picrosirius red staining. Upon fixation for 20 minutes in 4% paraformaldehyde, slides were washed for 5 minutes in demi water. After staining with picrosirius red (0.1% Sirius Red F3b in saturated picrine acid solution, pH=2.0), slides were three times 7 minutes washed in 0.2N HCl to remove any residual picrosirius red. After washing in demi water and dehydration with 70%, 96% and 99.6% ethanol, the slides were mounted in ClearVue Mountant. We determined mean collagen intensity and collagen area in pictures that were taken with polarized light and converted the signal to intensity values, using cellSens software. Next, collagen density was calculated by multiplying the mean collagen intensity with the collagen area and divided by total plaque area. Carotid arteries with plaque size <10 x 10e3 μm^2 were excluded from analyses, as constriction of the collar was not sufficient to promote lesion formation in these cases. When analyzing histochemistry data the left and right carotid artery of each mouse were averaged first after which a group average was calculated.

Flow cytometry

Blood and peritoneal cells were collected for flow cytometry analyses. A white blood cell count was measured on the Cell Dyn Sapphire Hematology Analyzer. Erythrocytes were lysed for 10 minutes on ice with erythrocyte lysis buffer (deionized water with 168 mM ammonium chloride (12125-02-9, Merck, Darmstadt, Germany), 9.99 mM potassium bicarbonate (298-14-6, Merck), 0.11 mM Na2EDTA (6381-92-6, Sigma-Aldrich, St. Louis, MO, USA) and phenol red (143-74-8, Sigma-Aldrich). The cell pellet was collected by centrifugation (unless otherwise mentioned, all centrifugation steps were at 350*g* for 5 minutes at 4°C). Cells were then washed with FACS buffer (PBS with 5% FBS (F2442, Sigma-Aldrich), centrifuged, resuspended in FACS buffer, and incubated for 30 minutes at 4°C in dark with antibodies to detect myeloid, T and B cell populations (**Supplemental table 1**). Next, unbound antibodies were washed away with FACS buffer by washing and centrifugation. Cells were resuspended in FACS buffer and measured with flow cytometry (Gallios, Beckman Coulter Fullerton, CA, USA). Flow cytometry data were analyzed with Kaluza Flow Analysis Software Version 1.5a (Beckman Coulter). Doublets were excluded based on TOF and forward scatter peak and integral measurements (data not shown). Any residual red blood cells or cell debris was excluded based on forward and sideward scatter (**Supplemental figure 3**). For blood cells, a myeloid panel was designed to select granulocytes ($CD11B^{high}Ly6G^{high}$), dendritic cells ($CD11B^{+}CD11C^{+}$), and monocytes were divided in $CD11B^{+}Ly6C^{high}$ and $CD11B^{+}Ly6C^{low}$ subsets (**Supplemental figure 3**). The T cell

panel was used to determine $CD4^+$ T helper (Th) cells, as well as Th1 ($CD4^+CXCR3^+$) and Th2 ($CD4^+CCR4^+$) subsets. From the $CD19^+$ B cells, we selected $IgM^{high}IgD^{low}$ B1 cells and $IgM^{low}IgD^{high}$ B2 cells. Subsequently, using CD5a expression, we identified B1a ($CD5a^-$) and B1b ($CD5a^+$) subtypes (**Supplemental figure 3**). Intraperitoneal cells were assessed for the expression of inflammatory (CD86 and MHC1II) and anti-inflammatory markers (CD206, CD301 and CD71) and CD200R.

Culturing of bone marrow-derived macrophages

Bone marrow-derived macrophages (BMDMs) were obtained from C57BL/6 mice. Bone marrow cells were flushed out of femurs and tibia with PBS and filtered over a 40 µm strainer. To induce differentiation into macrophages, bone marrow cells were cultured for 7 days in the presence of 100 ng/mL M-CSF (315-02 Peprotech, London, UK) in RPMI medium supplemented with 20% FBS, 1% L-glutamine (25030081, Gibco, Carlsbad, CA, USA), 1% non-essential amino acids (11140-035, Gibco), 1% sodium pyruvate (11360-039, Gibco) and 1% Pen/Strep (15070063, Thermo Fisher). To observe the effect on CD200R expression on BMDMs, cells were treated with PBS, isotype or OX110 (0.5 µg/mL) for 60 minutes and measured with flow cytometry as described before in the section flow cytometry.

Statistical analyses

As primary outcome, plaque size in OX110 treated mice was compared to isotype treated mice. Differences in normal distributions were compared using a one-way ANOVA with post-hoc Tukey's test. Non-normal distributions were compared with a Kruskal-Wallis test. All statistical analyses were performed using Graphpad Prism 7.02. We considered a p-value <0.05 as statistically significant.

RESULTS

To demonstrate that OX110 binds the CD200R on macrophages, we treated bone marrow-derived macrophages (BMDMs) with OX110, isotype or PBS, and measured CD200R expression using flow cytometry. Binding of OX110 antibodies to the CD200R was confirmed by flow cytometry, showing that, by use of an antibody directed against CD200R (clone OX110), the CD200R was being occupied (**Figure 1A** and **B**). Likewise, we show that OX110 antibodies bound to CD200R in vivo, as seen by reduced binding of flow cytometric OX110 antibodies on peritoneal cells (17 ± 11% ab binding in OX110 treated mice compared to 38 ± 12% in isotype and 50 ± 8% in PBS treated mice, $p<0.01$ for OX110 vs. isotype and PBS; **Figure 1C**). Though, isotype treated mice displayed also a slight decrease in the percentage of CD200R positive cells (isotype vs. PBS $p=0.03$; **Figure 1C**). In addition, peritoneal cells positive for CD301 and CD71, markers that have been associated with an anti-inflammatory M2-like phenotype, were higher in OX110 (15.8 ± 9.7%) than PBS (6.0 ± 5.8%) treated animals ($p=0.01$), and also tended to be higher than isotype (12.4 ± 9.1%)

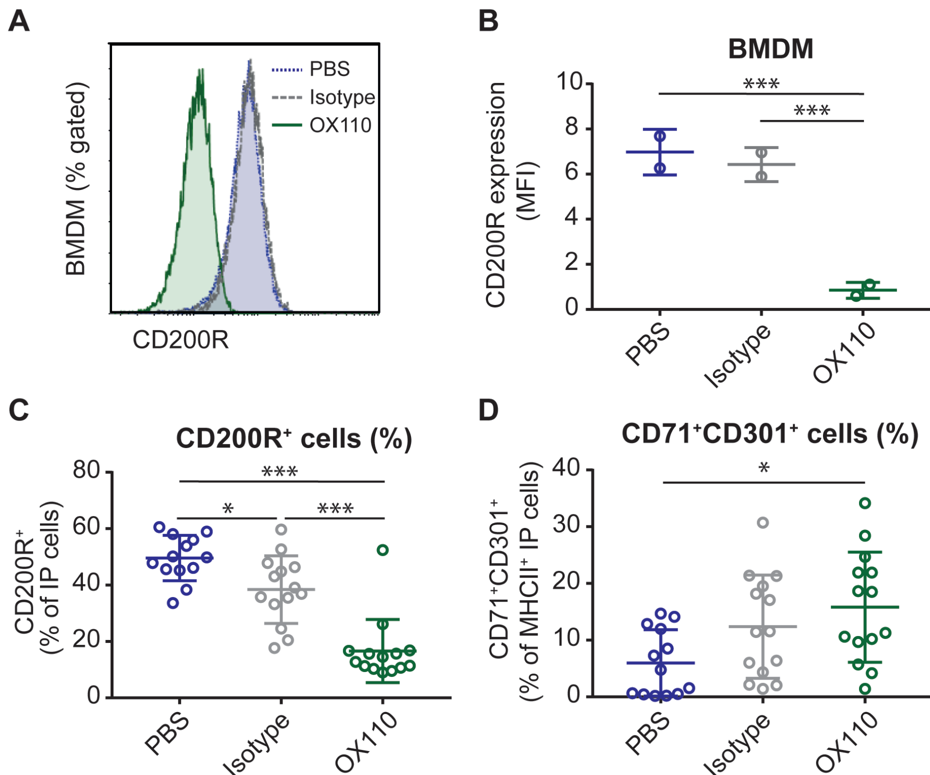

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Figure 1. OX110 binds to the CD200R. Bone marrow-derived macrophages (BMDMs) were incubated with OX110, isotype or PBS. OX110 antibodies bind to CD200R as upon incubation, the flow cytometry antibodies against CD200R (same clone: OX110) were no longer able to bind CD200R *in vitro* (A, quantification in B), and *in vivo* (C). In addition, peritoneal cells, positive for markers that have been associated with an M2-like phenotype, tended to be increased in OX110 treated mice as compared to isotype and PBS treated mice (C). * indicates $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

treated mice, although this was not statistically significant ($p=0.54$; **Figure 1D**).
Mice were matched on cholesterol levels and weight before treatment (T=49). First, we examined if OX110 treatment influences cholesterol levels or weight over time. OX110 treatment showed no effect on cholesterol levels or weight as compared to isotype or PBS treated mice (**Supplemental figure 4**).
To investigate the effect of OX110 on plaque progression, we analyzed the absolute maximal plaque area of both carotid arteries. The average plaque area of the left and right carotid artery was used from the HE stained arteries. We observed no statistically significant difference in plaque size between OX110 (45 ± 17 x 10e3 μm^2), isotype (36.8 ± 13 x 10e3 μm^2) or PBS (38 ± 22 x 10e3 μm^2) treated animals (p=ns, **Figure 2**). In addition, the percentage luminal stenosis of the carotid artery did not differ between OX110 (49 ± 17%) and isotype (42 ± 15%) or PBS (40 ± 19%) treated animals (p=ns; **Figure 2**).

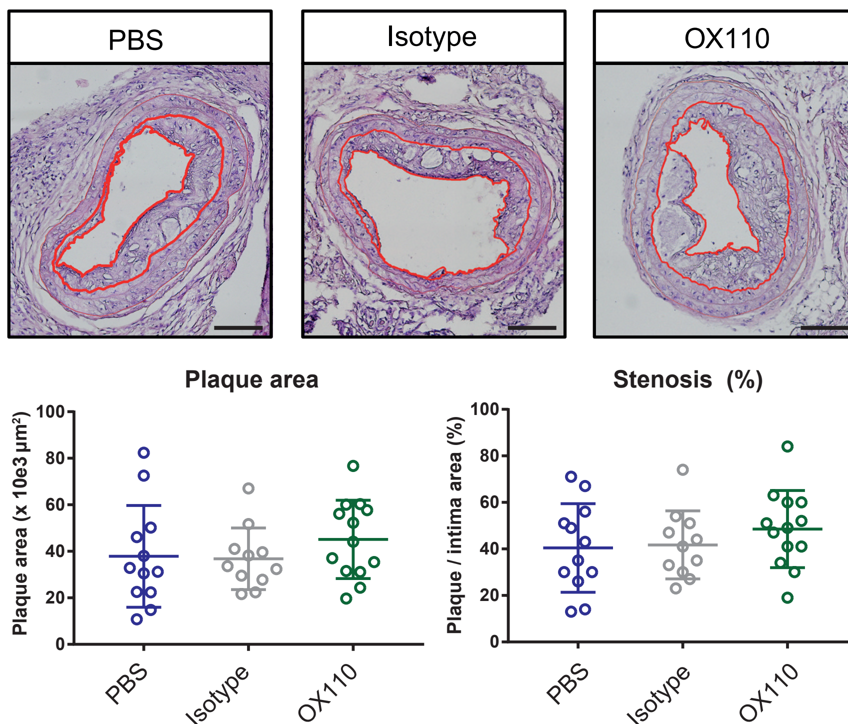

Figure 2. The effect of OX110 treatment on absolute and relative plaque size. Plaque size was determined using HE staining and depicted as absolute plaque area (x 10e3 µm^2) and percentage stenosis of the lumen (plaque area divided by the combined lumen and plaque area). Scale bar represents 100 µm.

Next, we investigated if OX110 treatment induced a more stable plaque phenotype, which is characterized by high collagen density and small necrotic core size. We observed however no statistically significant differences in plaque composition regarding collagen density in OX110 (median 0.53 [interquartile range (IQR) [0.02, 1.14]) as compared to isotype (0.53 [IQR 0.19, 2.55]) or PBS (0.57 [IQR 0.35, 1.80]) treated animals (*p*=ns; **Figure 3**). The necrotic core size tended to be smaller in OX110 (11 ± 6%) versus isotype (14 ± 7%) or PBS (17 ± 11%) treated mice, but this difference was not statistically significant (*p*=ns; **Figure 3**).
Lastly, we examined the profile of circulating inflammatory cells which are known to play an important role in plaque formation, and which might change upon OX110 treatment. Total white blood cell counts did not differ between OX110 (median 5.9 x 10e9 cells/L [IQR 4.1, 8.2]) and isotype (5.8 x 10e9 cells/L [IQR 4.1, 6.5]) or PBS (5.2 x 10e9 cells /L [IQR 4.2, 6.5]) treated animals (*p*=ns; **Supplemental table 2**). We next compared the percentages of inflammatory cell subsets, including myeloid, T and B cells. Within the myeloid population, percentages of $CD11B^{+}Ly6G^{high}$ granulocytes, $CD11B^{+}CD11C^{high}$

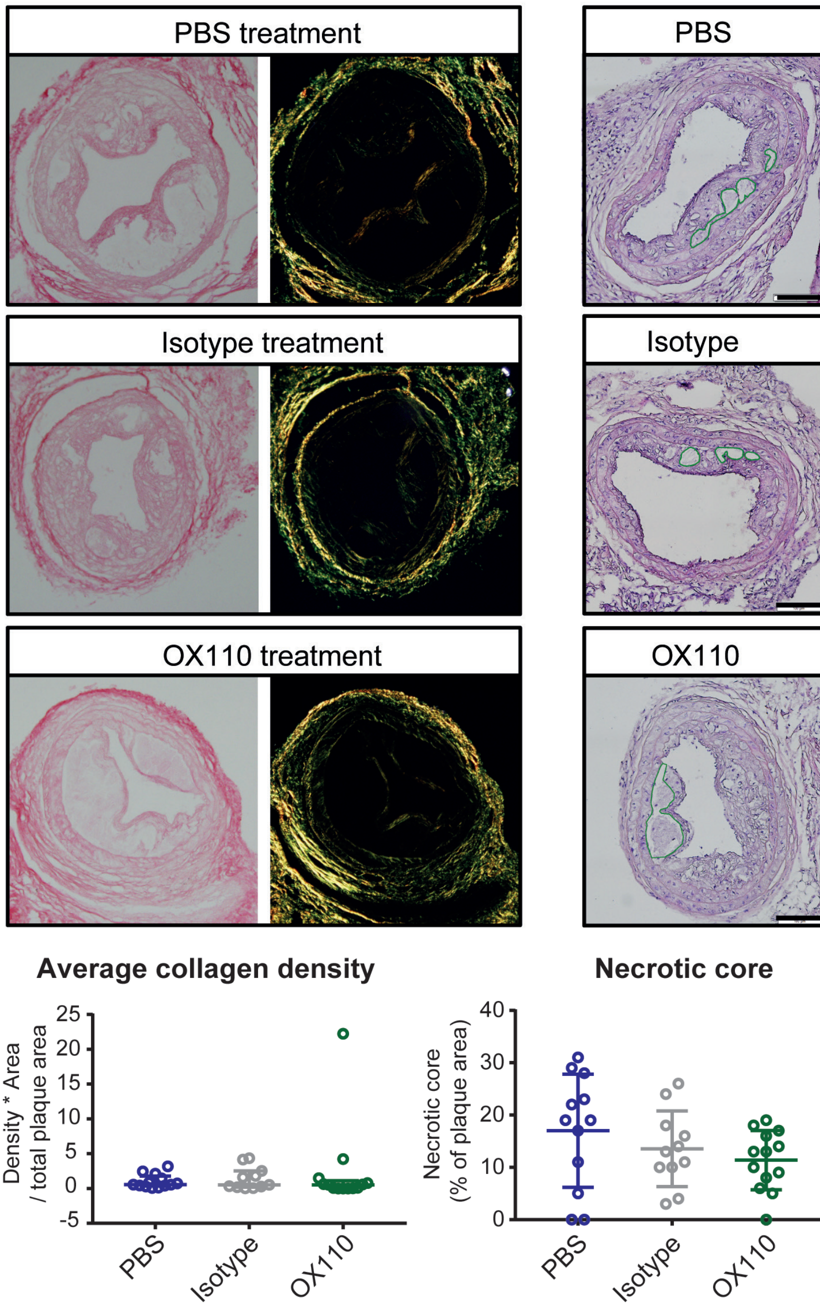

Figure 3. The effect of OX110 treatment on collagen and necrotic core content in the plaque. Collagen content was determined using polaroid converted images of picrosirius red staining (left panel) and necrotic core size was determined on HE stained plaques. OX110 treatment showed no significant difference in plaque collagen intensity or necrotic core formation as compared to isotype treatment. Scale bar represents 100 µm.

dendritic cells, $CD11B^+Ly6C^{high}$ and $CD11B^+Ly6C^{low}$ monocytes were not significantly different in OX110 treated animals as compared to isotype or PBS treated animals (**Supplemental table 2**). Likewise, $CXCR3^+$ Th1 and $CCR4^+$ Th2 cells did not differ significantly between OX110 and isotype treated animals (**Supplemental table 2**). Finally, the percentage of B2 and B1 B cells were also comparable between OX110 and isotype or PBS treated animals (**Supplemental table 2**).

DISCUSSION

In this study we investigated if stimulation of the CD200R with the agonistic antibody OX110 attenuated plaque progression in $LDLR^{-/-}$ mice. We hypothesized that stimulation of CD200R would dampen the chronic inflammatory response with subsequent attenuation of plaque progression. However, we observed no effect on plaque progression in OX110 treated animals as compared to control treated animals. In addition, plaque phenotype did not change upon OX110 treatment.

Studies investigating the role of co-stimulation and co-inhibition in atherosclerosis have indicated an important role for these molecules. Disruption of the OX40-OX40L and the CD40-CD40L pathways reduces atherosclerotic lesion formation and induces a stable plaque phenotype[17, 18]. In addition, deficiency of programmed cell death-1 (PD-1), an immune regulatory molecule, was associated with enhanced inflammation and increased lesion formation[19]. These studies indicate that these immune regulatory checkpoints are attractive therapeutic targets. The immune inhibitory role of CD200R has been well-established in other inflammatory disease models, for example in hind limb ischemia[16], experimental auto-immune encephalomyelitis and collagen induced arthritis[14], but not yet in atherosclerosis. One of the major differences between our model and other inflammatory disease models is the high lipid environment. The high cholesterol levels in atherosclerotic mouse models are highly inflammatory by themselves[20-22] and may have overruled the anti-inflammatory effect of CD200R stimulation on plaque formation. Studies in atherosclerotic mouse models with mild hypercholesterolemia, like $LDLR^{-/-}$ mice on regular chow, might shed more light on this matter. In addition, multiple inhibitory receptors that balance the immunological homeostasis have been described in literature[23]. Therefore, it might be possible that a combination of co-inhibitory and/or inhibitors of co-stimulatory factors works better than one factor on its own.

Intraperitoneal cells tended to display a M2-like polarization in OX110 treated mice, as compared to isotype and PBS treated mice. In line, a previous study showed also a shift towards macrophage M2 polarization in CD200-Fc stimulated macrophages[24]. We observed no differences with regard to white blood cell levels and subsets of lymphoid and myeloid cells. In agreement, $CD200^{-/-}$ mice have been reported to show no differences in the composition of lymphocyte numbers[11]. Others have shown that CD200 deficiency is associated with a switch from Th1 to Th2 cell responses, based on cytokine profiles[25]. In addition, activation of CD200R has been suggested to stimulate the differentiation to

regulatory T cells[26]. To further establish the effect of OX110 treatment in atherosclerotic mice, these cytokine profiles could be determined. In addition, the inflammatory content of the plaque, which is strongly correlated with plaque vulnerability, should also be investigated. In future experiments we will analyze the content of plaque monocytes and macrophages between the different groups. In addition, evaluation of M1 and M2 macrophage markers in the plaque would provide valuable information.
In conclusion, we found no reduced plaque formation in OX110 treated animals. In addition, no signs of increased plaque stability could be detected. These results suggest that the anti-inflammatory effect of CD200R stimulation with OX110 on atherosclerotic plaque formation is limited in male $LDLR^{-/-}$ mice on a high-cholesterol diet.

