**Novel pro- and anti-atherogenic effects of apolipoprotein B-containing lipoproteins: To feast or to fast?**

Marijke Akua de Vries

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Huisartsenpraktijk Stellendam

Layout, cover, printing: Optima Grafische Communicatie, Rotterdam

**Novel Pro- and Anti-Atherogenic Effects of Apolipoprotein B-containing Lipoproteins: To feast or to fast?**

**Nieuwe pro- en anti-atherogene effecten van apolipoproteïne B-bevattende lipoproteïnen**

**Proefschrift**

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. H.A.P Pols

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op

Vrijdag 3 maart 2017 om 13.30 uur

**Marijke Akua de Vries**
geboren te Rotterdam

**Erasmus University Rotterdam**

**PROMOTIECOMMISSIE**

| | |
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**TABLE OF CONTENTS**

# Part 1

## Plasma apolipoprotein B-containing lipoproteins cause atherosclerosis: role of postprandial inflammation

# Chapter 1

## General introduction

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Published in part in: Adv Exp Med Biol. 2014;824:161-70

## INTRODUCTION

Cardiovascular disease (CVD) is a global health issue. In 2012, an estimated 17.5 million people died of CVD worldwide, accounting for approximately 31% of all deaths [1]. About 80% of these deaths were due to coronary artery disease (7.6 million) and stroke (5.7 million) [1]. CVD is not only an important cause of mortality, but also of morbidity, with many individuals living with the consequences and limitations of coronary artery disease, cerebrovascular artery disease and peripheral artery disease. CVD is the single most important cause of lost health globally [1].

The underlying pathology of these conditions is atherosclerosis. Atherosclerosis may result from a combination of several risk factors, including smoking, diabetes mellitus, hyperlipidemia, hypertension, abdominal obesity, physical inactivity, a diet low in fruit and vegetables, regular alcohol consumption and psychosocial factors [2]. Collectively, these nine risk factors are estimated to account for 90% of the population attributable risk of myocardial infarction in men, and 94% in women [2].

## APOLIPOPROTEIN B AND ATHEROSCLEROSIS

One of the main lipid risk factors for atherosclerosis, is an increased apolipoprotein (apo) B / apo A-I ratio [2]. Apo B is the structural protein present on the surface of all atherogenic lipoproteins, including very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), chylomicrons and their respective remnants [3]. In contrast, apo A-I is the main apolipoprotein of high-density lipoproteins (HDL) [4].

The gene encoding for apo B is located on chromosome 2 [5]. In humans, two forms of apo B exist. Apo B100 is present on hepatically derived LDL, IDL, VLDL and their remnants. This protein of 4536 amino acids is synthesized in the liver, and plays a crucial role in the assembly of VLDL particles [3]. In addition, apo B serves as the ligand for the

uptake of atherogenic lipoproteins by the LDL-receptor [3]. Apo B48 is synthesized in the intestine. In the intestine, the single nucleotide for amino acid 2153 in the mRNA of the apo B gene is changed into a stop codon, resulting in an apo B protein of 2152 amino acids, which is 48% of the length of the apo B100 protein [3]. Apo B48 is crucial in the formation of chylomicrons [3].

Plasma apo B measurement includes both apo B100 and apo B48, with apo B100 making up the vast majority of all circulating apo B-containing lipoproteins [6]. Plasma apo B is a strong predictor of future CVD, with an estimated relative risk ratio (RRR) of 1.43 (95% confidence interval 1.35 to 1.51) [7]. In fact, apo B is considered to be an even better cardiovascular risk marker than LDL-cholesterol [7], which is currently one of the cornerstones of cardiovascular risk management guidelines [8].

## CHYLOMICRON AND VLDL SYNTHESIS

Lipids and fat-soluble vitamins ingested with the diet are transported from the intestine to the circulation by chylomicrons. In the intestine, dietary triglycerides are hydrolyzed into free fatty acids (FFAs) and 2-monoacylglycerols (MAG) [9]. These FFAs and MAG are then transported from the intestinal lumen to the smooth endoplasmatic reticulum (SER) of the enterocyte, where they are resynthesized into triglycerides [9,10]. The formation of chylomicrons starts in the rough endoplasmatic reticulum (RER), where nascent apo B48 is translocated into the lumen of the endoplasmatic reticulum (ER) [11]. The microsomal transfer protein (MTP) shuttles triglycerides from within the ER to an acceptor apo B48 molecule, giving rise to a primordial apo B-containing lipoprotein [11]. This particle is then enriched and expanded with lipids present within the ER, resulting in a prechylomicron, with a core containing triglycerides, cholesteryl esters and fat-soluble vitamins, and an outer layer with phospholipids, free cholesterol, apo B48 and apo A-IV [9,11]. The prechylomicron leaves the ER and is transported to the Golgi apparatus in prechylomicron transport vesicles. Several proteins, including apo B48 and CD36, are involved in this transport [12]. Within the Golgi, apo A-I is attached, resulting in the formation of a mature chylomicron, which is secreted into the lymphatic system [13,14].