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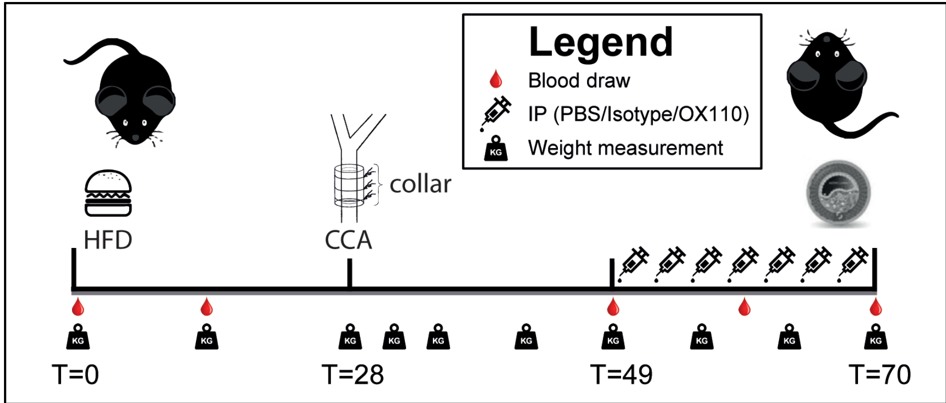
SUPPLEMENTAL DATA



Supplemental figure 1. Study design. At day 0, LDLR$^{-/-}$ mice were put on a high fat diet (HFD) and blood was collected for cholesterol measurements (also at day 14, 49, 59 and 70). At day 28, collars were placed around the common carotid arteries (CCA). Groups were made through matching on weight and cholesterol levels, and mice received intraperitoneal (IP) treatment with OX110, isotype or phosphate buffered saline (PBS) twice a week, from day 49 until termination at day 70.

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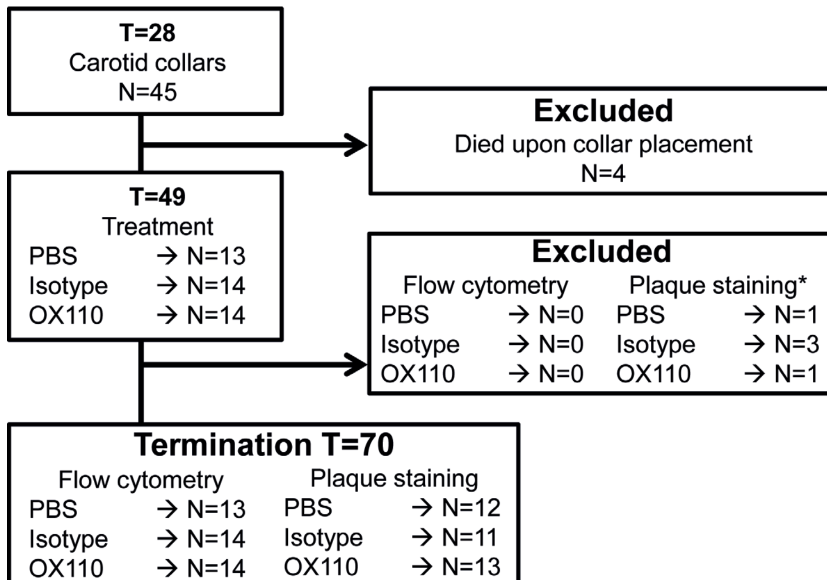
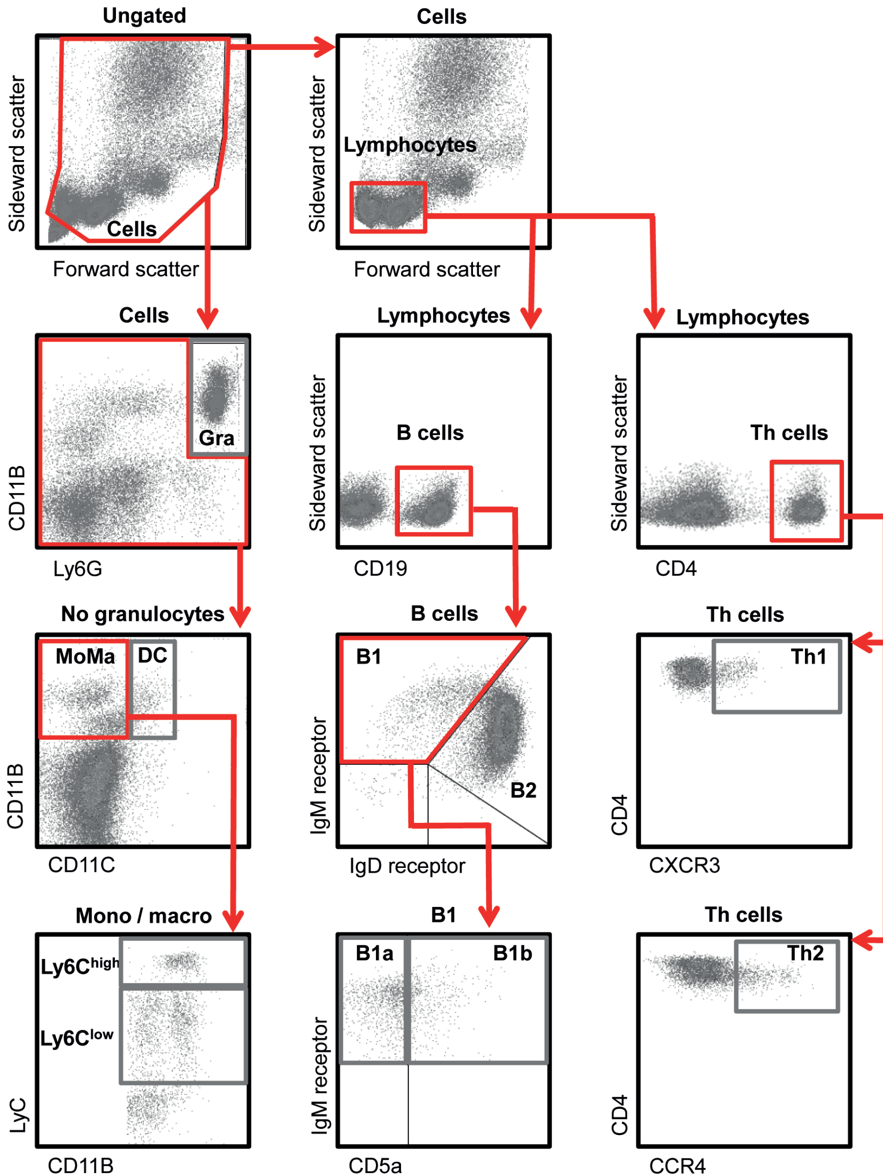
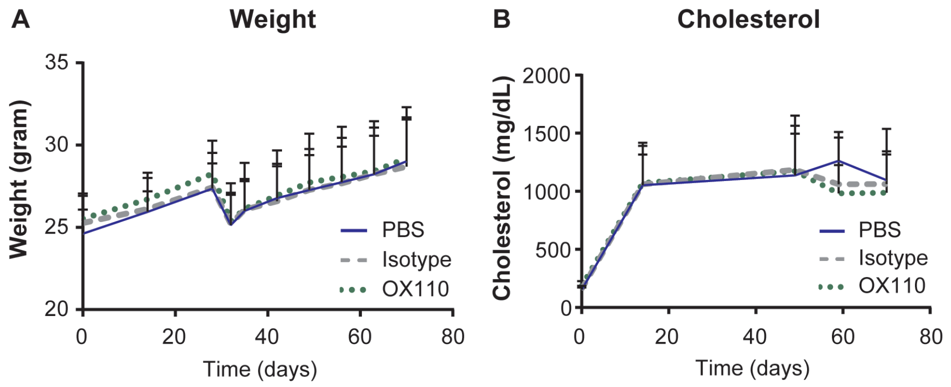


Supplemental figure 2. Overview of mice that were excluded from analyses during the experiment. *For plaque staining, 5 mice were excluded since both carotid arteries (total n=10 (5 left and 5 right carotid arteries)) were not available for plaque assessment because plaque area was too small <10 x 10e3 µm^2 (n=5), thrombus formation occurred (n=3) or technical issues occurred during work-up of the plaque sections (n=2).



Supplemental figure 3. Gating strategy B-lymphocytes, T-lymphocytes and myeloid cells. First, cells were discriminated from cell debris and remaining red blood cells using forward sideward scatter. Then, single cells were selected using time of flight and forward scatter peak and integral value (data not shown). Next, using different panels, subpopulations of myeloid cells (left), B lymphocytes (middle) and T lymphocytes (right) were identified. Within the myeloid panel, we identified Ly6G^{high}CD11B^{+} granulocytes and from the remaining cells, CD11C^{+} dendritic cells were identified. Within the CD11B^{+}CD11c^{-} cells, Ly6C distinguished Ly6C^{high} and Ly6C^{low} monocytes and macrophages (moma). From CD19^{+} B lymphocytes, we selected IgD^{+}IgM^{+} B2 cells, and from the IgM^{+}IgD^{-} B1 cells, we discriminated CD5^{+} B1a from CD5^{-} B1b cells. In the T lymphocyte panel CD4^{+} Th1 (CXCR3^{+}) and Th2 (CCR4^{+}) cells were identified.



Supplemental figure 4. OX110 treatment has no effect on weight or cholesterol levels. Mice were matched on cholesterol levels and weight at day 49. Weight (A) and cholesterol levels (B) did not differ between OX110 treated and PBS or isotype treated animals.

Supplemental table 1. Antibody mixes for different flow cytometry panels.

Marker	Fluorochrome	Clone	µL
T lymphocytes			
Marker	**Fluorochrome**	**Clone**	**µL**
CXCR3	FITC	CXCR3-173	0.5
CCR4	PE	2G12	5
CD4	PE-Dazzle	RM4-5	0.125
LAP	PE-Cy7	TW7-16B4	0.2
CCR6	APC	29-2L17	0.75
CD127	Alexa Fluor 700	A7R34	2.5
CD25	APC-eFluor 780	PC61.5	0.125
CD62L	BV510	ML-14	0.25
B lymphocytes			
Marker	**Fluorochrome**	**Clone**	**µL**
IgM	FITC	eB121-15F9	0.5
IgD	PE	11-26c	0.125
CD40	Pe-Cy5	IC10	0.7
CD27	APC	LG.7F9	0.15
CD86	Alexa Fluor 700	GL1	2
CD5	APC-eFluor 780	53-7.3	0.3
CD19	eFluor 450	eBio1D3	0.125
Myeloid cells			
Marker	**Fluorochrome**	**Clone**	**µL**
CD11c	PE	HL3	0.5
MHCII	PE-Cy5	M5/114.15.2	0.125
CD86	Alexa Fluor 700	GL1	2
CD11b	APC-eFluor 780	M1/70	0.125
LY6C	eFluor 450	HK1.4	0.125
CD62L	BV510	ML-14	0.25
Intraperitoneal cells			
Marker	**Fluorochrome**	**Clone**	**µL**
iNOS*	Alexa Fluor 488	CXNFT	0.25
CD301	PerCp/Cy5.5	MGL1/MGL2	5
CD206	PE/Cy7	C068C2	0.6
CD200R	APC	OX110	0.6
CD86	Alexa Fluor 700	PO3	0.25
MHCII	APC-eFluor 780	M5/114.15.2	1
CD71	BV421	C2	1
Viability	eFluor 506		0.5

Abbreviations: CXCR, C-X-C motif chemokine receptor; CCR, C-C chemokine receptor; CD, cluster of differentiation; LAP, latency-associated peptide; Ig, immunoglobulin; Cy5, cyanine-5; MHCII, major histocompatibility complex II. iNOS, inducible nitric oxide synthase; FITC, fluorescein; PE, phycoerythrin; Cy7, cyanine-7; APC, allophycocyanine; BV, brilliant violet; PerCp, peridinin-Chlorophyll-protein. *iNOS was the only intracellular marker used in this panel.

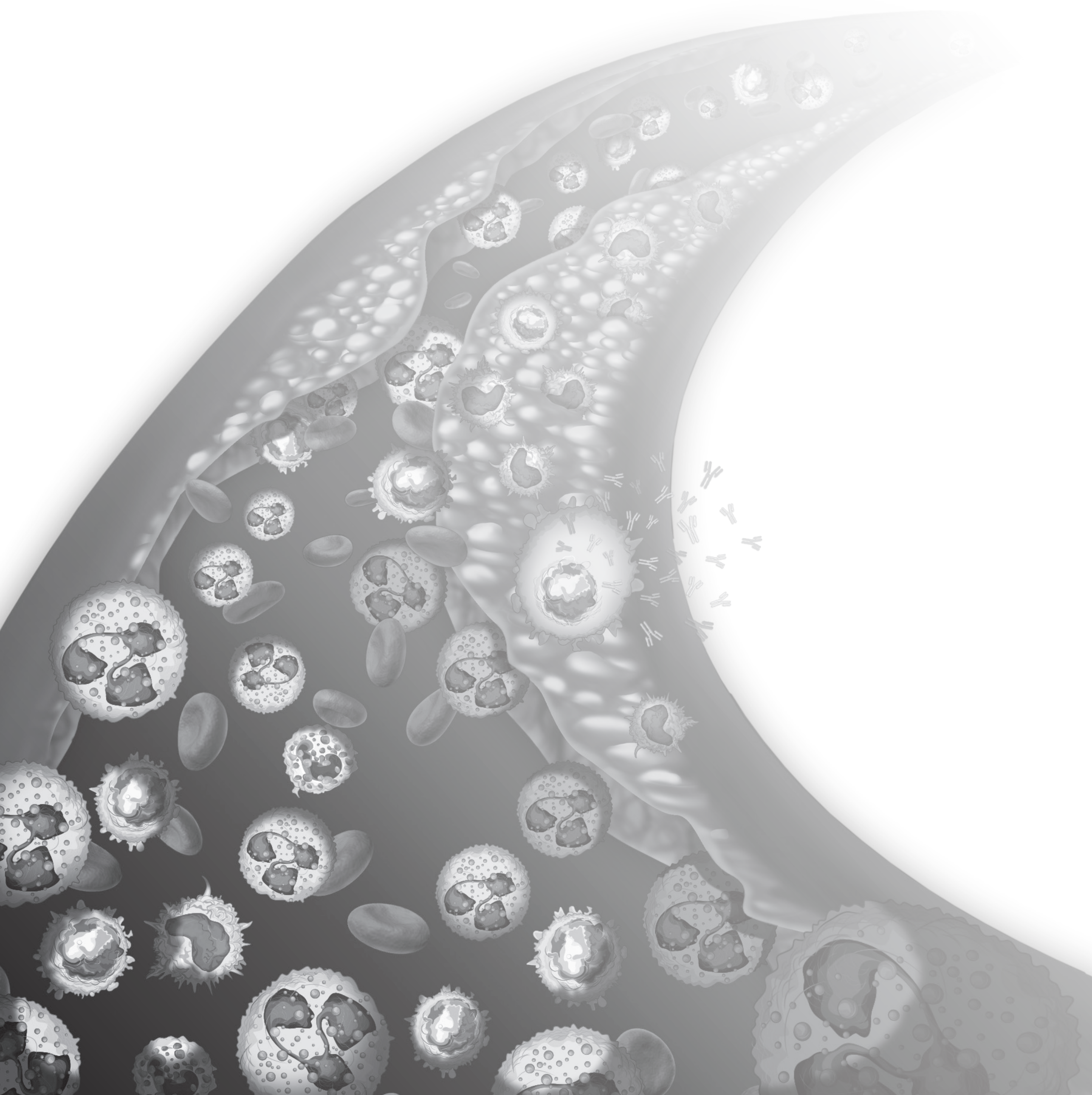
Supplemental table 2. Effect of OX110 treatment on circulating inflammatory cell profiles.

Cell type	**PBS** (n=13)	**Isotype** (n=14)	**OX110** (n=14)	***p***
White blood cells (10e9 cells/L)	5.2 [4.2, 6.5]	5.8 [4.1, 6.5]	5.9 [4.1, 8.2]	0.64
Ly6Ghigh granulocytes (% of WBC)	5.3 [3.6, 10.1]	7.7 [4.5, 11.8]	7.8 [4.2, 15.4]	0.98
Mono/mac total (% of WBC)	3.6 [2.9, 5.4]	4.3 [2.9, 5.5]	4.1 [2.8, 4.8]	0.64
Ly6Chigh (% of mono/mac)	19.1 [14.3, 28.1]	23.6 [18.5, 27.8]	29.2 [20.5, 32.6]	0.48
Ly6Clow (% of mono/mac)	42.4 [38.2, 45.4]	42.5 [40.0, 46.9]	40.7 [36.6, 47.4]	0.52
$CD11C^+$ dendritic cells (% of WBC)	0.8 [0.5, 1.2]	0.7 [0.5, 0.9]	0.6 [0.5, 1.1]	0.98
$CD19^+$ B lymphocytes (% of WBC)	34.9 [28.2, 38.9]	36.6 [30.0, 41.9]	40.3 [27.1, 46.9]	0.76
B1 (% of B cells)	7.6 [6.5, 9.1]	8.8 [7.4, 11.1]	8.9 [7.8, 12.4]	0.55
B1a (% of B1 cells)	68.6 [56.4, 75.3]	70.3 [61.2, 75.0]	71.8 [63.9, 75.7]	0.91
B1b (% of B1 cells)	31.3 [24.5, 43.1]	29.5 [24.7, 38.6]	28.2 [24.1, 35.9]	0.83
B2 (% of B cells)	90.1 [89.4, 91.5]	90.0 [87.3, 91.8]	89.6 [85.1, 90.8]	0.56
$CD4^+$ T lymphocytes (% of WBC)	20.4 [16.5, 21.6]	21.0 [16.2, 24.2]	20.5 [16.5, 21.7]	0.43
$CXCR3^+$ Th1 (% of T cells)	9.7 [6.8, 12.1]	7.9 [7.0, 10.3]	9.2 [6.1, 11.7]	0.54
$CCR4^+$ Th2 (% of T cells)	13.6 [9.4, 19.9]	15.8 [10.8, 20.3]	21.3 [12.0, 28.0]	0.29

Levels of circulating cells are presented as median [Interquartile range] because of their skewed distribution. *P*-values were calculated with Mann-Whitney *U* tests and derived from the comparison between OX110 and isotype treated animals. WBC indicates white blood cells; mono/mac, monocytes and macrophages.

PART TWO

INFLAMMATION AND CVD IN WOMEN WITH FORMER PREECLAMPSIA



CHAPTER

7

Preeclampsia and coronary plaque erosion: Manifestations of endothelial dysfunction resulting in cardiovascular events in women

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ABSTRACT

Atherosclerosis is the major underlying pathology of cardiovascular disease (CVD). The risk for CVD is increased in women with a history of preeclampsia. Multiple studies have indicated that accelerated atherosclerosis underlies this increased CVD risk. Furthermore, it has been suggested that endothelial dysfunction and inflammation play an important role in the increased CVD risk of women with preeclampsia. Rupture or erosion of atherosclerotic plaques can induce the formation of thrombi that underlie the onset of acute clinical CVD such as myocardial infarction and stroke. In relatively young women, cardiovascular events are mainly due to plaque erosions. Eroded plaques have a distinct morphology compared to ruptured plaques, but have been understudied as a substrate for CVD. The currently available evidence points towards lesions with features of stability such as high collagen content and smooth muscle cells and with distinct mechanisms that further promote the pro-thrombotic environment such as Toll Like Receptor (TLR) signaling and endothelial apoptosis. These suggested mechanisms, that point to endothelial dysfunction and intimal thickening, may also play a role in preeclampsia. Pregnancy is considered a stress test for the cardiovascular system with preeclampsia as an additional pathological substrate for earlier manifestation of vascular disease. This review provides a summary of the possible common mechanisms involved in preeclampsia and accelerated atherosclerosis in young females and highlights plaque erosion as a likely substrate for CVD events in women with a history of preeclampsia.

1. PLAQUE EROSION AS A SUBSTRATE FOR CVD IN YOUNG WOMEN

Cardiovascular disease (CVD) often manifests through an occluding thrombus as a consequence of a ruptured atherosclerotic plaque, or due to arterial plaque erosion. Interestingly, the current hypothesis is that the pathogenic mechanisms leading to either an atherosclerotic plaque rupture or erosion are different. Most of our knowledge on the pathology of plaque erosion is based on studies by the group of Virmani and colleagues that has elegantly described plaque erosion as a mechanism of sudden death in autopsy studies from the 1980s onwards[1,2]. Of all thrombi, approximately 31% is caused by plaque erosion[3,4] . Plaque erosion is especially common in young women[2,5], and accounts for approximately 80% of all thrombi in women under the age of 50 years[6]. Plaque histology is different for plaque rupture and plaque erosion. Ruptured plaques have a thin cap, macrophage infiltration, and lipid core that exposes to the blood upon plaque rupture. Eroded plaques are often characterized by a thick, intact cap and minor or no lipid core. In addition, eroded plaques have an altered subendothelial matrix that contains increased proteoglycans, hyaluronan, and smooth muscle cells. Exposure of this matrix to platelets and blood coagulation factors can cause thrombus formation[3]. In the carotid artery, histological analysis of atherosclerotic plaques consistently showed that women display plaques with more stable features and less inflammation, suggestive of plaque erosion as a more prevalent substrate for CVD as compared to men[7-9]. Interestingly, the finding that symptomatic women reveal more stable plaques is independent of cardiovascular risk profile and clinical presentation.

Eroded plaques, more common in women, show much less markers of inflammation compared to unstable and ruptured plaques[6]. Yet, inflammation may stimulate plaque erosion by negatively affecting the function and integrity of the endothelial lining of the atherosclerotic plaque. Proteases capable of degrading the basement membrane are produced in response to inflammation[3]. Different cytokines might have a role in the pathogenesis of plaque erosion, potentially via the induction of endothelial dysfunction. Although plaque erosion has been understudied as compared to plaque rupture, the potential mechanisms involved in the erosion of the plaque have been extensively reviewed. These generally consist of four components: (1) ***endothelial dysfunction and endothelial apoptosis***, (2) ***Toll Like Receptor signaling,*** (3) ***extracellular matrix changes*** and (4) ***changes in platelet adhesion***. With atherosclerotic plaque erosion being a more common substrate for acute CVD in younger women, and preeclampsia being a risk factor for CVD in women, we hypothesize that preeclampsia may predispose to plaque erosion via shared pathophysiological mechanisms.

2. PREECLAMPSIA AND CVD RISK, THE LINK WITH ATHEROSCLEROSIS

Preeclampsia is a hypertensive pregnancy disorder complicating around 1-5% of pregnancies and is characterized by *de novo* hypertension and proteinuria, maternal organ

dysfunction or uteroplacental dysfunction manifesting in the second half of pregnancy[10,11]. Preeclampsia is a major cause of maternal and fetal morbidity and mortality and may affect the health of the mother in the years directly following preeclampsia. Observational studies have consistently shown that women with former preeclampsia have a 2-fold increased risk to develop CVD later in life. In particular, there is cumulating evidence that preeclampsia predisposes to ischemic heart disease that occurs at younger age than in women who have uncomplicated pregnancies. In a study of 3658 women with preeclampsia, ≈ 50 % developed future hypertension with a 3.70 times higher risk compared to women with a normotensive pregnancy[12]. In addition, women with a history of preeclampsia have a 2.2 times higher risk of developing ischemic heart disease. The risk of fatal ischemic heart disease was increased in women with preeclampsia, and early onset preeclampsia (before week 37) significantly added to this risk, resulting in a relative risk of 7.71[12]. Similar, the risk of future stroke was 1.81 times higher in women with preeclampsia compared to normotensive pregnancy[12]. Finally, the risk of future thromboembolism is increased 1.79-fold for women with a preeclamptic pregnancy, although absolute risk remains low (0.1% at 4.7 years after delivery)[12]. Also, but to a lesser extent, there is a relationship between the severity of preeclampsia when presenting in pregnancy, and the long-term risk of CVD later in life. Whereas for patients with mild preeclampsia a relative risk of CVD of 2.00 was found, this was 2.99 for patients with moderate preeclampsia and 5.36 for patients with severe preeclampsia[13]. The exact mechanisms by which preeclampsia increases future cardiovascular risk are unknown although multiple similarities between the mechanisms responsible for CVD and preeclampsia are reported. For example, risk factors for both CVD and preeclampsia in young women include hypertension, obesity, insulin resistance, and hyperlipidemia[14]. However, these similarities do not fully explain the increased CVD risk in women with former preeclampsia as adjustment for CVD risk factors shows that preeclampsia is an independent risk factor for CVD[13,15]. It has been hypothesized that women who experience preeclampsia already have an increased vascular risk prior to developing CVD. The exposed vascular stress during pregnancy causes the body to reach the threshold for development of endothelial dysfunction, vascular inflammation and hampered vascular remodeling with insufficient placental oxygen supply, which have all been reported in the presence of preeclampsia[16]. Later in life, vascular risk factors rise for both the healthy population and women with a history of preeclampsia, but women with a history of preeclampsia are more likely to develop vascular disease earlier compared to the healthy female population pointing to a lower threshold for stressors to elicit vascular occlusive disease[16,17]. Several studies have revealed that ***endothelial dysfunction*** and ***angiogenic imbalance*** are the first indicators of vascular damage in preeclampsia patients. This in combination with the ***chronic inflammatory state*** in preeclampsia patients may lead to acceleration of atherosclerosis after preeclampsia.

3. VASCULAR DYSFUNCTION IN PREECLAMPSIA

While atherosclerosis is a slowly developing progressive condition that finds its origin in adolescence and progresses throughout life with atherosclerotic plaques as a final stage, vascular changes such as acute atherosis, occur relatively instantly in the placental blood vessels during preeclampsia. Atherosis in the spiral arteries is characterized by subendothelial lipid filled foam cells, fibrinoid necrosis of the arterial wall, perivascular lymphocytic infiltration, and it is histologically similar to early-stage atherosclerosis[18]. In the spiral artery remodeling study (SPAR) study, systematic screens of vascular pathology in placental bed biopsy samples were related to CVD risk factors in women with preeclampsia or normal pregnancy[19]. They showed that in women with preeclampsia fewer of the spiral arteries showed complete remodeling and the placenta contained not as much infiltrated $CD3^+$ T cells compared to health pregnancy. The authors speculate the latter may specifically relate to fewer infiltrating regulatory T cells, which have the ability to control for excessive inflammation. Besides it was suggested that the presence of acute atherosis in the placental bed associated to an unfavorable lipoprotein profile postpartum. Although this study provided valuable insight into the link between placental bed disorders and cardiovascular health, these findings remain to be established in the complete SPAR study cohort[19].
Carotid intima media thickness (cIMT) is a surrogate marker for atherosclerosis burden that is assessed non-invasively and associated with the presence of CVD. A recent meta-analysis showed that women who experienced preeclampsia had significantly increased carotid intima-media thickness compared to women without preeclampsia, both at the time of diagnosis and in the first decade postpartum[20,21]. In addition, impaired coronary flow reserve during preeclampsia has been documented. These measures of vascular dysfunction have been correlated with circulating levels of inflammatory biomarkers such as high sensitive C-reactive protein (hs-CRP) as well[22]. The time course of this vascular dysfunction after preeclampsia has not been established up to now. cIMT was increased the first year after severe preeclampsia, but was no longer elevated approximately 5 years postpartum[23]. This suggests that, despite evident vascular dysfunction during preeclampsia, vascular homeostasis may be restored after preeclampsia[23]. Despite the evident decrease in cIMT after 5 years it remains to be confirmed that vascular homeostasis is fully recovered. It may very well be that the endothelial integrity is not fully restored or that the vasculature may remain more sensitized to stress. As such the former preeclamptic vasculature may have an augmented response to stress-related or inflammatory stimuli as seen in atherosclerosis.

4. HYPOXIA, OXIDATIVE STRESS AND ANGIOGENESIS IN PREECLAMPSIA

Endothelial dysfunction is caused by a combination of oxidative stress, angiogenic and vasoresponsive imbalance, and inflammation (**Table 1**)[24]. As a result, women with endothelial dysfunction reveal decreased vasodilation[25,26], increased arterial stiffness[27]

and atherosclerosis[28] in the larger vessels. Skin microvascular density[29,30] and placenta microvascular density[31] were found to be decreased in women with preeclampsia, while others have shown that vascular growth was not significantly affected in preeclampsia[32]. A negative correlation in vasodilator response between the macro and microcirculation has been suggested and this negative correlation was also observed in women with preeclampsia[33-35]. Incomplete remodeling of the uterine spiral arteries causes placental ischemia-reperfusion episodes. During these episodes, reactive oxygen species are formed[24,36]. This leads to oxidative stress[37], shown by increased levels of marker lipid peroxide[38]. Reactive Oxygen Species decrease the bioavailability of pro-angiogenic nitric oxide (NO) via the suppression of NO synthase (NOS). In addition, peroxynitrite is formed when Reactive Oxygen Species and NO react. Peroxynitrate can in turn oxidize DNA, proteins, and lipids. As a consequence, NO balance is disturbed, which can result in impaired vasodilation and angiogenesis[39]. Other molecules that are upregulated by oxidative stress are NF-κB and superoxides, resulting in further activation of anti-angiogenic[24] and pro-inflammatory pathways[40].

Another result from placental ischemia is an angiogenic imbalance towards a more anti-angiogenic and vasoconstrictory state (**Table 1**)[24]. Vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) are important pro-angiogenic and vasodilatory molecules[17]. Serum levels of PlGF[41] and VEGF[42] are decreased in women who develop preeclampsia. Both VEGF and PlGF bind to VEGF-receptor 1, also known as Flt-1[17]. In a healthy pregnancy, VEGF and PlGF bind to Flt-1 on the endothelial cell surface, resulting in activation of anticoagulant, vasodilatory, and proangiogenic pathways. The soluble form of the Flt-1 receptor, sFlt-1, that is a natural antagonist for VEGF and PlGF, is increased in women with preeclampsia[41]. sFlt-1 binds VEGF and PlGF, thereby preventing binding to endothelial cell-surface Flt-1 receptors. As a result of decreased binding to the Flt-1 receptor, the anticoagulant, vasodilatory, and proangiogenic pathways are inhibited and endothelial cells become dysfunctional[43,44]. In addition to increased levels of sFlt-1, serum levels of soluble endoglin (sEng) were also found to be increased in patients with preeclampsia[45,46]. Endoglin is a TGF-β co-receptor highly expressed on the membranes of activated endothelial cells[47]. Membrane bound endoglin can either stimulate or inhibit cellular responses downstream of TGF-β and of its family members, the Bone Morphogenetic Protein (BMP) ligands. In endothelial cells endoglin stimulates angiogenesis[48]. sEng has different affinity for TGF-β and BMP. sEng interferes with TGF-β signaling by trapping circulating TGF-β1. TGF-β signaling stimulates vasodilation via the activation of NO[46], stimulates vascular homeostasis and can either stimulate or inhibit angiogenesis depending on the context[17]. As a result of increased sENG, NO induced vasodilation[46], angiogenesis and vascular homeostasis are diminished[17] in patients with preeclampsia. TGF-β binds with very low affinity to sEng and likely only does bind in the presence of a soluble TGFβ type II receptor while sEng binds with high affinity to BMP9 and inhibits BMP9 induced signaling[49]. Scavenging of BMP9 by sEng reduces the BMP9 induced ET-1 vascular stability and hypertension[50] It has been suggested that hypoxia-inducible factor 1 subunit α (HIF-1α) is responsible for the increased amounts of sEng and

sFlt-1 in preeclampsia[51]. The hypoxic environment of the preeclamptic placenta triggers the expression of HIF-1α, which indeed was increased in the placenta of preeclamptic women[52]. In pregnant mice, overexpression of HIF-1α increased the serum levels of sFlt-1 and sEng and resulted in hypertension and proteinuria, both hallmarks of preeclampsia. These human and animal data suggest that HIF-1α plays an important role in the pathogenesis of preeclampsia via the activation of antiangiogenic pathways[51,53]. This appears ambiguous, since HIF-1α normally activates the transcription of VEGF[54], PlGF[55], and other hypoxia- and angiogenesis-associated proteins[56] as a response to hypoxia. However, this increase is often brief. Long-term upregulation of HIF-1α has been described in the pathology of multiple diseases, including preeclampsia[57].

5. HYPOXIA, OXIDATIVE STRESS AND ANGIOGENESIS IN ATHEROSCLEROSIS AND PLAQUE EROSION

Angiogenic imbalance is less well described in atherosclerosis than in preeclampsia. General consensus is that plaque neovessels primarily derive from the preexisting dense vessel network of the adventitial vaso vasorum, rather than the arterial luminal[58,59] and increased neovascularization enhances atherosclerotic plaque progression via increasing macrophage infiltration and vessel wall thickening[60,61]. In addition, the newly formed vessels can rupture, causing intraplaque hemorrhage contributing in lipid rich necrotic core expansion and oxidative stress[61]. Although VEGF levels are sequentially increased with progressing atherosclerotic disease burden, the exact role of VEGF in atherosclerosis remains unclear, both in humans and in animal models[62,63]. In atherosclerosis, PlGF expression has been shown to be increased, especially in the shoulder region of the atherosclerotic plaque. In early stages of the disease, treatment with anti-PlGF antibodies was able to inhibit the inflammatory process and plaque progression. In later stages of the disease, anti-PlGF antibody treatment had no effect on the plaque development. This suggests that PlGF is primarily involved in plaque development by inducing an inflammatory response[64,65]. Indeed, PlGF has been shown to increase atherosclerotic development via the stimulation of intimal thickening and macrophage accumulation[66]. In addition to VEGF and PlGF, levels of Flt-1 are also increased in the shoulder regions of the atherosclerotic plaque[64]. The antagonist of VEGF and PlGF, sFlt-1 has been shown to inhibit plaque formation, possibly via the inhibition of intraplaque angiogenesis[67]. These processes are opposite to those witnessed in preeclampsia, where VEGF and PlGF levels are decreased and sFlt-1 levels are increased, causing an anti-angiogenic environment (**Table 1**). In plaque erosion, endothelial cell apoptosis is an important part of the pathologic process. Deprivation of growth factors such as VEGF contributes to this process[3]. In addition, decreased levels of VEGF increase oxidative stress and apoptosis[68,69]. Although the involvement of PlGF in plaque erosion has not yet been described, its involvement is likely similar to that of VEGF. The role of sFlt-1 in plaque erosion has also not been described yet. Given the function of sFlt-1, increased levels of sFlt-1 would decrease VEGF and PlGF

signaling, thereby stimulating endothelial cell apoptosis and plaque erosion. Another important protein in preeclampsia pathophysiology, endoglin, is also expressed by endothelial cells and smooth muscle cells in atherosclerotic vessels. Endoglin expression is associated with plaque neoangiogenesis, collagen deposition and thereby stabilizes the plaque[70]. Increased levels of sEng are proatherogenic via inhibition of endoglin function and inhibition of TGF-β signaling[71]. HIF-1α is expressed by various cell types in the atherosclerotic lesion and promotes the development and progression of atherosclerosis[72,73]. Inhibition of HIF-1α has been shown to decrease plaque size[74], reduce vascular inflammation[73,75] and overall inhibit the development of atherosclerosis[72,74,75].

Table 1. Overview of mechanisms of endothelial dysfunction and inflammation involved in atherosclerosis and preeclampsia.

	Atherosclerosis			Preeclampsia		
Endothelial dysfunction	**↑**		**Reference**	**↑**		**Reference**
Oxidative stress	↑	Plaque		↑	Placenta	37
Reactive Oxygen Species	↑	Plaque	81, 80	↑	Placenta	24, 36
NO imbalance	↑	Plaque	69	↑	Placenta	39
Superoxide				↑	Placenta	24
NF-κB	↑	Plaque	80	↑	Placenta	40
Angiogenic imbalance						
Pro-angiogenic	↑			↓		24
VEGF	↑	Plaque	64	↓	Systemic	42
PlGF	↑	Systemic, Plaque	64, 66	↓	Systemic	41
HIF-1α	↑	Plaque	72, 73	↑	Placenta	51, 52
sFlt-1	↑	Plaque	64	↑	Systemic	41
sEng	↑	Systemic, Plaque	71	↑	Systemic	45, 46
Vascular compliance						
Vasodilation	↓	Systemic	25, 26	↓ and ↑*	Systemic	33-35
Arterial stiffness	↑	Systemic	27	↑	Systemic	27
Microvascular density	↑	Plaque	58, 59	↓	Placenta	29-31
EndMT						
Myofibroblasts	↑	Plaque	84, 85, 86	?		
TGF-β	↑	Systemic, Plaque	88	?		
Inflammation	↑	Systemic, Plaque	96	↑	Systemic, Placenta	97
TLR	↑	Systemic PBMC	100, 106, 116	↑	Placenta PBMC	114 115
IL-6	↑	Systemic	117	↓ and ↑	Systemic	123, 124
TNFα	↑	Systemic	125	↓ and ↑	Systemic Placental	127-131 114, 133, 134

*Decreased in macrovasculature and increased in microvasculature.

Although no studies reported a role of sEng in plaque erosion, Matrix metalloproteinase 14 (MMP14) polymorphism was found to be related to a lower risk of vulnerable carotid plaque formation[76]. Furthermore, sEng induced Il-6 and NF-κB in endothelial cells, generating a pro-inflammatory state of the cells[77]. Finally, KLF6 was found to induce MMP14 in endothelial cells, resulting in increased levels of sEng, and reducing membrane bound Endoglin[78]. Reduced endoglin levels on the endothelial cell surface renders them more sensitive to endothelial to mesenchymal transition (EndMT).

6. REACTIVE OXYGEN SPECIES IN ATHEROSCLEROSIS AND PREECLAMPSIA

As well as in preeclampsia, endothelial dysfunction is a pivotal process in the development of atherosclerosis. In preeclampsia, endothelial dysfunction is characterized by oxidative stress, angiogenic imbalance, and vasodilatory imbalance. Endothelial dysfunction has also been described in plaque erosion. This endothelial dysfunction is suggested to be paired with endoplasmic reticulum stress and endothelial cell apoptosis causing detachment of the endothelial cell layer, exposing the sub endothelial matrix and activating thrombus formation[79]. Reactive Oxygen Species production is increased by the common risk factors of preeclampsia and CVD, for example hypertension, hypercholesterolemia, diabetes, cigarette smoking, ageing[80,81]. In preeclampsia, this is further driven by excessive Reactive Oxygen Species production in the placenta, and inadequate action of protective placental scavenging enzymes e.g. superoxide dismutase (SOD)[37]. This sustained elevation of Reactive Oxygen Species levels leads to oxidative stress, with endothelial dysfunction as a consequence[82]. In addition, Reactive Oxygen Species stimulate inflammation via the activation NF-κB pathway and activation of the macrophages in the plaque[80]. In plaque erosion, Reactive Oxygen Species cause the production of oxidized lipoproteins. These lipoproteins induce apoptosis of endothelial cells, resulting in plaque erosion[68,69]. In addition, Reactive Oxygen Species induced endothelial dysfunction might induce the production and activation of proteases and matrix metalloproteinases (MMP), which in turn can degrade the basement membrane. Basement membrane degradation has been described as a process involved in plaque erosion[3]. Also, oxidative stress causes a decrease in NO, thereby decreasing its anti-apoptotic effect[69].

7. NOVEL MANIFESTATIONS OF ENDOTHELIAL DYSFUNCTION IN ATHEROSCLEROSIS

Another process that may add to the endothelial dysfunction seen in both atherosclerosis and preeclampsia is endothelial to mesenchymal transition (EndMT). Endothelial to mesenchymal transition (EndMT) is a complex, dynamic and reversible biological process that can change endothelial cell integrity and can contribute to the pathogenesis of many

diseases including hypertensive disorder[83]. EndMT can be stimulated by differences in several metabolic and inflammatory factors, which are all important in the pathogenesis of atherosclerosis. EndMT is a common phenomenon in atherosclerotic lesion, contributes to plaque progression and lesion calcification[84,85], and is more common in advanced vulnerable plaques[86]. As such it has been suggested that the presence of endMT may associate with clinical events[86] and that the process of endMT may be a promising therapeutic target for atherosclerosis[87].

8. ENDOTHELIAL TO MESENCHYMAL TRANSITION (ENDMT)

When endothelial cells undergo EndMT, they loosen cell-cell contact and change from a cobblestone-like well-structured monolayer into a more chaotic mesenchymal elongated phenotype. Endothelial cells undergoing EndMT down-regulate their endothelial specific markers like Pecam-1, VE-cadherin, and FLK-1, and start to express protein characteristics for a mesenchymal gene signature cells such as α smooth muscle actin (α-SMA), fibroblast specific protein-1 (FSP-1) and type I collagen[88]. The EndMT derived mesenchymal cells are often called myofibroblasts as they express both smooth muscle cell and fibroblast specific genes. EndMT is initiated by activation of the endothelium by e.g. inflammatory cytokines which loosens the cell-cell contacts. One of the key regulators of the mesenchymal transition is TGF-β in a variety of endothelial cells. TGF-β can directly influence EndMT by the expression of the transcription factors Snail and Slug, or indirectly by inducing EndMT regulating microRNAs like microRNA-21[89]. Disturbed flow, can induce a morphological switch in endothelial cells. Endothelial cells in high shear areas lose their primary cilia making them more sensitive to TGF-β induced EndMT[90-92]. Also in the context of atherosclerosis it has been shown that EndMT can be induced by shear stress modifications[93,94] and plaque foam cells can initiate EndMT trough the release of the chemokine CCL4/Macrophage Inflammatory Protein 1β[95]. As mentioned, these cells are prominent in atherosclerosis, due to disturbed flow at the boundaries of the plaque[92] with key functions including regulation of inflammation, matrix and collagen production, and plaque structural integrity (**Table 1**). 'Transitioning' cells are readily detected in human atherosclerotic plaques co-expressing endothelial and fibroblast/mesenchymal proteins, indicative of EndMT[86] .

9. INFLAMMATION AS COMMON PATHWAY FOR PREECLAMPSIA AND ATHEROSCLEROTIC PLAQUE EROSION

Besides endothelial dysfunction, inflammation is a major contributor to the onset of preeclampsia and atherosclerosis[96,97]. Endothelial dysfunction causes increased adhesiveness and permeability of the endothelial layer allowing for leukocytes and platelets to adhere and transmigrate through this damaged endothelium. In addition, the dysfunctional endothelium produces cytokines, vasoactive molecules, and growth factors.

The inflammatory response initiated in preeclampsia shows many similarities to the inflammatory response of atherosclerosis (**Table 1**). Atherosclerotic lesion formation is characterized by massive macrophage accumulation in the intimal area, but, as the lesion progresses, can actually contain almost all inflammatory cell types. The placentas of preeclamptic women contain higher numbers of macrophages and the infiltration of macrophages has been associated with impaired trophoblast infiltration[98]. Similar to that observed in atherosclerosis, these macrophages are predominantly of the pro-inflammatory M1 like phenotype[99]. The persisting inflammatory response results in activation of macrophages and lymphocytes in the affected area where they release hydrolytic enzymes, chemokines, cytokines, and growth factors. Although many cytokines and chemokines have been implicated in the development of atherosclerosis and preeclampsia, here we review those most studied in both pathologies.