In the circulation, the triglycerides present in chylomicrons are hydrolyzed into MAG and FFAs by the endothelium-bound enzyme lipoprotein lipase (LPL), giving rise to chylomicron remnant particles [15]. The dietary FFAs and chylomicron remnants can be taken up by the liver, to be used for the synthesis of VLDL [16]. The assembly of VLDL begins in the ER of hepatocytes. In humans, the liver lacks an editing enzyme complex such as in the intestine and therefore, hepatic triglyceride-rich lipoproteins (TRLs) contain solely apo B100 as structural apolipoprotein [17]. Apo B100 is synthesized in the RER and under the influence of MTP, the nascent lipoprotein becomes enriched with triglycerides

which are synthesized in the SER [17]. This gives rise to pre-VLDL, which can be further processed to form triglyceride-poor $VLDL_2$. The VLDL synthesis in the liver is a continuous process, and VLDL secretion is controlled mainly by intracellular degradation, depending largely on lipidation of the nascent VLDL particles [18]. Therefore, pre-VLDL that is not transformed to $VLDL_2$ is subject to posttranslational degradation [18]. Otherwise, $VLDL_2$ is transported to the Golgi, where it is either secreted into the circulation, or converted to triglyceride-rich $VLDL_1$, which is then released into the circulation.

The large VLDL particles in the circulation are hydrolyzed by LPL, resulting in the formation of intermediate-density lipoproteins (IDL) and eventually low-density lipoproteins (LDL) [19]. However, since chylomicrons and VLDL largely share the same lipolytic pathway, competition at the level of several steps involved in the lipolysis and removal of TRLs from the circulation may occur. These include not only LPL [15], but also hepatic lipase, the low-density lipoprotein receptor (LDL-R), the LDL receptor-related protein 1 (LRP-1) and heparan sulfate proteoglycans [19,20]. This competition may lead to accumulation of TRLs in the circulation.

## INFLAMMATION AND LEUKOCYTE ACTIVATION IN ATHEROSCLEROSIS

In the past decades, it has become evident that inflammation plays a key role in the development of atherosclerosis. Several inflammatory markers, including C-reactive protein (CRP), total leukocyte count and the third component of complement (C3) have been associated with CVD [21-24].

Postprandial inflammation starts with the increase of remnant lipoproteins and glucose in the circulation. This leads to activation of circulating leukocytes, which interact with the endothelium [25-29]. Endothelial dysfunction, due to for instance hypercholesterolemia, smoking, hypertension or diabetes mellitus, may induce the expression of endothelial adhesion molecules, such as vascular cell adhesion molecule 1 (VCAM-1) [25,29]. An important step in the development of atherosclerosis is the activation of leukocytes in the circulation. Unstimulated neutrophils have some adherence to endothelial cells, but enhanced adherence is almost entirely dependent on the expression of integrins on the leukocyte surface [30]. These integrins are stored in intracellular granules and are brought to the surface upon stimulation [31,32]. CD11b, also known as complement receptor 3 or Mac-1, is expressed on activated neutrophils and monocytes [30]. The most important ligand for CD11b on endothelial cells is intercellular adhesion molecule-1 (ICAM-1) [33,34]. CD66b, also known as CEACAM8, is a marker of neutrophil degranulation [32,35]. Once leukocytes have adhered to the endothelium, they are stimulated by chemoattractant signals, in particular monocyte chemotactic protein-1 (MCP-1), to migrate into the arterial wall [29]. Subendothelial migrated monocytes dif-

ferentiate into macrophages, which can take up modified lipoproteins, leading to foam cell formation [25]. This process results in the recruitment of even more leukocytes to the lesion site, and in the production of cytokines, chemokines and growth factors, further damaging the endothelial wall, and eventually leading to necrosis [25].