9.1 Toll Like Receptors

Toll-like receptors (TLRs) are highly conserved receptors of the innate immune arm that are instrumental in atherosclerotic plaque inflammation by recognizing pathogen- and damage-associated molecular patterns that can be upregulated on, for example, tissue damage or cell stress[100-104]. In atherosclerosis, TLR function is traditionally linked to its effect on plaque macrophages and foam cells, but nowadays it is also recognized that certain TLRs can modify endothelial cell function [105]. TLR2 and TLR4 are predominantly expressed in endothelial cells of the vascular wall and have been associated to atherosclerotic lesion development in different murine models[100,106]. Genetic variation in the TLR2 and TLR4 gene have been associated to early onset preeclampsia[107,108], while the association of TLR2 and TLR4 SNPs to cardiovascular risk is debated on. Genetic variations in the TLR4 gene, Asp299Gly and Thr399Ile have been associated to increased risk for cardiovascular events[109,110], that may be the consequence of modified efficacy of statin treatment in the Asp299Gly carriers[109]. On the other hand, it has also been reported that the TLR4 Asp299Gly variant associates to decreased CRP levels and increased carotid artery compliance, suggesting that this variant may also reduce cardiovascular risk[111,112]. In an elegant study of Li and colleagues, first trimester primary cytotrophoblast and first trimester decidual macrophages were isolated during normal pregnancy and subsequently stimulated with LPS. Trophoblast are placental epithelial cells that are important for proper implantation of the fertilized eggs. Cytotrophoblasts can differentiate into syncytiotrophoblast which are important in transport of nutrients to the fetal system, or into extravillous trophoblast which are important in uterine vasculature remodeling to ensure proper blood supply. Decidual macrophages are placenta specific macrophages that accumulate in close proximity to the implantation site of spiral arteries with the maternal uterine lining (decidua). Decidual macrophages play an important role in play important roles in spiral artery remodeling and angiogenesis but also in extravillous trophoblast invasion and in modulation of the inflammatory response[113]. LPS dose-dependently induced cytokine release, including TNFα, IL-6 and IL-1β, from trophoblasts. In addition, LPS stimulation inhibited trophoblast invasion while it increased macrophage

accumulation in a TLR4 dependent manner[114]. In line with these findings it was shown that the inflammatory response to the TLR ligands is stronger in women with a history of preeclampsia and remain stronger over time when compared to women without preeclampsia [115]. Similar effects were observed in patient with stable coronary artery disease compared to healthy controls[116].

9.2. Interleukin-6 (IL-6)

IL-6 predicts cardiovascular events and has been associated with endothelial dysfunction and arterial stiffness[117]. IL-6 is involved in the causal pathway of atherosclerotic disease progression. The biological function of IL-6 is pleiotropic and includes increased platelet production, differentiation, migration and proliferation of T cells, migration and proliferation of smooth muscle cells, recruitment of neutrophils and the production of matrix metalloproteinases (MMPs), which are all processes driving the inflammatory process in atherosclerosis and preeclampsia. Experimental atherosclerosis studies have shown that in absence of IL-6 atherosclerotic lesion development in $ApoE^{-/-}$ mice is accelerated, which rather appeared to be a consequence of increased plasma lipid levels and not of inflammation, as the latter was decreased in IL-6 deficient animals[118]. Genetic variation in the IL6 gene is associated with reduced CVD risk as shown in a large mendelian randomization study and a meta-analysis[119,120]. It has been suggested that genetic variation in the promotor region of IL6 also is a genetic regulator of early onset preeclampsia[121], while there is no association to the IL6 SNPs described to be associated to cardiovascular risk[122]. In addition, although circulating IL-6 levels did not predict for the onset of preeclampsia[123], IL-6 levels were shown to be significantly increased in severe preeclampsia as compared to normotensive pregnant women[124]. Thus, IL-6 is a major player in several shared pathologic mechanisms of both preeclampsia and atherosclerotic disease.

9.3. Tumor Necrosis Factor-α (TNFα)

TNFα is a classical inflammatory cytokine that is increased in patients with atherosclerosis[125]. It attributes to the development of atherosclerosis by inducing endothelial dysfunction as a consequence of decreased NOS expression and endothelial cell apoptosis[126]. In addition, TNFα increases oxidative stress, inflammation and vascular remodeling. Data regarding circulating TNFα levels in preeclampsia yielded conflicting results. Some studies showed increased TNFα levels during all trimesters[127,128], or the last trimester only[129], while others have found TNFα levels to be unchanged or even decreased in 2nd trimester preeclamptic pregnancy[130,131]. One prospective study even found that TNFα levels, in combination with mean uterine artery doppler, could have additional value in predicting women at risk for preeclampsia[132]. TNFα is expressed in trophoblasts and its expression is increased in the preeclamptic placenta, predominantly in early preeclampsia[133]. Exposure of Human primary trophoblast to LPS increased TNFα production in these cells[114]. In addition, mononuclear cells of women with preeclampsia were more sensitive to stimulation with a mitogen or antigen and consequently produced more TNFα[134]. TNFα (G-308A) polymorphism is enriched in preeclampsia patients[135,136] and presence of this

polymorphism increased disease risk[137], again suggestive of a genetic component in the inflammatory arm of preeclampsia.
Despite the fact that the exact mechanisms involved in plaque erosion are largely unknown, it is generally accepted that plaque erosion mainly occurs in relatively stable plaques. These plaques are typically characterized by a low amount of plaque inflammation. As such, it has been postulated that plaque inflammation may not be so important for plaque erosion. Systemic inflammatory responses however may affect the process of plaque erosion directly, as a consequence of promoting thrombus formation, or indirectly through induction of endothelial dysfunction or apoptosis.

10. FUTURE PERSPECTIVES

In summary, many of the determinants that are involved in atherosclerotic plaque erosion are also observed in preeclampsia related vascular disease (**Figure 1**). The mechanisms that underlie the predisposition of atherosclerotic CVD early in life in women with a history of preeclampsia is poorly understood. The concept of a pre-existent lowered threshold to withstand vascular stressors is intriguing and positions pregnancy as a clinically relevant vascular stress test. Research on the mechanisms leading to preeclampsia may also unravel the mechanisms that result in early manifestations of cardiovascular disease with plaque erosion as the pathological substrate. If common mechanisms are unraveled it may well become reality that prevention of arterial thrombosis in young females may be directed towards stabilization of endothelial function and subsequent prevention of plaque erosion.

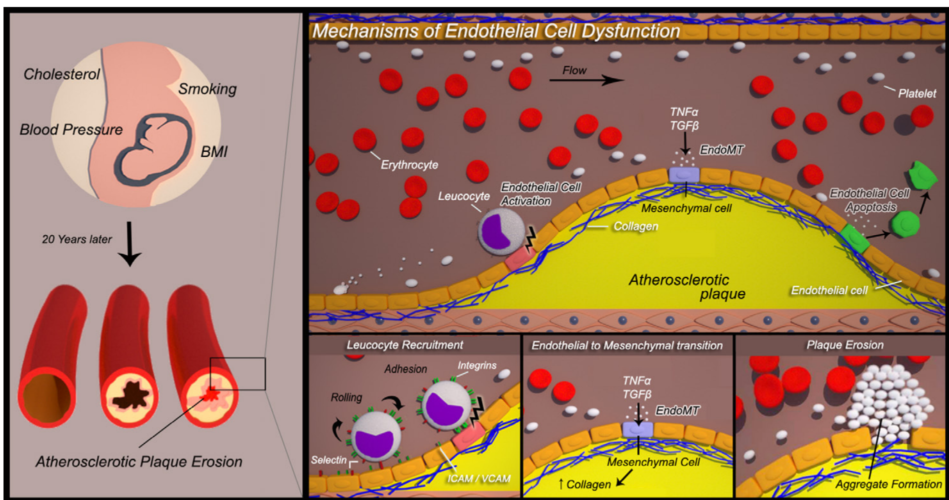

Figure 1. Mechanisms involving endothelial dysfunction and inflammation in the pathology of preeclampsia that may expedite cardiovascular disease development later in life.

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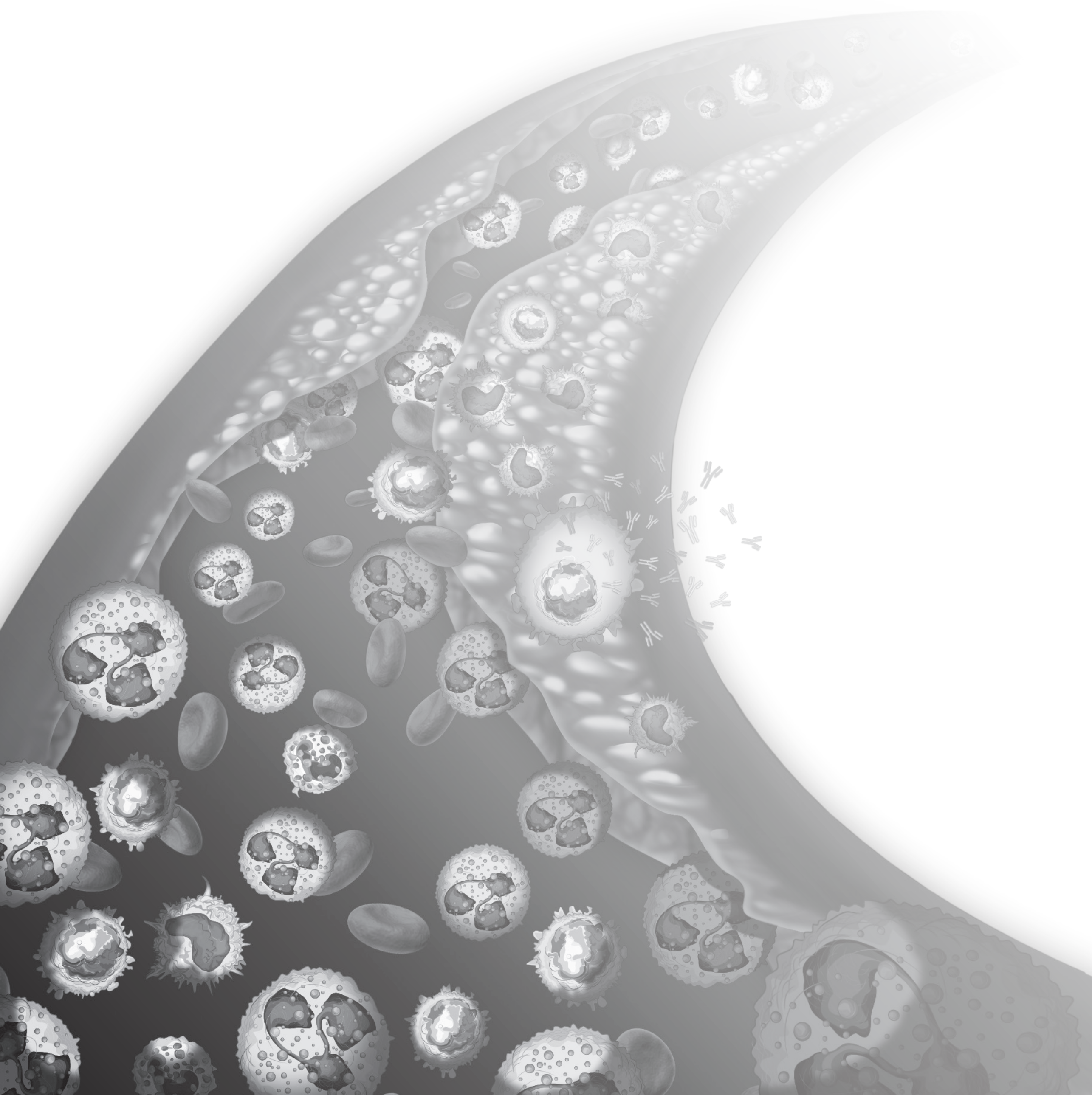

CHAPTER

8

Circulating neutrophils do not predict subclinical coronary artery disease in women with former preeclampsia

Submitted

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ABSTRACT

Introduction

Preeclampsia (PE) represents a hypertensive pregnancy disorder associated with increased cardiovascular disease (CVD) risk. This increased risk has been attributed to accelerated atherosclerosis, with inflammation being a major contributor. Neutrophils play an important role in the onset and progression of atherosclerosis and have been associated with vascular damage in the placenta as well as the chronic inflammatory state in women with PE. We therefore investigated whether circulating neutrophil numbers or reactivity were associated with the presence and severity of subclinical atherosclerosis in women with a history of PE.

Methods

Women aged 45-60 years with a history of PE (n=90), but without symptomatic CVD burden were screened for the presence of subclinical coronary artery disease (CAD) using both contrast-enhanced and non-contrast coronary CT angiography. Subclinical CAD was defined as a coronary artery calcium (CAC) score ≥100 Agatston Units (AU) and/or ≥50% coronary luminal stenosis. We assessed whether the numbers and activity of circulating neutrophils were associated with the presence of subclinical CAD, and as secondary outcome measurements, with the presence of any calcium (CAC score >0 AU), or luminal stenosis, categorized as absent (0%), minimal to mild (>0 and <50%), and moderate to severe (≥50%) narrowing of the coronary artery. Blood was drawn just before CT and neutrophil numbers were assessed by flow cytometry. In addition, the presence of the C-X-C chemokine receptor type 2 (CXCR2) and CXCR4, which are known to be instrumental in neutrophil recruitment, and neutrophil activity upon stimulation with the bacterial peptide formyl-methionyl-leucyl phenylalanine (fMLF) was assessed by flow cytometry.

Results

Of the participating women, 13% (12 out of 90) presented with signs of subclinical CAD and 37% (33 out of 90) had a positive CAC score. Total white blood cell counts and neutrophil counts were not associated with the presence of subclinical CAD or with CAC presence. When assessing the presence of the chemokine receptors CXCR4 and CXCR2, we observed a slight decrease of neutrophil CXCR2 expression in women with CAC (median MFI 22.0 [interquartile range (IQR) 20.2, 23.8]) compared to women without CAC (23.8 [IQR 21.6, 25.6], *p*=0.02). We observed no differences regarding neutrophil CXCR4 expression. In addition, expression of the early activity marker CD35 was slightly lower on neutrophils of women with subclinical CAD (median MFI 1.6 [IQR 1.5, 1.9] compared to 1.9 [IQR 1.7, 2.1] in women without CAD, *p*=0.02). However, for all findings, statistical significance disappeared after adjustment for multiple testing.

Conclusion

Our findings indicate that neutrophil counts and (re)activity are not directly associated with silent CAD disease burden and as such are not suitable as biomarkers to predict the presence of subclinical CAD in a high-risk population of women with a history of preeclampsia.

INTRODUCTION

Cardiovascular disease (CVD) is a major health problem and causes 17.7 million deaths, annually[1]. Women with a history of preeclampsia (PE), a hypertensive pregnancy disorder responsible for severe maternal and fetal morbidity and mortality[2, 3], are at high risk of developing premature CVD, including coronary artery disease (CAD)[4-7]. Therefore, risk stratification is important to help prevent CAD in high-risk patients as recommended in clinical guidelines[8-11]. However, adequate biomarkers to identify women with former PE who are at high risk for CAD are currently not available.

Atherosclerosis and preeclampsia (PE) share common pathophysiologic mechanisms such as endothelial dysfunction and increased inflammation[12]. In PE patients, hypertension and neutrophil activation enhance the development of endothelial dysfunction[13]. Indeed, in several studies, neutrophil activity was profoundly increased during pregnancy in women with PE as compared to women with uncomplicated pregnancy[14-19].

The pathology of atherosclerosis is characterized by inflammation in the context of high lipids, and reflected by the presence of circulating inflammatory cells[20-24]. The short-lived neutrophils are sparsely detected in atherosclerotic plaques, possibly because of their short lifespan and rapid clearance by macrophages[24]. Nevertheless, neutrophils still play a major role in the development and rupture of atherosclerotic plaques[25-27]. Infiltrating neutrophils contribute to necrotic core formation[24], and induce plaque destabilization by the release of metalloproteinases and myeloid peroxidase[27, 28]. In addition, neutrophils can enhance recruitment and local activation of monocytes[29], thereby increasing the necrotic core and enhancing plaque vulnerability[30]. Peripheral neutrophil counts have been significantly associated with lesion size and risk of recurrent cardiovascular events in patients[25, 31-34], indicating that regulation of neutrophil homeostasis affects atherosclerosis[24]. Neutrophil migration (i.e. retention and release from the bone marrow and return of senescent neutrophils for clearance in the bone marrow) is regulated by chemokine receptors CXCR2 and CXCR4. Increased CXCR2 and decreased CXCR4 expression is associated with enhanced neutrophil release from the bone marrow and impaired homing towards the bone marrow. In contrast, high CXCR4 expression in combination with low CD62L expression stimulates homing to the bone marrow and the clearance of senescent neutrophils.[24] Interestingly, reduced expression of CXCR4, which increases neutrophil mobilization and reduces homing to the bone marrow, was associated with increased atherosclerotic lesion size in mice[35, 36]. In line with these findings, CXCR4 expression on circulating neutrophils was lower in carotid endarterectomy patients and patients with unstable angina pectoris as compared to healthy controls[36].

Although an important role for neutrophils has been established in the pathology of both atherosclerosis and preeclampsia, little is known about the predictive value of neutrophils for subclinical CAD in women with former preeclampsia. In the current study, we investigated if neutrophil counts, neutrophil recruitment, and neutrophil activity were associated with subclinical CAD in women with a history of PE.

METHODS

Participants

The current cross-sectional study comprises women from the CREW-IMAGO study[37, 38]. As part of the study, we included women aged 45 to 60 years old with a history of early preeclampsia (delivery <34 weeks of gestation). Participants underwent a cardiovascular risk screening and coronary computed tomography (CCT) in the University Medical Center Utrecht. Preeclampsia was defined according to the 2014 definition of the International Society for the Study of Hypertension in Pregnancy as hypertension developing after 20 weeks of gestation in combination with either proteinuria, maternal organ dysfunction or uteroplacental dysfunction[39]. Women with any serious illness that compromised study participation were excluded, as well as patients with high risk for contrast nephropathy (renal dysfunction with an estimated glomerular filtration rate <60 mL/min/1.73m2) or patients with a history of myocardial infarction. The study protocol conforms to the Declaration of Helsinki and has been approved by the local ethics committee on research on humans. All participants provided written informed consent upon inclusion.

Measurement of coronary artery calcium and stenosis

The imaging methods for the visualization of calcification and stenosis are described in detail before[37] and analyzed by an experienced cardiovascular radiologist (BKV) using a standardized protocol. Briefly, a non-contrast CCT was performed to calculate the coronary artery calcium (CAC) score with the Agatston scoring method[40]. Presence of CAC was defined as >0 Agatston Units (AU). Next, coronary CT angiography was performed to assess plaque burden and luminal stenosis according to the American Heart Association classification[41, 42]. Primary outcome was subclinical CAD, defined as (CAC score ≥100 AU and/or ≥50% luminal stenosis). Secondary outcomes included the presence of any coronary calcification (CAC score >0 AU), which were converted to MESA percentiles[43], and the presence of luminal stenosis, categorized as absent (0%), minimal to mild (>0 and <50%), and moderate to severe (≥50%) narrowing, based on diameter measurements comparing diameters of the maximal stenosis to a reference diameter proximal and distal to the stenotic area[44].

Blood collection, stimulation of neutrophils and red blood cell lysis

Prior to the CT imaging procedure, 10 mL blood was collected in Sodium-Heparin anticoagulant tubes. A complete blood count profile was determined by a general hematology cell counter (Cell Dyn 1800 Abbott, Minesota, USA). We assessed both baseline activity of neutrophils, and neutrophil activity response upon whole blood stimulation. In order to stimulate the neutrophils, 2 mL of blood was incubated with the chemotactic peptide N-Formylmethionyl-leucyl-phenylalanine (fMLF, end concentration 1 μM, F3506, Sigma, St. Louis, MO, USA) for 5 minutes at 37°C, within 10 minutes after blood draw. Activation was confirmed with flow cytometry analyses (described below) by increased expression of degranulation markers CD35, CD11B and CD66B. As expected,

CD62L surface receptors shed upon fMLF stimulation ($p<0.001$ for all markers) (**Supplemental figure 1A**). To indicate the response of neutrophils upon stimulation with fMLF, we used the index (MFI after fMLF stimulation / MFI before stimulation) (**Supplemental figure 1B**).

After stimulation, red blood cells (RBCs) were lysed with RBC lysis buffer (deionized water supplemented with 155mM NH_4Cl, 10mM $KHCO_3$ and 0.1 mM EDTA, osmolality 305-310, and pH 7.4 at 4°C) for 20 minutes on ice. To remove any remaining RBCs, the pellet was resuspended in RBC lysis buffer and centrifuged at 350*g* for 5 minutes at 4°C. To wash the cells, the pellet was resuspended and centrifuged at 350*g* for 5 minutes at 4°C twice with PBS^{2+} (PBS supplemented with Albuman 10% w/v and trisodium citrate 0.32% w/v).

Flow cytometry

To identify different neutrophil subsets and activity markers, the cells were incubated with antibodies (**Supplemental table 1**) for 60 minutes, at 4°C, protected from light. To remove any unbound antibodies, cells were washed with PBS. To distinguish dead cells, the cells were then incubated with fixable viability dye (eFluor 506, eBioscience, San Diego, CA, USA) for 30 minutes on ice, protected from light. After washing with PBS^{2+}, the cells were measured by flow cytometry (Gallios, Beckman Coulter). Viable neutrophils were identified using forward and sideward scatter measurements in combination with markers for viability and CD9 (to exclude eosinophils). From the selected neutrophils, we measured the surface expression of chemokine receptors CXCR2 and CXCR4, markers of early (CD35), intermediate (CD11B) and late stage (CD66B) degranulation, and activity marker CD62L, which is shed upon activation. Both neutrophils from normal blood samples and activated neutrophils were measured by flow cytometry and analyzed with statistical methods as described below.

Statistical analyses

Continuous variables were depicted as mean ± SD or median and interquartile range. Variables with normal distributions were compared using Student's *t* tests or one-way ANOVA, and non-normal distributed variables were compared with the non-parametric Mann-Whitney *U* or Kruskal-Wallis tests. Categorical variables were analyzed with Chi-squared or Fisher's exact tests. Data management and statistical analyses were performed with RStudio[45] and the R software package (version 3.2.0. Vienna, Austria)[46]. Since several comparisons were performed, *p*-values were corrected for multiple testing using the false discovery rate (FDR) method[47]. We considered adjusted *p*-values <0.05 as statistically significant. Graphs were produced using the Graphpad Prism 7.02 software (La Jolla, CA, USA).

RESULTS

Baseline characteristics

The current study comprised 90 women with former preeclampsia, of which 12 (13%) had subclinical coronary artery disease (CAD), defined as a CAC score ≥100 Agatston Units (AU) and/or ≥50% luminal stenosis. Baseline characteristics were stratified by the presence of subclinical CAD (**Table 1**). Mean age was 49 years and did not differ between groups. Women with subclinical CAD tended to have a higher waist circumference (mean 94.6 ± 11.6 cm) compared to controls (88.4 ± 11.9 cm, p=0.09). A positive family history of premature CVD was slightly more common in women with subclinical CAD (66.7%) than in women without CAD (39.7%), although this difference did not reach statistical significance (p=0.15). The frequency of other CVD risk factors, such as hypertension, diabetes and metabolic syndrome was comparable between women with and without CAD. Fifty-seven participants (63%) had a CAC score of zero. The median CAC score in the group with CAC >0 AU was 35 AU (range 0.5 – 1989), and the median CAC percentile based on MESA classification was 93 (range 61 – 99)[43].

Total neutrophil numbers in subclinical CAD

We first investigated the white blood cell (WBC) composition in our population. WBC counts and WBC subtypes were within the clinical reference values (data not shown). Similarly, in our patient population we did not observe any statistically significant differences in total WBC, lymphocyte and monocyte counts between women with and without CAC (defined as >0 AU), among women with different stenosis categories, or between women with and without subclinical CAD (**Figure 1A-C**). Although the granulocyte count was slightly increased in women with subclinical CAD (4.6 x 10e9 cells/L [IQR 3.6, 5.7]) as compared to women without subclinical CAD (4.1 x 10e9 cells/L [IQR 3.3, 5.3]), statistical significance was not reached (p=0.45).

Neutrophil Chemokine Receptor expression and subclinical CAD

Next, we explored if neutrophil migration receptors CXCR2 and CXCR4 were associated with the presence of any coronary calcium, stenosis or subclinical CAD. No differences were observed when the percentage of cells negative for CXCR2 or positive for CXCR4 were compared between women with or without CAC, stenosis or subclinical CAD (**Figure 2A-C**). While the percentage of CXCR2 negative cells was not different, CXCR2 expression was slightly lower in women with CAC (median 22.0% [IQR 20.2, 23.8] as compared to women without (median 23.8% [IQR 21.6, 25.6], p=0.02) (**Figure 2D**). However, after adjustment for multiple testing, the results were not statistically significant (p=0.13). Neutrophil CXCR4 expression was not associated with CAC presence (**Figure 2D**). Surface expression of neutrophil migration markers CXCR2 and CXCR4 was not significantly associated with the presence of coronary stenosis or subclinical CAD (**Figure 2E-F**).

Table 1. Baseline characteristics of subclinical CAD cases and controls.

	Controls (n=78)	Subclinical CAD cases (n=12)	*p*
Patient characteristics			
Age (years)	49.4 ± 3.7	49.0 ± 5.0	0.72
GA delivery (days)	213.0 ± 28.4	205.6 ± 20.4	0.39
Clinical measurements			
Systolic blood pressure (mmHg)	130.8 ± 16.0	127.5 ± 15.1	0.50
Diastolic blood pressure (mmHg)	80.1 ± 10.0	77.7 ± 10.6	0.43
BMI (kg/m2)	27.6 ± 5.1	29.7 ± 6.9	0.20
Waist circumference (cm)	88.4 ± 11.9	94.6 ± 11.6	0.09
Total cholesterol (mmol/L)	5.3 [4.8, 6.0]	5.6 [4.8, 5.8]	0.87
Triglycerides (mmol/L)	1.1 [0.8, 1.5]	1.2 [0.9, 1.5]	0.54
HDL-cholesterol (mmol/L)	1.5 [1.3, 1.6]	1.5 [1.3, 1.6]	1.00
LDL-cholesterol (mmol/L)	3.3 [2.8, 3.9]	3.4 [2.7, 3.8]	0.64
Glucose (mmol/L)	5.5 ± 1.3	5.3 ± 0.5	0.57
CVD risk factors			
Family history of premature CVD (no, %)	31 (39.7)	8 (66.7)	0.15
Hypertension[a] (no, %)	42 (54.5)	8 (66.7)	0.64
Obesity (no, %)	23 (29.5)	4 (33.3)	0.75[†]
Diabetes (no, %)	3 (3.8)	0 (0.0)	1.00[†]
Current smoking (no, %)	8 (10.5)	0 (0.0)	0.59[†]
Metabolic syndrome[b] (no, %)	25 (32.1)	4 (33.3)	1.00[†]
FRS (percentage)	6.4 ± 4.0	5.5 ± 3.5	0.48
Intermediate–high risk, FRS ≥10% (no, %)	12 (15.8)	1 (9.1)	1.00[†]
Whole blood count			
WBC (10e9/L)	6.8 [5.5, 7.8]	6.6 [5.5, 8.4]	0.79
Lymphocytes (10e9/L)	1.8 [1.4, 2.1]	1.8 [1.3, 2.1]	0.97
Monocytes (10e9/L)	0.7 [0.5, 0.8]	0.7 [0.6, 0.7]	0.72
Granulocytes (10e9/L)	4.1 [3.3, 5.3]	4.6 [3.6, 5.7]	0.45

Demographic characteristics are stratified by presence of subclinical coronary artery disease. The values are presented as mean ± SD for normal distributions, number of patients (frequency in percentage) for categorical variables, and median [interquartile range] for non-normal distributions. *P*-values are calculated using the Student's *t* test, Chi-squared or Fisher's exact test (†), and Mann-Whitney *U* test, respectively. CAD indicates subclinical coronary artery disease defined as ≥100 Agatston Units and/or ≥50% stenosis; GA, gestational age; BMI, body-mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; FRS, Framingham Risk Score; WBC, white blood cells. [a] Blood pressure ≥140/90 mmHg or current use of antihypertensive treatment. [b] According to NCEP ATP-III criteria.

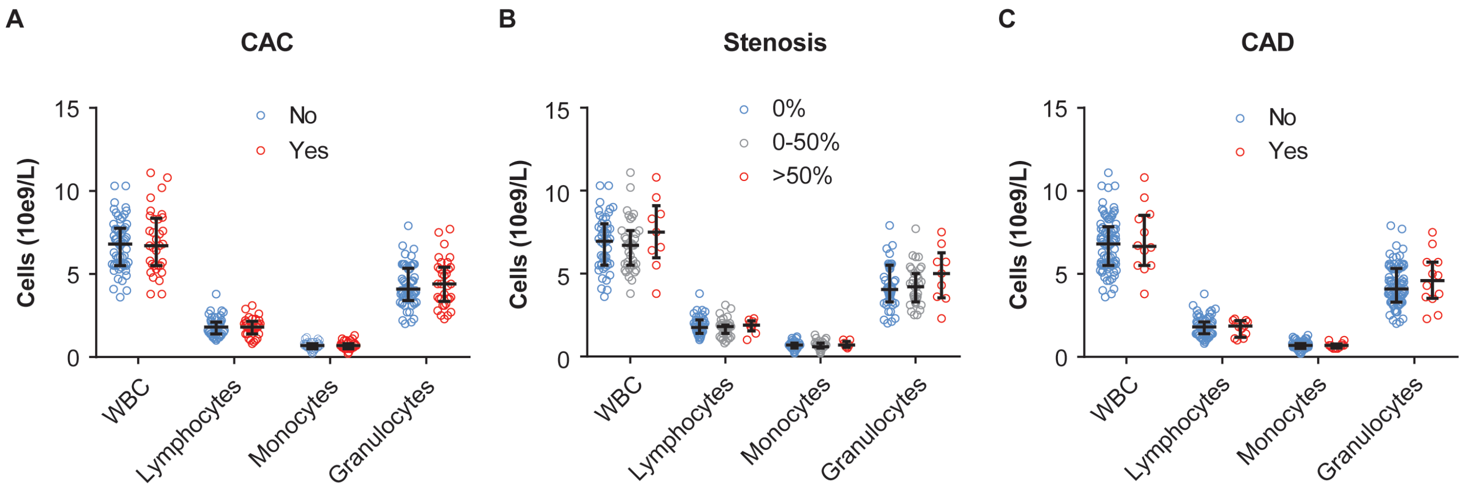

Figure 1. WBC, lymphocyte, monocyte and neutrophil counts of the total study population stratified by presence of coronary artery calcification (CAC) (A), stenosis (B) and subclinical coronary artery disease (CAD) (C). Although the granulocyte count is slightly increased in former PE women with ≥50% stenosis and subclinical CAD, these differences were not statistically significant. CAC indicates coronary artery calcification defined as >0 Agatston Units (AU); CAD, subclinical coronary artery disease defined as ≥100 AU and/or ≥50% stenosis.

Figure 2. Presence of chemokine receptors CXCR2 and CXCR4 is shown in women with a history of PE. The percentages of cells expressing CXCR2 or CXCR4 were comparable between women with or without presence of CAC, stenosis and subclinical CAD (A-C). While the percentage of CXCR2 negative cells was comparable (A), CXCR2 surface expression was lower in women with CAC as compared to women without CAC (D). We observed no differences for CXCR2 or CXCR4 regarding the presence of stenosis or subclinical CAD (E-F). CAC indicates coronary artery calcification defined as >0 Agatston Units (AU); CAD, subclinical coronary artery disease defined as ≥100 AU and/or ≥50% stenosis. * indicates $p<0.05$ (Mann-Whitney U test).

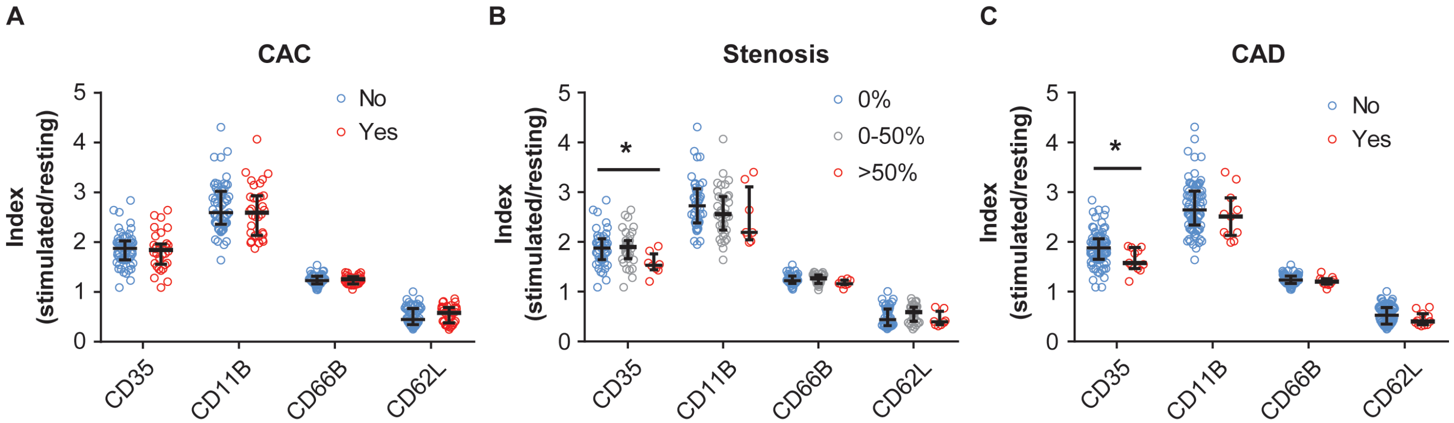

Figure 3. Neutrophil activity is shown in women with former PE. Neutrophil activity was not different between women with and without CAC (A). In women with ≥50% of coronary stenosis and with subclinical CAD, the neutrophil CD35 index was lower, indicating a lower response to stimulation with the chemotactic peptide fMLF (B-C). A similar trend was observed for degranulation marker CD11B, but not for CD66B and CD62L (B-C). CAC indicates coronary artery calcification defined as >0 Agatston Units (AU); CAD, subclinical coronary artery disease defined as ≥100 AU and/or ≥50% stenosis. * indicates $p<0.05$ (Kruskal-Wallis test and Mann-Whitney *U* test).

Neutrophil activity and subclinical CAD

Neutrophil activity was comparable between patients with and without CAC (**Figure 3A** and **supplemental table 2**). We then investigated whether neutrophil activity markers were associated with the degree of coronary stenosis in women with former PE. Upon fMLF stimulation, the response of neutrophils was lower for CD35 in PE patients with ≥50% stenosis (1.5 [IQR 1.5, 1.6]) as compared to PE patients without stenosis (1.9 [IQR 1.7, 2.0], p=0.03) (**Figure 3B** and **supplemental table 3**). However, after adjustment for multiple testing, the results were not statistically significant (p=0.16). In line, the CD35 response was also lower in patients with subclinical CAD (1.6 [IQR 1.5, 1.9]) than in patients without CAD (1.9 [IQR 1.7, 2.1], p=0.02), although the results were not statistically significant when multiple testing correction was applied (p=0.11). A similar trend was observed for CD11B in women with coronary stenosis (**Figure 3B** and **supplemental table 3**) and with subclinical CAD (**Figure 3C** and **supplemental table 4**).

DISCUSSION

According to the European guidelines on cardiovascular disease prevention of 2016, a periodic screening for hypertension and diabetes mellitus should be considered in women with a history of pre-eclampsia[11]. However, recent data from the CREW-IMAGO study showed that between 45 and 55 years of age, 30% of the women with a history of PE had an elevated CAC score as compared to 18% in the control group[38], demonstrating the importance of early identification of women with a history of PE that are at high risk for cardiovascular complications. Finding a biomarker to identify these women at high risk is crucial, and such a marker may be found in players that are involved in both PE and CVD. Therefore, we investigated if women after PE with silent CAD had a different profile of neutrophil subsets or neutrophil (re)activity compared to women without evidence of subclinical CAD. Despite the well-established role of neutrophils in both PE and atherosclerosis, we found no differences in neutrophil numbers or (re)activity in cases compared to controls, except for a slight decrease in expression of the early neutrophil activity marker, CD35. These results suggest that neutrophil numbers or (re)activity are not useful to distinguish subclinical CAD cases from controls in women with a history of PE at middle-age.

Neutrophils have been shown to play an important role in the pathophysiology of PE[14-16, 19]. In addition, it has been well-established that neutrophil counts are associated with the presence of CAD and the risk of recurrent CVD events[32-34, 48, 49]. Moreover, experimental studies have identified a crucial role for neutrophils in atherosclerotic development and progression[24, 28, 29]. Neutrophils contribute to the oxidation of lipoproteins and recruit monocytes to the atherosclerotic lesion[24] and neutrophil counts are correlated with atherosclerotic lesion area[25]. Mice lacking neutrophil granule protein cathelicidin (CRAMP or LL37 in human), an important activator and recruiter of immune cells, show reduced lesion size and less macrophage infiltration[29]. Recruited neutrophils from the bone marrow

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show low CXCR4 and high CXCR2 expression. Inhibition of CXCR2 led to impaired neutrophil recruitment in atherosclerotic lesions[25]. We found that women with coronary CAC tended to exhibit lower neutrophil CXCR2 expression, which might seem counterintuitive. However, since decreased neutrophil CXCR2 surface is associated with increased sequestration in the bloodstream and increased neutrophil activity[50], these results might suggest that activated neutrophils tend to remain in the bloodstream in women with CAC. Interestingly, rheumatoid arthritis patients, being at increased risk of CVD, also exhibited low neutrophil CXCR2 expression in the synovial fluid as compared to the peripheral blood, which might be the result of recruitment of neutrophils via IL-8 signaling or the local presence of TNF-α in the synovial fluid[51]. In addition, (non-neutrophil specific) CXCR2 expression was decreased in preeclamptic placentas[52]. Further research elaborating the pathways that involve CXCR2 signaling, for example the Akt pathway, might shine new light on the role of CXCR2 in PE patients that develop CAD.

We also found that upon stimulation with fMLF, neutrophil surface expression of degranulation marker CD35 (CR1) was marginally lower in women with subclinical CAD as compared to controls. These results might indicate that neutrophils become less activated in response to fMLF stimulation, which was also reflected by a trend of decreased neutrophil CD11B expression in formerly PE women with subclinical CAD. On the other hand, one may speculate that decreased expression of early degranulation marker CD35 indicates accelerated activation of neutrophils, but that should be accompanied with increased expression of the later degranulation markers CD11B and CD66B or by decreased CD62L expression, what we however not observed. In other studies, the role of CD35 remains to be fully elucidated. CD35 was higher in sepsis patients and also found in a soluble form in the synovial fluid of patients with rheumatoid arthritis[53, 54]. Studies on CD35 polymorphisms in CVD patients have found contrasting results. In one study, CD35 polymorphisms were associated with increased inflammation and increased risk of MI[55]. In contrast, another study reported a higher frequency of a CD35 polymorphism in healthy controls than in CAD patients[56]. However, no functional evidence for neutrophil involvement was provided in these studies, as the authors linked the underlying biological mechanism mainly to CD35 on erythrocytes.

The lack of differences in neutrophil (re)activity among women with former PE with or without CAD might have different explanations. First, it might be possible that neutrophils are activated in all women with former PE, but that these differences are not associated with presence of subclinical CAD. Although not the goal of the current study, a control population of women with a normal pregnancy might elaborate this hypothesis. Second, gender differences regarding neutrophil counts and activity have been reported in both experimental models and human studies[57-61], which may complicate the translation from previous atherosclerosis studies to the current study. For example, female mice had lower circulating neutrophil numbers[57], and reproductive female rats showed higher phagocytic response than males and pre- or postreproductive females[59]. In humans, neutrophil extracellular traps, represented by myeloperoxidase-DNA complexes were also found to be higher in males than females[60]. These differences might complicate the proper

understanding of a possible role for neutrophils in women with former PE at high risk for CAD. Third, the available human evidence for neutrophil involvement in both PE and atherosclerosis was predominantly derived from acute manifestations of the diseases. Therefore, the involvement of neutrophils in PE might be temporal and not present during the subclinical phase. Indeed, when neutrophil activity was assessed in PE patients, CD11B expression was higher in PE patients compared to controls, but the differences disappeared 6 weeks and 6 months postpartum[19]. In line, neutrophil superoxide production was increased in PE patients as compared to controls during pregnancy, but disappeared after delivery. In a control group of pregnant women with essential hypertension, superoxide production was also increased, and remained high postpartum, indicating that neutrophil activity might rather result from hypertension, than from PE[16]. In the current study, we could not test this hypothesis since we could not expose healthy individuals to CCT with a radiation dose of 3.0 mSv for research purposes, because of regulatory and ethical restrictions. When comparing former PE women with and without hypertension, however, neutrophil count, migratory capacity and activity did not differ (data not shown).

The current study showed no predictive value at cross-sectional level for subclinical CAD, and therefore, long-term follow-up to appreciate the prognostic value of neutrophil counts and neutrophil activity for the occurrence of cardiovascular events in these women at high risk remains to be established. Biomarkers enabling risk stratification before the onset of clinical symptoms would be useful to develop further. Other inflammatory cells or markers that are involved in the pathology of both PE and atherosclerosis[12], or sex-specific markers involved in atherosclerosis such as growth differentiation factor 15[62], may be of specific interest.

Thus, although neutrophils are important in both atherosclerosis and preeclampsia, these findings indicate that neutrophil counts and (re)activity are not directly associated to subclinical CAD disease burden and as such are not suitable as biomarkers to predict the presence of subclinical CAD in women with a history of preeclampsia.