## POSTPRANDIAL LIPIDS INDUCE INFLAMMATION

Lipids induce a state of inflammation, with increased levels of adhesion molecules, cytokines, oxidative stress and leukocyte activation. An *in vitro* study demonstrated that TRLs with a high triglyceride and cholesterol content stimulate human aortic endothelial cells to express VCAM-1 [36], and during hypertriglyceridemia, the adhesion of monocytes to VCAM-1 is increased [37]. Not only TRLs itself, but also the FFAs released by hydrolysis of these particles stimulate endothelial cells to express adhesion molecules and to produce inflammatory cytokines [38]. In an *in vivo* study, ingestion of a high-fat meal resulted in an increase in neutrophil count, interleukin-6 (IL-6) and hydroperoxides, with a simultaneous decrease in endothelial function, reflected by flow-mediated endothelium-dependent dilatation [39]. Others showed that ingestion of a high-fat meal by healthy volunteers increased serum bacterial endotoxin, or lipopolysaccharide (LPS), which may also lead to leukocyte activation and inflammation [40]. The ingestion of fat therefore results in increased leukocyte activation, which is reflected by an increase of surface expression of CD11b, CD11c and CD14 on monocytes, and CD11b, CD66b and CD62L on neutrophils, as has been shown *in vitro* [41,42] and *in vivo* [27,37,43,44]. These data point at a pro-inflammatory effect of dietary lipids on circulating inflammatory cells with detrimental effects on the vessel wall.

Apo B-containing lipoproteins have been shown to adhere to postprandial leukocytes in the circulation [26]. By adding labeled palmitic acid to an oral fat load, it was demonstrated that leukocytes become enriched with dietary FFAs [26]. The uptake of TRLs by leukocytes is most likely mediated by different receptors, such as the LDL-R, LRP-1 and the apo B48 receptor [37,45,46]. Opsonization of remnants in the circulation may be directly related to leukocyte activation.

The molecular mechanism behind lipid-induced inflammation probably involves activation of the transcription factor nuclear factor kappa B (NF-κB). The remnants of TRLs migrate into the subendothelial space, where they induce the production of reactive oxygen species (ROS). This may lead to the oxidative modification of LDL, and these oxidized lipoproteins (oxLDL) are easily taken up by macrophages, smooth muscle cells and endothelial cells via scavenger receptors, such as CD36, SR-AI/II, SR-BI and the lectin-like oxidized LDL receptor 1 (LOX-1) [47,48]. Internalized lipoproteins can induce activation of the protein kinase C (PKC) pathway, resulting in activation of the IκB

kinase complex and subsequently NF-κB. Activated NF-κB will induce the transcription of genes encoding for several cytokines, including tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), interleukin-1 beta (IL-1β), IL-6 and IL-8, chemokines, such as MCP-1 and macrophage inflammatory protein-1 alpha (MIP-1α) and cellular adhesion molecules, such as VCAM-1 (Figure 1.1).