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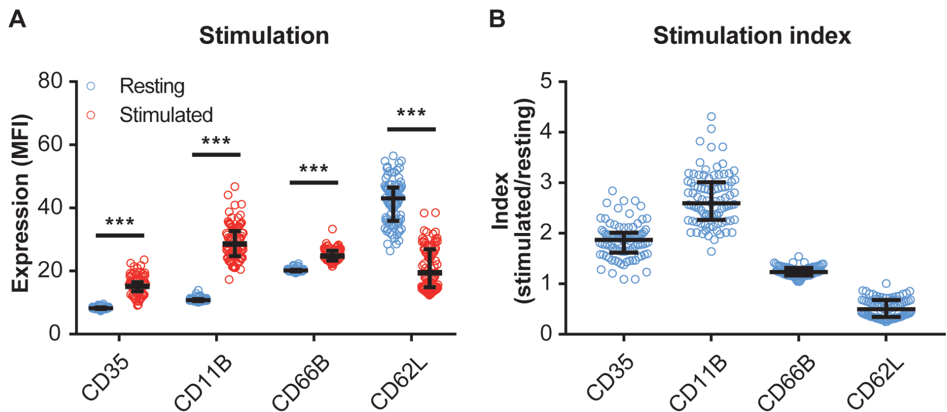
SUPPLEMENTAL DATA

Supplemental table 1. Flow cytometry antibodies.

Marker	Alternative name	Fluorochrome	Clone	Supplier	Amount (µL)
CD182	CXCR2	AF488	5E8	Biolegend	0.5
CD35	CR1 or C3b/C4b	PE	E11	BD biosciences	5
CD16	FcγRIII	ECD	3G8	Beckman Coulter	0.5
CD14		RPE/Cy7	Rmo52	Beckman Coulter	2.5
CD66b	CEACAM8	APC	80H3	Beckman Coulter	1
CD62L	L-selectin	AF700	DREG56	BD biosciences	0.5
CD11b	CR3A	APC-eFluor 780	ICRF44	eBioscience	2.5
CD184	CXCR4	BV421	12G5	BD biosciences	5
CD9	TSP-29	BV510	M-L13	BD biosciences	5

CD indicates cluster of differentiation; CXCR, C-X-C motif receptor; CR, complement receptor; FcγR, Fc gamma receptor; CEACAM, carcinoembryonic antigen-related cell adhesion molecules; TSP-29, tetraspanin protein 29; AF, Alexa Fluor; PE, (R-) phycoerythrin; ECD, phycoerythrin-Texas Red conjugate (energy coupled dye); RPE/Cy7, (R-) Phycoerythrin: Cy-7 Tandem Conjugate; APC, Allophycocyanin; BV, Brilliant Violet.



Supplemental figure 1. Stimulation of neutrophils with N-Formylmethionyl-leucyl-phenylalanine (fMLF). Upon stimulation with fMLF, surface expression of activity markers CD35, CD11B and CD66B increases, whereas CD62L surface expression decreases (A). Also, the index values (after / before stimulation with fMLF) are shown (B). Data are presented as median with interquartile ranges from all patients (n=90). *** indicates p<0.001 (Mann-Whitney *U* test).

Supplemental table 2. Neutrophil activity is shown for former PE women with or without CAC.

		CAC				CAC		
	% cells	**No (n=57)**	**Yes (n=33)**	***p***	**MFI**	**No (n=57)**	**Yes (n=33)**	***p***
CD35+	Resting	1.9 [1.1, 3.4]	1.7 [1.0, 3.2]	0.60	Resting	8.2 [8.0, 8.4]	8.2 [8.0, 8.5]	0.79
	Stimulated	97.0 [93.7, 98.5]	97.5 [91.4, 98.9]	0.79	Stimulated	15.2 [13.7, 16.4]	14.8 [13.0, 16.6]	0.72
CD11B+	Resting	1.3 [0.7, 2.6]	0.9 [0.5, 1.5]	0.16	Resting	10.8 [10.5, 11.2]	10.6 [10.5, 10.9]	0.39
	Stimulated	99.8 [99.3, 99.8]	99.7 [99.4, 99.8]	0.64	Stimulated	29.4 [25.2, 32.8]	27.9 [23.8, 32.1]	0.39
CD66B+	Resting	0.9 [0.4, 1.9]	1.0 [0.6, 2.7]	0.20	Resting	20.2 [19.9, 20.5]	20.1 [19.9, 20.3]	0.21
	Stimulated	49.2 [42.6, 60.0]	54.2 [42.8, 64.1]	0.41	Stimulated	24.6 [23.4, 26.2]	25.2 [23.4, 26.4]	0.80
CD62L-	Resting	1.4 [0.5, 2.1]	0.7 [0.4, 3.0]	0.50	Resting	7.2 [4.8, 7.9]	7.1 [5.1, 7.8]	0.89
	Stimulated	56.9 [48.2, 64.7]	54.2 [46.8, 64.0]	0.25	Stimulated	2.1 [1.9, 2.4]	2.0 [1.8, 2.6]	0.72

Neutrophil activity is shown as the median percentage or the MFI and [interquartile range] of cells positive for CD35, CD11B or CD66B and negative for CD62L while resting and upon stimulation with fMLF. Neutrophil activity was not different between women with and without CAC. CAC indicates coronary artery calcification, defined as >0 Agatston Units. Indicated *p*-values derived from the Mann-Whitney *U* test.

Supplemental table 3. Neutrophil activity is shown in women with former PE with different degrees of luminal coronary stenosis.

		Luminal stenosis					Luminal stenosis			
	% cells	**0% (n=46)**	**0-50% (n=35)**	**≥50% (n=9)**	***p***	**MFI**	**0 % (n=46)**	**0-50% (n=35)**	**≥50% (n=9)**	***p***
CD35+	Resting	1.9 [1.1, 4.0]	1.7 [1.2, 3.2]	1.2 [0.9, 2.2]	0.79	Resting	8.2 [8.0, 8.4]	8.2 [7.9, 8.4]	8.2 [8.0, 8.6]	0.99
	Stimulated	97.1 [93.5, 98.7]	97.5 [93.2, 98.8]	94.2 [90.0, 96.9]	0.32	Stimulated	15.3 [13.7, 16.8]	15.4 [13.7, 16.8]	12.8 [12.1, 14.6]	**0.03**
CD11B+	Resting	1.4 [0.7, 2.5]	0.9 [0.5, 1.5]	1.3 [0.4, 5.1]	0.14	Resting	10.8 [10.5, 11.0]	10.8 [10.5, 11.1]	10.6 [10.5, 10.8]	0.74
	Stimulated	99.8 [99.5, 99.8]	99.7 [99.2, 99.8]	99.6 [99.5, 99.8]	0.48	Stimulated	29.7 [25.3, 32.8]	28.6 [24.4, 31.5]	24.6 [22.7, 29.6]	0.31
CD66B+	Resting	0.9 [0.5, 1.9]	1.0 [0.6, 2.1]	0.9 [0.6, 3.6]	0.67	Resting	20.2 [19.9, 20.5]	20.1 [19.8, 20.3]	20.2 [20.1, 20.4]	0.16
	Stimulated	48.9 [44.1, 59.8]	57.4 [41.7, 64.2]	47.5 [41.7, 51.6]	0.42	Stimulated	24.5 [23.4, 26.1]	25.5 [23.5, 26.7]	23.6 [23.1, 24.5]	0.16
CD62L-	Resting	1.4 [0.5, 2.1]	1.2 [0.4, 2.5]	0.7 [0.5, 2.6]	0.70	Resting	6.4 [4.8, 7.8]	7.2 [4.6, 7.9]	7.6 [6.8, 8.0]	0.41
	Stimulated	58.1 [49.1, 65.4]	51.5 [46.0, 56.9]	64.4 [55.2, 67.1]	**0.01**	Stimulated	2.2 [1.9, 2.4]	2.0 [1.8, 2.2]	2.4 [2.0, 2.7]	0.08

Neutrophil activity is shown as the median percentage or the MFI and [interquartile range] of cells positive for CD35, CD11B or CD66B and negative for CD62L while resting and upon stimulation with fMLF. In women with ≥50% coronary stenosis, the neutrophil CD35 surface expression (MFI) was lower upon stimulation with fMLF. A similar trend was observed for degranulation marker CD11B, but not for CD66B and CD62L. Indicated *p*-values were calculated with the Kruskal-Wallis test.

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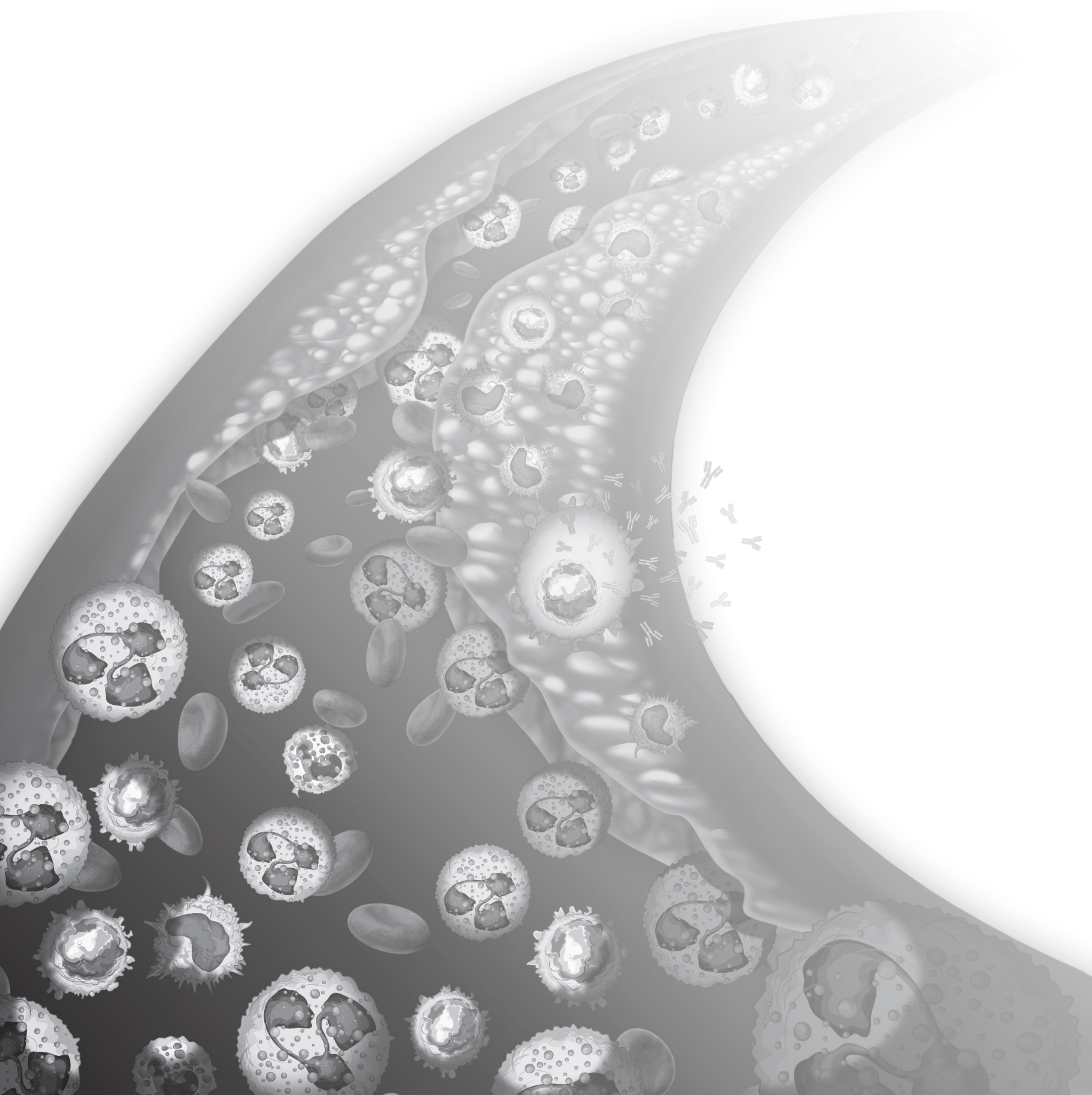
Supplemental table 4. Neutrophil activity is shown in women with former PE with or without subclinical CAD.

	Subclinical CAD				Subclinical CAD			
	% cells	**No (n=78)**	**Yes (n=12)**	***p***	**MFI**	**No (n=78)**	**Yes (n=12)**	***p***
CD35+	Resting	1.9 [1.1, 3.3]	1.2 [1.0, 2.6]	0.56	Resting	8.2 [8.0, 8.4]	8.2 [7.9, 8.5]	0.98
	Stimulated	97.3 [93.3, 98.8]	95.4 [91.8, 97.8]	0.17	Stimulated	15.3 [13.7, 17.0]	13.7 [12.5, 14.8]	**0.03**
CD11B+	Resting	1.1 [0.7, 2.0]	1.0 [0.5, 2.3]	0.66	Resting	10.8 [10.5, 11.1]	10.6 [10.5, 10.9]	0.57
	Stimulated	99.8 [99.4, 99.8]	99.7 [99.5, 99.8]	1.00	Stimulated	29.2 [25.1, 32.8]	26.0 [23.2, 29.9]	0.29
CD66B+	Resting	0.9 [0.5, 1.9]	1.1 [0.6, 3.1]	0.32	Resting	20.2 [19.9, 20.4]	20.1 [19.9, 20.2]	0.97
	Stimulated	50.8 [42.5, 63.0]	49.7 [44.9, 60.0]	0.88	Stimulated	24.8 [23.4, 26.6]	24.1 [23.2, 25.2]	0.32
CD62L-	Resting	1.3 [0.4, 2.4]	0.6 [0.4, 2.8]	0.32	Resting	7.2 [4.7, 7.9]	6.9 [6.2, 7.8]	0.66
	Stimulated	56.1 [48.2, 64.5]	57.4 [47.6, 65.7]	0.79	Stimulated	2.1 [1.9, 2.4]	2.0 [1.8, 2.6]	0.92

Neutrophil activity is shown as the median percentage or the MFI and [interquartile range] of cells positive for CD35, CD11B or CD66B and negative for CD62L while resting and upon stimulation with fMLF. In women with subclinical CAD, the neutrophil CD35 surface expression (MFI) was lower upon stimulation with fMLF. A similar trend was observed for degranulation marker CD11B, but not for CD66B and CD62L. CAD indicates subclinical coronary artery disease defined as ≥100 Agatston Units and/or ≥50% stenosis. Indicated *p*-values are derived from the Mann-Whitney *U* test.

PART THREE

SUMMARY AND DISCUSSION



CHAPTER

9

General summary and discussion

INTRODUCTION

We introduced CVD as a major cause of mortality, accounting for approximately 30% of the deaths worldwide[1]. Comprehensive research has been performed with the aim to understand the underlying pathology, to find biomarkers that predict which patients are at risk and to develop novel treatments. We have described inflammatory cells as pivotal players in atherosclerosis development, progression and plaque destabilization. In the current thesis, we have evaluated different inflammatory players in relation to vulnerable plaque characteristics and CVD presence and prognosis. One of the questions I asked myself at the end of my PhD project is: what is the benefit for the patient? The honest answer is: there is no benefit. At least, no direct benefit, as patients coming into the clinic are not differently treated. Nonetheless, this work has contributed to a better understanding of inflammation and the role of specific immune cell subsets in atherosclerotic disease. Together with future research that is necessary to establish our findings, novel inflammation related biomarkers might enroll the clinical arena of CVD.

THE FUTURE OF CIRCULATING IMMUNE CELLS IN CVD

In **chapter 2**, we reviewed several articles, describing a strong association between circulating immune cells and the risk of recurrent events in patients with stable and acute coronary syndromes. The total white blood cell count, and especially the ratio between neutrophils and lymphocytes (NLR) displayed a strong prognostic association with secondary CVD events. Hence, its biomarker potential is promising. In combination with known and easily available risk factors, including age, sex, cholesterol, heart rate, blood pressure and creatinine levels, the NLR has great potential to identify patients at risk for secondary CVD events. Proper risk stratification might reduce inappropriate treatment of patients at low risk, and instead, lead to more appropriate interventions in patients at high risk for secondary cardiovascular events.

Meanwhile, additional information can be derived from more specific cell subsets. Research on the prognostic value of specific cell subtypes for the occurrence of secondary CVD outcome was limited (**Chapter 2**). However, experimental mice studies have shown that within the monocyte and lymphocyte pool many subtypes exist, having distinct atheroprotective and atherogenic properties[2-7]. Compared to the relative easy blood test needed to determine the NLR, extensive flow cytometry panels assessing cell subsets are more complicated. Though, with advanced multicolor flow cytometry[8], routine assessment of specific cell subsets becomes easier, and future studies assessing the prognostic value of specific cell subtypes warrant further attention. Moreover, studies examining specific cell subtypes can increase the understanding of the underlying mechanisms and might provide novel therapeutic targets for patients with cardiovascular disease.

An example of a specific atherogenic monocyte subset is the murine Ly6C^{high} monocyte. The pro-inflammatory Ly6C^{high} monocytes invade the plaque, and subsequently develop into inflammatory M1 macrophages, thereby enhancing atherosclerotic plaque formation and progression[5, 9-13]. Also in humans, different monocyte subsets have been related to the presence and severity of CVD[7]. In **chapter 3**, we investigated the role of circulating monocyte subsets in patients with severe atherosclerosis from the Athero-Express. Although in animal studies circulating classical Ly6C^{high} monocytes have been frequently associated with plaque characteristics[5, 9-12, 14], we found that human classical monocyte subtypes were not associated with vulnerable plaque characteristics in patients with severe atherosclerosis. Given the fact that these patients have severe atherosclerosis, the role of monocytes may be less prominent as compared to earlier stages of atherosclerosis. Indeed, in a series of comprehensive analyses it was shown that monocytes play a major role in early atherosclerosis and are the main contributors to plaque macrophages[15]. However, in later stages of atherosclerosis, plaque macrophages mainly derive from local proliferation of macrophages. A limitation of our study was that we only examined the characteristics of one plaque, while it is likely that more atherosclerotic plaques were present in these patients.

In addition to the plaque analyses, we evaluated if circulating monocytes were associated with the occurrence of secondary adverse manifestations. Although in other studies classical and intermediate monocytes prognosticated CVD events[16-18], we found no prognostic value for monocyte subsets in severe atherosclerotic patients during follow-up. Explanations might include that our patients were older and had more severe atherosclerosis (**Chapter 3**).

Besides monocytes, other immune cells have also been investigated in atherosclerosis. With the development of Rituximab[19], a drug that specifically targets B cells, experiments depleting B cell subsets showed a pro-atherogenic role for B2 lymphocytes[4, 20, 21]. In contrast, B1a lymphocytes were considered atheroprotective via the secretion of natural IgM antibodies[21-24]. The evidence for the role of B cell subsets in human CVD is limited[25, 26]. In a profiling study of B cell subsets in patients from the Athero-Express, we found that high levels of switched and unswitched memory B cells prognosticated better CVD outcome during follow-up (**Chapter 4**). In contrast to their atheroprotective role in animal studies, human B1 cells showed no association with the occurrence of secondary CVD events.

Notably, animal studies exploring the role of B cells in atherosclerosis were published in 2002[27, 28]. To our knowledge, the first study on circulating B cell subsets in relation to human CVD was published in 2014[26], indicating a long gap between animal and human research. Although I believe that animal models are important and indispensable to better understand the complex mechanisms of atherosclerotic disease, the translation from mice to humans could be made earlier. Preferably, when possible, the order would be

reversed. Targets found in humans should be further studied in animal models in order to understand the underlying mechanisms and to find approaches for drug development. In this way, unnecessary time investment in pathways that are promising in animals, but fail to have any relation with human disease may be avoided. In addition, when we focus our animal experiments on the understanding and interference of pathways identified in human studies, the development and validation of therapeutic targets might take less time.

To further understand the role of B cell subsets in human atherosclerosis, we investigated the antibodies that are produced by B cells. Several antibodies have been related to the presence and severity of CVD as shown by a recent meta-analysis[29]. Although we observed a significant association between switched memory B cells and IgG-α-oxLDL antibodies, anti-oxLDL antibody levels were not associated with the risk of secondary manifestations (**Chapter 4**). Given that the main function of B cells is antibody production, we further investigated the antibodies in the serum of atherosclerotic patients. Amongst the different isotypes, we found that IgG4 levels were significantly associated with the occurrence of secondary major cardiovascular events in females, but not in males. On the other hand, the levels of an inflammatory protein, c-reactive protein (CRP) were associated with the occurrence of CVD events in males, but not in females (**Chapter 5**). The question why IgG4 prognosticates secondary manifestations specifically in females remains unanswered. It is likely that differences in the underlying inflammatory responses between males and females might also underlie the different prognostic value of biomarkers between sexes in CVD[30-32]. We further investigated the role of IgG4 by determining the presence of IgG4 deposits in different plaque stages. Although IgG4 serum and plaque levels were higher in males, the abundance of IgG4 was comparable among different plaque stages (intimal thickening, pathological thickening, thick fibrous cap atheroma and intraplaque hemorrhage). However, in females, we found that IgG4 deposits were mainly present in the thick cap fibrous atheroma. As thick cap fibrous atheroma are associated with plaque erosion, we speculated that IgG4 may play a role in plaque erosion through the exertion of pro-fibrotic, anti-inflammatory and pro-thrombotic properties[33-36]. To further elucidate the role of IgG4 in females with CVD, large genetic studies on SNP's associated with IgG4 serum levels in relation to the presence and severity of CVD might indicate if IgG4 is causally involved. In addition, epitope mapping of IgG4 antibodies may shine light on the role and possible targets of IgG4 antibodies in females with CVD.