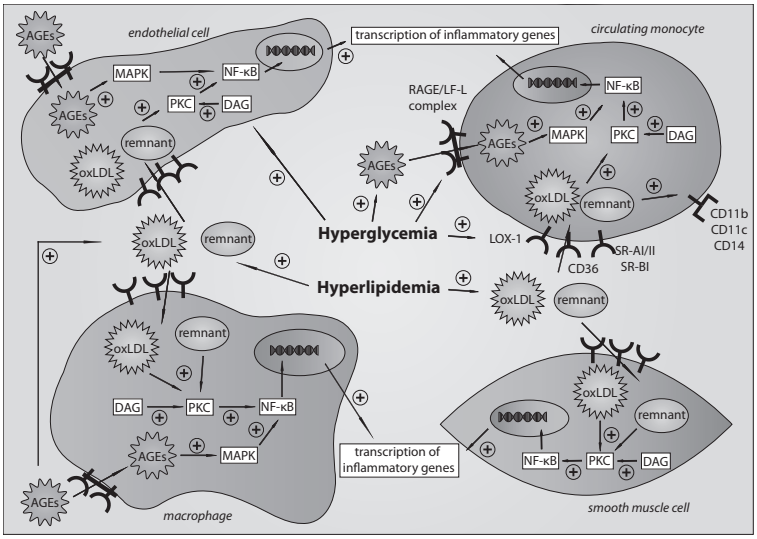


**Figure 1.1.** The cellular processes involved in lipid- and glucose-induced inflammation

The remnants of triglyceride-rich lipoproteins and oxidized low-density lipoprotein (oxLDL) are taken up by circulating leukocytes, macrophages, endothelial cells and smooth muscle cells via several scavenger receptors, including CD36, SR-AI/II, SR-BI and lectin-like oxidized LDL receptor 1 (LOX-1). Intracellular, remnants and oxLDL activate the protein kinase C (PKC) pathway, resulting in activation of nuclear factor kappaB (NF-κB). NF-κB induces the transcription of several inflammatory genes, including genes encoding for cytokines, chemokines and adhesion molecules. During hyperglycemia, the intracellular synthesis of diacylglycerol (DAG) in endothelial cells, smooth muscle cells, monocytes and macrophages is increased, leading to further activation of the PKC pathway. Activation of the PKC pathway in monocytes also results in the release of the integrins CD11b, CD11c and CD14 stored in intracellular vesicles. In addition, during hyperglycemia, advanced glycation end products (AGEs) are formed. These AGEs are taken up by endothelial cells, monocytes and macrophages via the receptor for AGE (RAGE) and lactoferrin-like polypeptide (LF-L) complex, resulting in activation of mitogen-activated protein kinase (MAPK) and subsequently NF-κB activation. AGEs also enhance the formation of oxLDL, and during hyperglycemia the expression of LOX-1 on monocytes and macrophages increases. These processes further facilitate the uptake of oxLDL by macrophages and thus enhance the inflammatory process.

## GLUCOSE AND POSTPRANDIAL INFLAMMATION

Not only lipids, but also glucose may be involved in postprandial inflammation. In fact, the generation of oxidative stress due to hyperglycemia has been proposed to be a key event in the development of diabetic complications [49]. *In vivo* studies have demonstrated that ingestion of glucose results in increased production of TNF-α and IL-1β by peripheral blood mononucleated cells [50], reduced flow-mediated endothelium-dependent dilatation [39,51], increased formation of ROS and oxidative stress [51] and increased leukocyte activation [28].

Hyperglycemia may induce inflammation via several mechanisms. One is the increased activation of the PKC pathway in endothelial cells, macrophages and smooth muscle cells. Activation of PKC results in increased cytokine production via activation of NF-κB [52]. This increased PKC activity is probably stimulated by an increased *de novo* synthesis of diacylglycerol in endothelial cells during hyperglycemia, which is an important activator of PKC [53].

Glucose can modify proteins to form advanced glycation end products (AGEs), which exert a pro-inflammatory effect in several ways [54]. AGEs can interact with cellular binding sites, such as the receptor for AGE (RAGE) and lactoferrin-like polypeptide (LF-L) on endothelial cells and macrophages. This AGEs-RAGE interaction results in uptake of AGEs by these cells and subsequently generation of ROS and activation of NF-κB via activation of mitogen-activated protein kinase (MAPK) [55-58]. The interaction between AGEs and RAGE on circulating monocytes also induces chemotaxis, leading to increased migration of these cells into the subendothelial space [57]. The presence of AGEs induces increased expression of RAGE on endothelial cells [59], which functions as a receptor for the integrin CD11b on monocytes and neutrophils, promoting leukocyte adhesion to the vessel wall [60].

Furthermore, AGEs induce the glycosylation of apo B and phospholipids on LDL particles, making these lipoproteins more susceptible to oxidative modifications, resulting in enhanced uptake by macrophages via scavenger receptors [56]. During hyperglycemia, the surface expression of LOX-1 on macrophages is increased, thereby enhancing the uptake of oxLDL by these cells [52].

Moreover, glycation of the regulatory membrane protein CD59 on endothelial cells, which inhibits the deposition of the membrane attack complex (MAC) of complement, leads to reduced activity of this protein and thus a higher susceptibility of the endothelium for the MAC-induced release of pro-inflammatory cytokines [61].

Finally, hyperglycemia results in a reduced antioxidant capacity. Hyperglycemia leads to increased reduction of glucose to sorbitol by aldose reductase, and during this process NADPH is consumed [54]. Since the cellular antioxidant capacity depends on the energy provided by NADPH to the antioxidants glutathione and thioredoxin, reduction

of NADPH will result in increased oxidative stress [54,62]. The cellular processes involved in inflammation induced by lipids and glucose are summarized in Figure 1.1.