In the future, evaluation of plaque inflammation and the risk for subsequent cardiovascular events might include the use of modern imaging techniques. Many imaging modalities exist and have been used to identify plaque composition in order to predict vulnerable plaques to rupture. Noninvasive techniques like computed tomography (CT) positron emission tomography (PET) and magnetic resonance imaging (MRI) and invasive techniques, including (intravascular ultrasound (IVUS) or optical coherence tomography (OCT), allow researchers to identify plaque burden, plaque morphology, cap thickness,

macrophage infiltration and plaque angiogenesis[37-41]. With MRI techniques, ongoing processes of plaque inflammation can be followed over time. This was achieved by studying a surrogate for plaque inflammation: the uptake of ultrasmall super paramagnetic particles of iron oxide (USPIO) in the plaque. Interestingly, upon statin treatment, a significant reduction in USPIO-defined inflammation was observed[42]. Although a small follow-up trial was not able to demonstrate a significant association between USPIO-defined inflammatory plaques at baseline and the occurrence of CVD events[43], these studies show that USPIO based MRI is a useful technique to study the kinetics of vulnerable inflammatory plaques *in vivo*. Future imaging studies with higher resolutions may have great potential to predict high risk patients based on plaque vulnerability. In addition, imaging studies can be used to monitor plaque progression and might provide useful information regarding the decision for surgical intervention. However, there are also limitations for these techniques, like radiation exposure, claustrophobia and harm due to contrast agents[37, 44]. In addition, MRI is time-consuming and not available for patients with metal implants. Moreover, it is difficult to identify plaques that will give rise to a cardiovascular event. Vulnerable plaques, often considered as thin-capped inflammatory plaques with a large lipid core, underlie only a part of cardiovascular events[45]. Plaque erosion is also a major driver of cardiovascular events, but the plaque composition of eroded plaques differs significantly from ruptured plaques. Plaque erosion typically occurs on the surface of plaques with a thick fibrous cap having high collagen and smooth muscle cell content[46-48]. It remains to be established if imaging modalities will be able to distinguish plaques that are prone to erode from stable plaques[41]. Therefore, more research into the underlying mechanisms of plaque erosion might help to find specific predictors of plaque erosion. It has been suggested that neutrophils, located at the luminal plaque surface, are implicated in plaque erosion[48]. To speculate, a combination of plaque imaging together with markers predictive of plaque rupture and/or erosion, like VCAM-1[49], neutrophils[48] or IgG4 antibodies (**Chapter 5**), might have potential in the prediction of future cardiovascular events.

CARDIOVASCULAR DISEASE IN WOMEN

Differences between males and females in CVD become increasingly evident[32, 50, 51]. With regard to CVD symptoms and pathology, researchers should thoroughly evaluate these differences and, if necessary, perform sex-stratified analyses and develop sex-specific treatments. Women experiencing preeclampsia (PE) during pregnancy are known to be at higher risk for cardiovascular disease later in life[52-54]. As described in **chapter 7**, we hypothesized that the shared pathological mechanisms between preeclampsia and atherosclerosis might underlie the development of cardiovascular disease and subsequent clinical manifestations in women with former PE. Inflammation is one of the pathological mechanisms that is involved in PE and atherosclerosis. Amongst these inflammatory players, neutrophils are described to be activated and to induce endothelial dysfunction

in the placentas of women with PE[55-59]. In addition, neutrophils have been shown to be crucial in atherosclerotic development, progression and destabilization. In collaboration with the Queen of Hearts consortium and the Cardiovascular RiskprofilE - IMaging and gender-specific disOrders (CREw-IMAGO) consortium[60], we investigated if circulating neutrophils were associated to subclinical coronary artery disease in women with a history of PE (**Chapter 8**). We observed no significant differences in neutrophil activity or neutrophil response in women with coronary calcification or stenosis as compared to women without. Nevertheless, women with early PE are at high risk of future CVD and should be closely monitored and treated (if applicable) to prevent early clinical CVD manifestations.
Besides preeclampsia, other female-specific risk factors include polycystic ovarian syndrome (PCOS) and early menopause[61-63]. As treatment in CVD predominantly relies on observations in men, increased awareness for the differences in the underlying pathology of CVD in women is important. Moreover, the participation of women in clinical trials has been hampered, probably because women are more likely to be excluded due to their child-bearing potential and due to the fact that they are older when they develop CVD[64-66]. It is difficult to pinpoint a solution. The first step is increasing awareness of the fact that not only men, but also women have a high risk to develop CVD and that most drugs and treatments are based on findings in men[67, 68]. This does not mean that these findings do not apply to women, but rather that biomarkers, treatments and drugs for cardiovascular disease need to be firmly established in women. Future clinical trials should take into account the differences in the underlying pathology between men and women and, if necessary, perform sex stratified analyses.

CERTAINTY IN SCIENCE

One thing that I have learned the past years, is that the only certainty in science, is that everything is uncertain. There is no certainty when performing experiments, for example due to technical challenges that may influence your results. Or you wait in uncertainty because of the simple fact that a compound that you need is not delivered in time. In addition, there is no certainty due to the complex biology in many diseases. For example, a large body of evidence is available for the role of monocytes in atherosclerosis. However, in **chapter 3** we found no association between monocytes and plaque phenotype or the occurrence of secondary cardiovascular events. Likewise, although CD200R stimulation was earlier shown to inhibit inflammation and the subsequent development of auto-immune diseases[69-71], we observed no effect on plaque progression and stabilization (**Chapter 6**). In addition to complex and multifactorial diseases, statistical methods cannot deliver certainty in science. For example, the *p*-value, the most commonly used value to report statistical significance in biomedical research, gives no certainty. When comparing two groups, a *p*-value below 0.05 only tells us that there is less than a 5 percent chance of seeing these differences in a world where the null hypothesis (there is no difference)

is true. There are interesting discussions about the *p*-value and how it should (not) be used[72-74], but here I would like to point out that although many scientists rely heavily on *p*-values below 0.05, this *p*-value guarantees no certainty.

In order to increase the certainty in science, awareness about the complexity of diseases and the limitations of research methods is inevitable. In addition, I believe that thorough observation and evaluation of raw data, in combination with comprehensive validation and replication of findings, are key components of proper biomedical research. In relation, it is important to reward reports with 'neutral' or 'negative' findings that are properly conducted, thereby avoiding the search for significant *p*-values. Lastly, education and individual coaching of young scientists to define precise research questions and to develop proper scientific methods will further increase the certainty in science. Eventually, increased certainty may lead to enhanced understanding of complex diseases, being accompanied by the accelerated development of new biomarkers and drugs.

CONCLUSION

It is amazing what a simple blood drop can tell us. In this thesis we have investigated different subtypes of circulating white blood cells. We have shown that components of the white blood cell count can harbor information that holds prognostic value to identify men and women at high risk for secondary cardiovascular events. Beyond its biomarker potential, future research might evaluate treatment options that stimulate protective mechanisms of anti-inflammatory cells, or inhibit excessive and chronic inflammation, thereby preventing the occurrence of CVD events.

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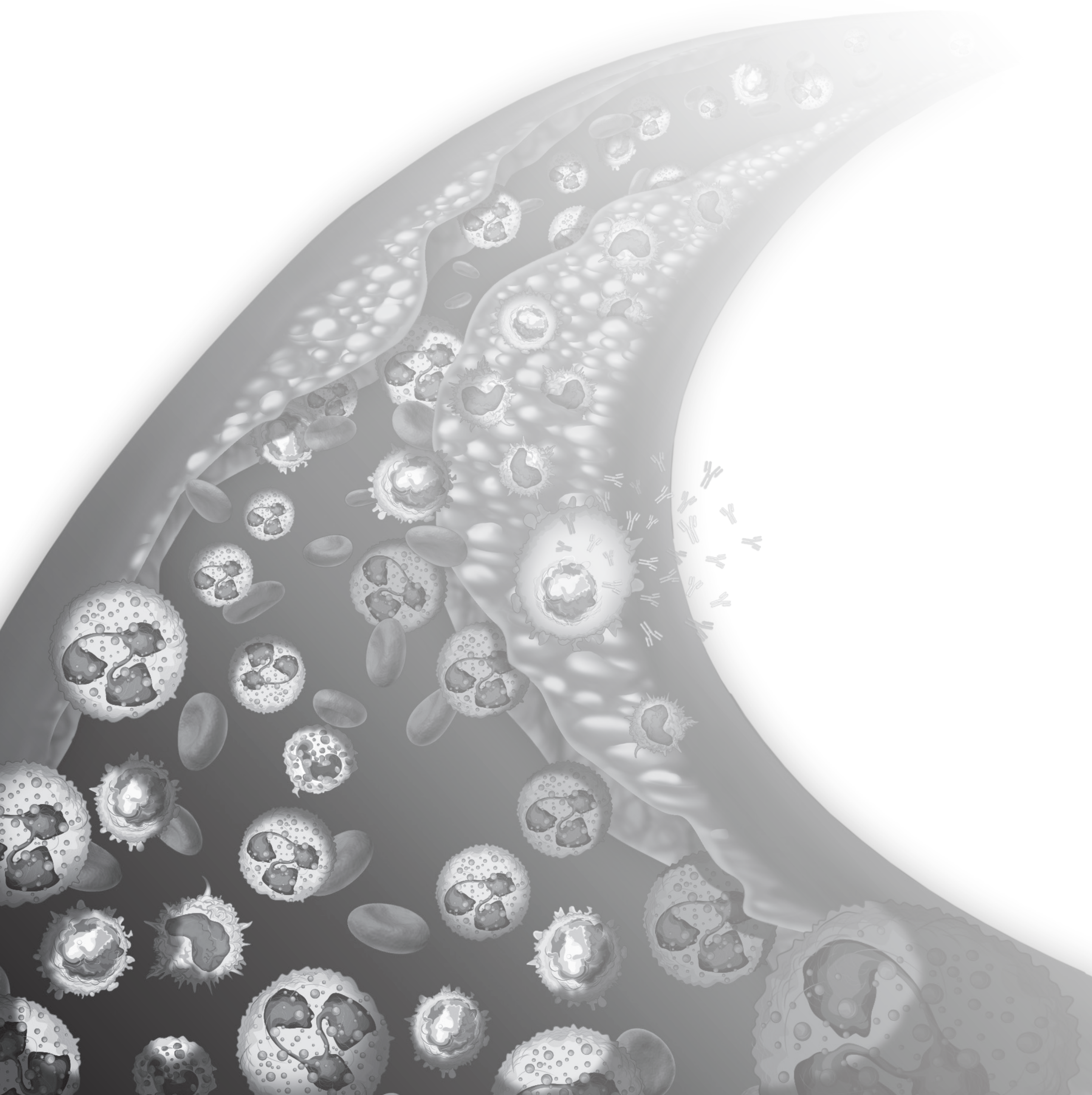

CHAPTER

10

Dutch summary and discussion

INTRODUCTIE

Hart- en vaatziekten (HVZ) zijn verantwoordelijk voor ongeveer 30% van alle wereldwijde sterfgevallen[1]. Er is veel onderzoek gedaan om de onderliggende pathologie, slagaderverkalking, te begrijpen. Dit onderzoek kan bijvoorbeeld leiden tot de ontwikkeling van medicijnen of helpen bij het voorspellen van een risico op hart- en vaatziekten, zoals een hart- of herseninfarct.

Slagaderverkalking is een aandoening van de slagaders met als belangrijkste oorzaken: te veel vetten en een chronische ontsteking in de bloedvaten. Slagaderverkalking begint al op zeer jonge leeftijd, zoals blijkt uit een belangrijk onderzoek onder jonge Amerikaanse soldaten[2]. Slagaderverkalking begint met beschadiging aan de binnenzijde van de bloedvatwand, het endotheel. De beschadigde wand zorgt ervoor dat low-density lipid (LDL-)cholesterol kan binnendringen, en daardoor wordt een vettige aanslag gevormd aan de binnenkant van het bloedvat[3]. In de loop van de tijd neemt de hoeveelheid LDL-cholesterol verder toe en worden LDL-deeltjes geoxideerd door lokale moleculen of door factoren die worden uitgescheiden door infiltrerende ontstekingscellen, zoals macrofagen[3-5]. Macrofagen nemen het geoxideerde LDL op en ontwikkelen zich tot zogenaamde schuimcellen. Een combinatie van hoog LDL-cholesterol en langdurige ontsteking stimuleert de voortgang van slagaderverkalking[4, 6, 7]. Slagaderverkalking kan gedurende lange tijd onopgemerkt in het lichaam aanwezig zijn, zonder presentatie van klinische symptomen. Lokale slagaderverkalking, ook wel plaque, kan over tijd groter en kwetsbaarder worden. Een kwetsbare plaque kan daardoor scheuren, dit verschijnsel heet plaque ruptuur. Een stolsel dat zich vervolgens vormt kan het bloedvat ter plekke of verderop verstoppen en daarmee bijvoorbeeld een hart- of herseninfarct veroorzaken. Ook schade aan de oppervlakte van de plaque, zonder dat de plaque dus scheurt, kan leiden tot bloedstolling en daarmee een infarct veroorzaken. Dat verschijnsel heet plaque-erosie.

Naast andere risicofactoren, zoals ouder worden, roken en hoge bloeddruk, speelt ontsteking een belangrijke rol bij het groeien van plaques en het scheuren van plaques. Daarom zijn de ontstekingsprocessen bij slagaderverkalking de afgelopen decennia grondig onderzocht. Verschillende wetenschappelijke publicaties hebben aangetoond dat cellen van het afweersysteem (immuuncellen) belangrijk zijn bij de ontwikkeling en voortgang van slagaderverkalking. In dit proefschrift hebben we bestudeerd hoe verschillende immuuncellen in relatie staan tot kenmerken van kwetsbare plaques en of deze stoffen de aanwezigheid en het verloop van hart- en vaatziekten kunnen voorspellen. Dat is belangrijk, want bij mensen die al eens een herseninfarct hebben gehad is de kans op nieuwe symptomen van slagaderverkalking groter, maar niet iedereen krijgt opnieuw een hart- of herseninfarct. Daarom willen we nauwkeurig voorspellen welke patiënten weinig of juist veel risico hebben op nieuwe (secundaire) cardiovasculaire complicaties. Dat risico probeerden we te voorspellen door het soort en de hoeveelheid immuuncellen te meten

op het laboratorium. Stoffen of cellen die worden gebruikt om bijvoorbeeld het risico op HVZ te voorspellen worden ook wel biomarkers genoemd.

Een van de vragen die ik mezelf aan het einde van mijn promotieonderzoek stelde is: wat is na dit onderzoek nou het voordeel voor de patiënt? Het eerlijke antwoord is: dat is er niet. Tenminste, geen direct voordeel, omdat de patiënten die in het ziekenhuis komen niet anders worden behandeld. Toch heeft dit werk bijgedragen aan het beter begrijpen van de rol van ontsteking en specifieke types immuuncellen bij patiënten met slagaderverkalking. Samen met toekomstig onderzoek, dat nodig is om onze bevindingen te bevestigen, kunnen nieuwe ontstekingsgerelateerde biomarkers mogelijk een belangrijke rol spelen op het gebied van HVZ.

DE TOEKOMST VAN CIRCULERENDE IMMUUNCELLEN IN HVZ

In **hoofdstuk 2** hebben we een overzicht gemaakt van de bestaande literatuur over circulerende immuuncellen in relatie tot HVZ. Het bleek dat er een sterke associatie was tussen circulerende immuuncellen en het risico op terugkerende cardiovasculaire complicaties in patiënten met stabiele en acute coronaire aandoeningen. Het totale aantal witte bloedcellen, en met name de ratio tussen neutrofielen en lymfocyten (NLR) blijkt een goede maat te zijn om te voorspellen welke patiënten hoog risico lopen om opnieuw complicaties van hart en vaatziekten te ontwikkelen. Het potentieel van de NLR als biomarker is daarom veelbelovend. In combinatie met bekende en standaard beschikbare risicofactoren, waaronder leeftijd, geslacht, cholesterol, hartslag en bloeddruk, kan de NLR een grote bijdrage leveren aan de identificatie van risicopatiënten. Een goede risicostratificatie kan bijdragen aan optimale therapie voor patiënten met een hoog of laag risico op secundaire cardiovasculaire complicaties.

Tegelijkertijd kunnen specifieke cel subtypes meer aanvullende informatie geven over het risico op HVZ. Onderzoek naar de voorspellende waarde van specifieke cel subtypes in relatie tot het optreden van terugkerende cardiovasculaire complicaties was beperkt (**Hoofdstuk 2**). Experimentele muizenstudies hebben echter aangetoond dat er binnen de monocyten- en lymfocyten populaties veel subtypes voorkomen, met verschillende beschermende en schadelijke eigenschappen in relatie tot slagaderverkalking[8-13]. Vergeleken met de relatief gemakkelijke bloedtest die nodig is om de NLR te bepalen, zijn de methodes waarmee we verschillende cel subtypes kunnen identificeren ingewikkeld en tijdrovend. Toch wordt met moderne, geavanceerde technieken, waaronder multicolor flow cytometrie,[14] de routinematige beoordeling van specifieke subgroepen van cellen wel gemakkelijker, en daarom verdienen toekomstige studies die de voorspellende waarde van specifieke subtypes van cellen onderzoeken nadere aandacht. Bovendien kunnen onderzoeken naar specifieke cel subtypes het begrip van de onderliggende mechanismen vergroten en nieuwe therapeutische doelen opleveren voor patiënten met hart- en vaatziekten.

Een voorbeeld van een specifiek monocyt subtype die belangrijk is in het ontstaan van slagaderverkalking is de Ly6C^{high} monocyt in muizen. De pro-inflammatoire Ly6C^{high} monocyten dringen de plaque binnen en ontwikkelen zich vervolgens tot ontsteking stimulerende M1-macrofagen, waardoor het proces van slagaderverkalking bevorderd wordt [11, 15-19]. Ook bij mensen zijn verschillende monocyten subtypen gerelateerd aan de aanwezigheid en ernst van HVZ[13]. In **hoofdstuk 3** hebben we de rol onderzocht van circulerende monocyten subtypes bij patiënten met ernstige slagaderverkalking uit de Athero-Express biobank. Hoewel in dierstudies circulerende klassieke Ly6C^{high}-monocyten vaak in verband werden gebracht met kenmerken van een kwetsbaar fenotype van de plaque[11, 15-18, 20], ontdekten we dat menselijke klassieke monocyten-subtypen die zich in het bloed bevinden niet geassocieerd waren met kwetsbare plaque-kenmerken bij patiënten met ernstige slagaderverkalking. Gezien het feit dat deze patiënten ernstige slagaderverkalking hebben, kan de rol van monocyten minder prominent zijn in vergelijking met eerdere stadia van slagaderverkalking. In een reeks uitgebreide analyses werd inderdaad aangetoond dat monocyten een belangrijke rol spelen bij vroege slagaderverkalking en de belangrijkste bijdrage leveren aan plaque-macrofagen[21]. In latere stadia van het ziekteproces zijn plaque-macrofagen echter voornamelijk afkomstig van lokale vermenigvuldiging en minder van nieuwe infiltratie. Een beperking van onze studie was dat we alleen de kenmerken van één plaque onderzochten, terwijl het waarschijnlijk is dat er meerdere plaques bij deze patiënten aanwezig waren.

Naast plaque analyses hebben we ook geëvalueerd of circulerende monocyten geassocieerd waren met het optreden van secundaire cardiovasculaire complicaties. Hoewel in andere onderzoeken bepaalde soorten monocyten voorspellend waren voor cardiovasculaire complicaties [22-24], vonden we geen voorspellende waarde voor monocyt subtypes in patiënten met ernstige slagaderverkalking gedurende de drie jaar dat ze gevolgd zijn. Mogelijke verklaringen zijn dat onze patiënten ouder waren en ernstigere vormen van slagaderverkalking hadden (**Hoofdstuk 3**). In het latere stadium zijn de monocyten in de circulatie mogelijk minder betrokken bij het verergeren van de slagaderverkalking.

Naast monocyten zijn er ook andere immuuncellen onderzocht bij slagaderverkalking, bijvoorbeeld B-lymfocyten. Vroeger werd altijd gedacht dat B-lymfocyten beschermend waren doordat ze antilichamen maakten die het slechte cholesterol bij slagaderverkalking opruimen. Met de ontwikkeling van Rituximab[25], een medicijn dat specifiek gericht is tegen B-cellen, werd in proefdieren aangetoond dat B2-lymfocyten een beschadigende rol hadden bij aderverkalking[10, 26, 27]. Daarentegen werden B1a-lymfocyten beschouwd als beschermend door het uitscheiden van natuurlijke IgM-antilichamen[27-30]. Het bewijs voor de rol van B-cel subsets in mensen met HVZ is beperkt[31, 32]. In een onderzoek naar de profielen van B-cel subtypen bij patiënten van de Athero-Express ontdekten we dat hoge niveaus van B-geheugencellen beschermen tegen het optreden van toekomstige cardiovasculaire complicaties de drie jaar dat ze gevolgd werden (**Hoofdstuk 4**).

B-geheugencellen staan met name bekend om hun geheugen: als ze hetzelfde antigen opnieuw 'zien' kunnen ze de reactie en werking van het immuunsysteem versnellen. In tegenstelling tot hun beschermende rol bij aderverkalking in dierstudies vertoonden humane B1-cellen geen associatie met het optreden van secundaire cardiovasculaire complicaties.

Dierstudies die de rol van B-cellen in slagaderverkalking onderzochten werden al gepubliceerd in 2002[33, 34]. Voor zover ons bekend was, werd het eerste onderzoek over circulerende B-cel subsets in relatie tot humane HVZ gepubliceerd in 2014[32], wat aangeeft dat er een lange tijd zit tussen dierlijk en menselijk onderzoek. Hoewel ik geloof dat diermodellen belangrijk en onmisbaar zijn om de complexe mechanismen van slagaderverkalking beter te begrijpen, kan de vertaling van muizen naar mensen eerder worden gemaakt. Bij voorkeur wordt de volgorde omgekeerd als dat mogelijk is. Targets die bij de mens worden gevonden moeten in diermodellen nader worden bestudeerd om de onderliggende mechanismen te begrijpen en om de ontwikkeling van geneesmiddelen te stimuleren. Op deze manier kunnen onnodige tijdsinvesteringen in mechanismen die veelbelovend zijn in dieren, maar die geen relatie hebben met menselijke ziekten, worden vermeden. Wanneer we onze dierexperimenten richten op het begrip en de interventie van de mechanismen die in studies bij mensen zijn geïdentificeerd, zou de ontwikkeling en validatie van therapeutische targets mogelijk minder tijd kosten.

Om de rol van B-cel subtypes bij menselijke slagaderverkalking beter te begrijpen, hebben we antilichamen onderzocht die door B-cellen worden geproduceerd. Verschillende antilichamen, waaronder ox-LDL-antilichamen zijn gerelateerd aan de aanwezigheid en ernst van HVZ, zoals blijkt uit een recente meta-analyse[35]. Hoewel we een significante associatie tussen B-geheugencellen en IgG-a-oxLDL-antilichamen hebben waargenomen, waren de niveaus van anti-oxLDL-antilichamen niet geassocieerd met het risico op terugkerende cardiovasculaire complicaties (**Hoofdstuk 4**). Aangezien de belangrijkste functie van B-cellen de productie van antilichamen is, hebben we de antilichamen in het serum van patiënten met slagaderverkalking verder onderzocht. We vonden dat een van de verschillende types antilichamen, IgG4, het optreden van terugkerende cardiovasculaire complicaties kon voorspellen bij vrouwen, maar niet bij mannen. Aan de andere kant waren de niveaus van een inflammatoir eiwit, c-reactief proteïne (CRP), juist geassocieerd met het optreden van cardiovasculaire complicaties bij mannen, maar niet bij vrouwen (**Hoofdstuk 5**). De vraag waarom IgG4 secundaire verschijnselen specifiek bij vrouwen voorspelt blijft onbeantwoord. Het is aannemelijk dat verschillen in de onderliggende ontstekingsreacties tussen mannen en vrouwen ook ten grondslag liggen aan de verschillen in voorspellende waarde van biomarkers tussen mannen en vrouwen met HVZ[36-38]. We hebben de rol van IgG4 verder onderzocht door de aanwezigheid van IgG4 in verschillende plaquestadia te bepalen. Hoewel serum en plaque IgG4-niveaus hoger waren bij mannen, was de hoeveelheid IgG4 vergelijkbaar tussen verschillende stadia van slagaderverkalking. Bij vrouwen vonden we echter dat ophoping van IgG4 voornamelijk aanwezig is in plaques

met een dikke kap (fibreuze atheroma's). Omdat fibreuze atheroma's met een dikke kap geassocieerd zijn met plaque-erosie, en omdat verschillende eigenschappen van IgG4 plaque-erosie kunnen bevorderen, speculeerden we dat IgG4 mogelijk voorspellend is voor cardiovasculaire complicaties in vrouwen via het optreden van plaque-erosie[39-42]. Om de rol van IgG4 bij vrouwen met CVD nader toe te lichten, kunnen grote genetische onderzoeken naar SNP's, die geassocieerd zijn met IgG4-serumlevels, in relatie tot de aanwezigheid en de ernst van HVZ, aangeven of IgG4 oorzakelijk betrokken is. Bovendien kan het in kaart brengen van de stoffen waar IgG4-antilichamen tegen gericht zijn, de rol van IgG4-antilichamen bij vrouwen met HVZ verduidelijken.

In de toekomst kan de evaluatie van ontstekingsreacties in de plaque, en het risico op daaropvolgende cardiovasculaire complicaties, ook gebruikmaken van moderne beeldvormende technieken. Er zijn veel beeldvormingsmodaliteiten gebruikt om de samenstelling van de plaque te identificeren en om daarmee te voorspellen of kwetsbare plaques scheuren. Niet-invasieve technieken, zoals computertomografie (CT), positron emissie tomografie (PET) en magnetische resonantie beeldvorming (MRI), en invasieve technieken, waaronder intravasculaire echografie (IVUS) of optische coherentie tomografie (OCT), stellen onderzoekers in staat om de hoeveelheid plaque, plaquemorfologie, kapdikte, infiltratie van macrofagen en plaque-doorbloeding te beoordelen[43-47]. Met MRI-technieken kunnen processen van plaqueontsteking over tijd worden gevolgd. In een onderzoek werd dit gedaan door een surrogaat van plaqueontsteking te onderzoeken: de opname van ultrakleine superparamagnetische deeltjes van ijzeroxide (USPIO) in de plaque. In een vervolgonderzoek konden ze na statinebehandeling een significante afname van de surrogaatontsteking waarnemen[48]. Hoewel een kleine vervolgstudie geen significante associatie kon aantonen tussen door de surrogaat ontsteking in de plaques aan het begin van de studie en het optreden van secundaire cardiovasculaire complicaties[49], laten deze studies zien dat op USPIO gebaseerde MRI een bruikbare techniek is om de kinetiek van kwetsbare inflammatoire plaques *in vivo* te bestuderen. Toekomstige beeldvormingsstudies met hogere resoluties hebben goede mogelijkheden om te voorspellen welke patiënten verhoogd cardiovasculair risico hebben op basis van de kwetsbaarheid van plaques. Bovendien kunnen beeldvormingsstudies worden gebruikt om plaque-groei te volgen en kan het nuttige informatie opleveren met betrekking tot de beslissing voor chirurgische interventie. Er zijn echter ook beperkingen voor deze technieken, zoals blootstelling aan straling, claustrofobie en schade door contrastmiddelen[43, 50]. Daarnaast is MRI tijdrovend en niet beschikbaar voor patiënten met metalen implantaten. Bovendien is het moeilijk om plaques te identificeren die aanleiding zullen geven tot een cardiovasculaire complicaties. Kwetsbare plaques, vaak beschouwd als plaques met veel ontstekingscellen, een dunne kap en een grote lipide kern, liggen ten grondslag aan slechts een deel van de cardiovasculaire complicaties[51]. Plaque-erosie is ook een belangrijke oorzaak van cardiovasculaire complicaties, maar de plaquesamenstelling van geërodeerde plaques verschilt aanzienlijk van gescheurde plaques. Plaque-erosie vindt vaak plaats op het oppervlak van plaques met een dikke

vezelige kap en een hoog collageen- en gladde spiercelgehalte [52-54]. Er moet nog worden vastgesteld of beeldvormingsmodaliteiten in staat zullen zijn plaques die geneigd zijn te eroderen te onderscheiden van stabiele plaques[47]. Daarom kan meer onderzoek naar de onderliggende mechanismen van plaque-erosie helpen om specifieke voorspellers van plaque-erosie te vinden. Er is gesuggereerd dat neutrofielen, gelokaliseerd in het lumen tegen het oppervlak van de plaque, betrokken zijn bij plaque-erosie[54]. Het zou kunnen dat een combinatie van plaquebeeldvorming met markers voorspellend voor plaqueruptuur en/of -erosie, zoals VCAM-1[55], neutrofielen[54] of IgG4-antilichamen (**Hoofdstuk 5**), nuttig zijn bij het voorspellen van toekomstige cardiovasculaire complicaties.

VROUWEN MET HART- EN VAATZIEKTEN

Verschillen tussen mannen en vrouwen in HVZ worden steeds duidelijker[38, 56, 57]. Met betrekking tot symptomen en pathologie bij HVZ moeten onderzoekers deze verschillen mijns inziens grondig evalueren en, indien nodig, analyses apart voor mannen en vrouwen uitvoeren en sekse specifieke behandelingen ontwikkelen. Vrouwen die pre-eclampsie (PE) krijgen tijdens de zwangerschap hebben een hoger risico op hart- en vaatziekten later in het leven[58-60]. Zoals beschreven in **hoofdstuk 7** veronderstelden we dat de gedeelde pathologische mechanismen tussen pre-eclampsie en slagaderverkalking de basis zouden kunnen vormen voor de ontwikkeling van hart- en vaatziekten en daaropvolgende klinische complicaties bij vrouwen met voormalig PE. Ontsteking is een van de pathologische mechanismen die betrokken is bij zowel PE als slagaderverkalking. Een voorbeeld van een ontstekingscel die betrokken is bij PE en slagaderverkalking is de neutrofiel. Tijdens PE worden neutrofielen geactiveerd en ze kunnen ook endotheeldisfunctie induceren in de placenta van vrouwen met PE[61-65]. Bovendien is aangetoond dat neutrofielen belangrijk zijn bij de ontwikkeling, het verloop, en het scheuren van plaques. In samenwerking met de consortia Queen of Hearts en Cardiovascular RiskprofilE - IMaging and gender-specific disorders (CREw-IMAGO)[66], onderzochten we of circulerende neutrofielen geassocieerd waren met subklinisch kransslagaderverkalking bij vrouwen met een voorgeschiedenis van PE (**Hoofdstuk 8**). We observeerden geen significante verschillen in de activiteit of de respons van de neutrofielen bij vrouwen met slagaderverkalking vergeleken met vrouwen zonder slagaderverkalking. Niettemin hebben vrouwen met vroege PE verhoogd risico op toekomstige HVZ en moeten ze nauwlettend worden gevolgd, en indien nodig behandeld, om vroegtijdige klinische cardiovasculaire complicaties te voorkomen.

Naast pre-eclampsie zijn er ook andere vrouwspecifieke risicofactoren voor HVZ, zoals polycysteus ovariumsyndroom (PCOS) en vroege menopauze[67-69]. Aangezien de behandeling van HVZ voornamelijk berust op observaties bij mannen, is een groter bewustzijn voor de verschillen in de onderliggende pathologie van HVZ bij vrouwen belangrijk. Bovendien nemen relatief weinig vrouwen deel aan klinische onderzoeken, waarschijnlijk omdat vrouwen meer kans hebben om te worden uitgesloten vanwege de

mogelijkheid om zwanger te worden en vanwege het feit dat zij ouder zijn wanneer ze HVZ ontwikkelen[70-72]. Het is moeilijk om een oplossing te vinden. De eerste stap is het vergroten van het bewustzijn van het feit dat niet alleen mannen, maar ook vrouwen een hoog risico hebben om HVZ te ontwikkelen en dat de meeste geneesmiddelen en behandelingen gebaseerd zijn op bevindingen in mannen[73, 74]. Dit wil niet zeggen dat deze bevindingen niet van toepassing zijn op vrouwen, maar dat biomarkers, behandelingen en geneesmiddelen voor HVZ bij vrouwen nadrukkelijk moeten worden bevestigd. Toekomstige klinische onderzoeken moeten rekening houden met de verschillen in de onderliggende pathologie tussen mannen en vrouwen en indien nodig, sekse gestratificeerde analyses uitvoeren.

ZEKERHEID IN DE WETENSCHAP

Een ding dat ik de afgelopen jaren heb geleerd is dit: de enige zekerheid in de wetenschap, is dat alles onzeker is. Er is geen zekerheid bij het uitvoeren van experimenten, bijvoorbeeld vanwege technische uitdagingen die je resultaten kunnen beïnvloeden. Of je wacht in onzekerheid vanwege het simpele feit dat een stof die je nodig hebt niet op tijd wordt geleverd. Bovendien is er geen zekerheid vanwege de complexe biologie bij veel ziekten. Er is bijvoorbeeld een grote hoeveelheid bewijs beschikbaar voor de rol van monocyten bij slagaderverkalking. In **hoofdstuk 3** vonden we echter geen verband tussen monocyten en de kenmerken van kwetsbare plaques of het optreden van secundaire cardiovasculaire complicaties. Een ander voorbeeld: hoewel eerder is aangetoond dat CD200R-stimulatie ontstekingen remt en de daaropvolgende ontwikkeling van auto-immuunziekten[75-77], hebben we geen effect op groei of stabiliteit van de plaques waargenomen (**Hoofdstuk 6**). Ook statistische methoden kunnen geen zekerheid bieden in de wetenschap. De *p*-waarde, de meest gebruikte waarde om statistische significantie te rapporteren in biomedisch onderzoek, geeft bijvoorbeeld geen zekerheid. Wanneer we twee groepen vergelijken, vertelt een *p*-waarde kleiner dan 0,05 ons alleen dat er minder dan 5 procent kans is om deze verschillen te zien in een wereld waar de nulhypothese (er is geen verschil) waar is. Er zijn interessante discussies over de *p*-waarde en hoe deze (niet) gebruikt moet worden[78-80], maar hier wil ik erop wijzen dat, hoewel veel wetenschappers sterk leunen op *p*-waarden onder 0,05, deze *p*-waarde geen zekerheid biedt.

Om de zekerheid in de wetenschap te vergroten, is bewustzijn voor de complexiteit van ziekten en de beperkingen van onderzoeksmethoden onvermijdelijk. Daarnaast ben ik van mening dat grondige observatie en evaluatie van onbewerkte gegevens, in combinatie met uitgebreide validatie en replicatie van bevindingen, belangrijke voorwaarden zijn voor degelijk biomedisch onderzoek. In verband hiermee is het belangrijk om publicaties met 'neutrale' of 'negatieve' bevindingen, die goed worden uitgevoerd te waarderen, waarbij het zoeken naar significante *p*-waarden wordt vermeden. Tenslotte zal onderwijs en individuele coaching van jonge wetenschappers om precieze onderzoeksvragen te

definiëren en goede wetenschappelijke methoden te ontwikkelen de zekerheid in de wetenschap verder vergroten. Uiteindelijk kan verhoogde zekerheid leiden tot een beter begrip van complexe ziekten, vergezeld met de versnelde ontwikkeling van nieuwe biomarkers en medicijnen.

CONCLUSIE

Het is verbazingwekkend wat een simpele bloeddruppel ons kan vertellen. In dit proefschrift hebben we verschillende subtypes van circulerende witte bloedcellen onderzocht. We hebben aangetoond dat witte bloedcellen belangrijke informatie kunnen bevatten die voorspellende waarde heeft om mannen en vrouwen met hoog risico op terugkerende cardiovasculaire complicaties te identificeren. Toekomstige onderzoeken zijn nodig om onze resultaten te bevestigen. Desondanks ben ik ervan overtuigd dat immuuncellen in de toekomst een belangrijke bijdrage kunnen leveren aan het ontwikkelen van nieuwe medicatie en het identificeren van risicopatiënten met slagaderverkalking.

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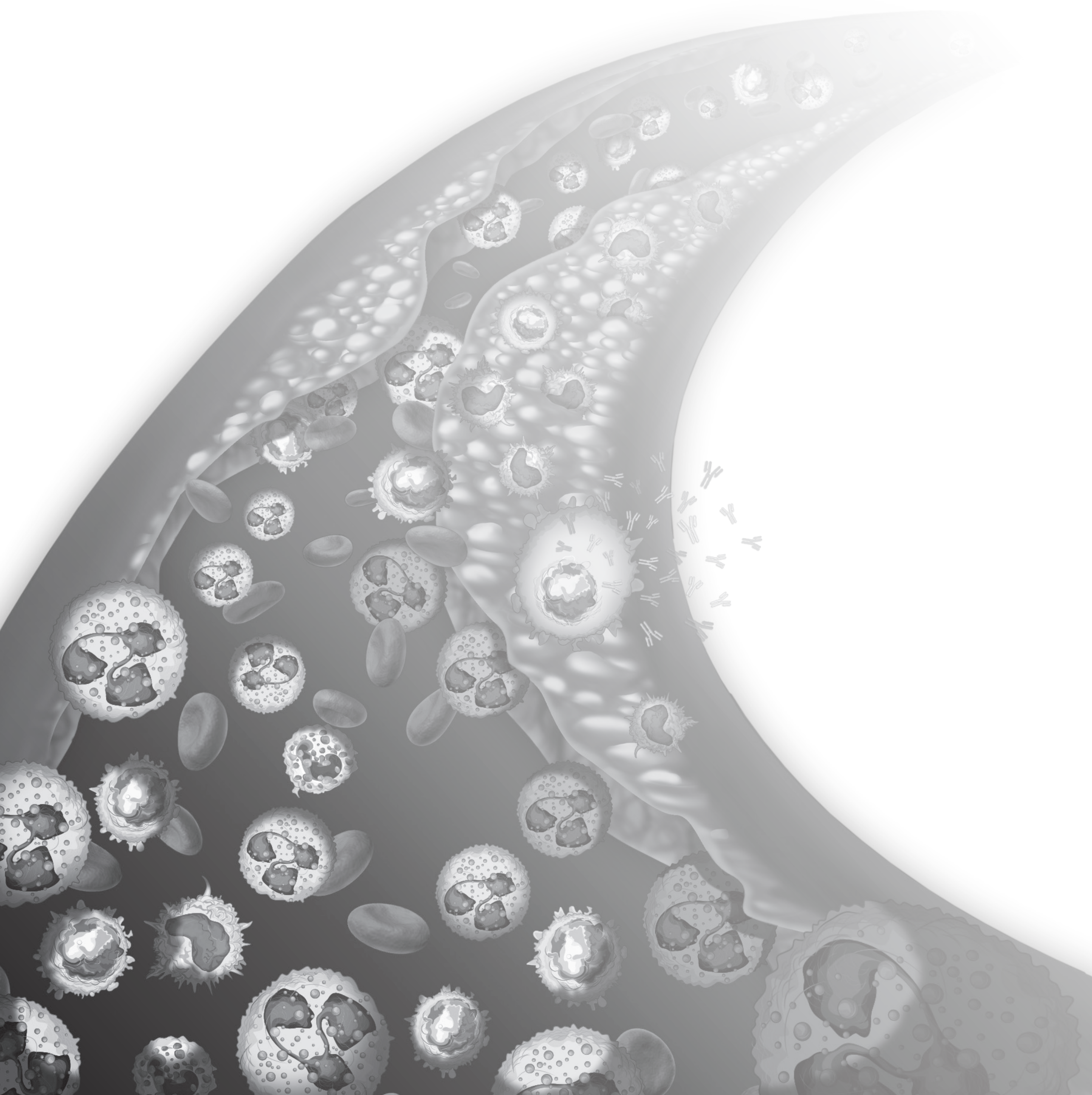

APPENDIX

Review committee
List of publications
Acknowledgements
Curriculum vitae

REVIEW COMMITTEE

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LIST OF PUBLICATIONS

Maracle CX, Agca R, Helder B, Meeuwsen JAL, Niessen HWM, Biessen EAL, de Winther MPJ, de Jager SCA, Nurmohamed MT, Tas SW. Noncanonical NF-kappaB signaling in microvessels of atherosclerotic lesions is associated with inflammation, atheromatous plaque morphology and myocardial infarction. *Atherosclerosis* **270**, 33-41 (2018).

Meeuwsen JAL, van Duijvenvoorde A, Gohar A, Kozma MO, van de Weg SM, Gijsberts CM, Haitjema S, Bjorkbacka H, Fredrikson GN, de Borst GJ, den Ruijter HM, Pasterkamp G, Binder CJ, Hoefer IE, de Jager SCA. High levels of (un)switched memory B cells are associated with better outcome in patients with advanced atherosclerotic disease. *J. Am. Heart Assoc*. **6**, 10.1161/JAHA.117.005747 (2017).

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Meeuwsen JAL, Wesseling M, Hoefer IE, de Jager SCA. Prognostic value of circulating inflammatory cells in patients with stable and acute coronary artery disease. *Front. Cardiovasc. Med.* **4**, 44 (2017).

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Meeuwsen JAL, de Vries JJ, Zoet GA, Franx A, Fauser BCJM, Velthuis BK, Visseren FL, Pasterkamp G, Hoefer IE, van Rijn BB, den Ruijter HM, de Jager SCA. Circulating neutrophils do not predict subclinical coronary artery disease in women with former preeclampsia. (*Submitted*).

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A

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CURRICULUM VITAE

John was born on October 4th, 1991 in Reimerswaal, the Netherlands. After completing secondary school in Goes, John moved to Utrecht to study Biomedical Sciences at Utrecht University. He obtained his bachelor's degree in Biomedical Sciences in 2012 and graduated from the master's program Biology of Disease in 2014. During these studies, John developed a strong interest for cardiovascular disease. After his master's, John first worked as junior policy adviser Biomedical Sciences within the Graduate School of Life Sciences for a year. In 2015, he started his PhD candidacy at the laboratory of Experimental Cardiology. He investigated the role of circulating immune cells in atherosclerotic disease under supervision of Gerard Pasterkamp, Imo Hoefer and Saskia de Jager. The results of this research are summarized in the present thesis. John is currently working as a junior teacher and researcher in Biomedical Sciences and Medicine at the UMC Utrecht and Utrecht University.