## LIFESTYLE AND PHARMACEUTICAL INTERVENTIONS MODULATING POSTPRANDIAL INFLAMMATION

Evidence from clinical trials is starting to emerge, demonstrating that postprandial inflammation can be reduced by lifestyle modifications. Several studies have established a positive effect of dietary antioxidants on postprandial inflammation. Foods rich in polyphenols, such as strawberries and black raspberries, reduce postprandial inflammation [63,64]. The daily consumption of strawberry beverages during six weeks reduced the postprandial oxidative modification of LDL in hyperlipidemic patients [63]. The consumption of black raspberries for four days attenuated the high-fat meal-induced production of IL-6 [64]. Flavonoids seem to reduce postprandial inflammation as well. An *in vitro* study demonstrated that the flavonoid epigallocatechin-3-gallate suppressed the hyperglycemia-induced expression of VCAM-1 on human umbilical vein endothelial cells and reduced the adhesion of monocytes to these cells, via inhibition of PKC and NF-κB activation [65]. This beneficial effect of flavonoids on postprandial inflammation has been confirmed *in vivo*. In a randomized trial with 30 healthy volunteers, the consumption of orange juice, which contains flavonoids, with a high-fat meal reduced the postprandial production of ROS and prevented the postprandial increase in LPS [66]. Food containing carotenoids may also decrease postprandial inflammation. The consumption of tomatoes containing this antioxidant reduced postprandial IL-6 production and prevented LDL oxidation, even though the tomatoes exaggerated postprandial lipemia [67]. This anti-inflammatory effect, despite an increase in serum lipids, has also been observed with the consumption of red wine. Although the addition of red wine to a meal resulted in a higher increase in serum lipids, red wine prevented the production of NF-κB by peripheral blood mononucleated cells [68]. In contrast, addition of vodka to the meal did not attenuate the NF-κB activation, indicating that this effect was not due to the alcohol content of the wine [68]. The anti-inflammatory effect of red wine is more likely the result of the antioxidants quercetin and α-tocopherol succinate, since treatment of human mononuclear cells with VLDL in the presence of these antioxidants inhibited the activation of NF-κB [68]. Collectively, these data suggest a beneficial effect of dietary antioxidants, such as polyphenols, flavonoids, carotenoids, quercetin and α-tocopherol succinate, on postprandial inflammation.

In addition to antioxidants, a diet rich in monounsaturated fatty acids (MUFA), as opposed to saturated fatty acids (SFA), reduces postprandial inflammation. In a small study, the consumption of a meal based on extra virgin olive oil, which is rich in MUFA, did not

elicit NF-κB activation in peripheral blood mononucleated cells, in contrast to ingestion of a meal based on butter, rich in SFA, or walnuts, which contained an equal amount of MUFA, SFA and polyunsaturated fatty acids (PUFA) [69]. This beneficial effect of MUFA was confirmed in another trial with 20 elderly healthy subjects. In this study, a Mediterranean diet with a high virgin olive oil content not only reduced total cholesterol, LDL-C and apo B, but also the postprandial expression of MCP-1, matrix metalloproteinase 9 (MMP-9) and the NF-κB subunit p65 in peripheral blood mononucleated cells [70]. In another small study, the addition of avocado, rich in MUFA, to a hamburger meal attenuated the postprandial increase of IL-6, via reduced activity of the NF-κB pathway [71].

The effect of exercise on postprandial inflammation remains controversial. In a study among 20 cigarette smokers, those who reported to exercise two or more hours a week had lower postprandial levels of malondialdehyde than untrained smokers [72]. However, in a small randomized trial, exercise before a high-fat meal did not reduce postprandial serum lipid hydroperoxides [73]. In a cross-sectional study among middle-aged men, active men had lower fasting levels of IL-6 and CRP than sedentary men, but no difference in postprandial inflammation was observed [74].

Weight loss seems to have a favorable effect on postprandial inflammation. Moderate weight loss reduced the postprandial soluble intercellular adhesion molecule (s-ICAM), MCP-1 and high-sensitivity CRP (hs-CRP) increment in 11 normolipidemic moderately obese men [75]. In another study, weight loss reduced postprandial IL-6 levels in eight men with impaired glucose tolerance [76].

Several lipid-lowering drugs are effective in lowering postprandial inflammation as well. Simvastatin, atorvastatin and pitavastatin have all been shown to attenuate postprandial inflammation [77-79]. In addition, fenofibrate reduces the production of TNF-α, IL-1β, IL-6, MCP-1 and MIP-1α [80,81]. Glucose-lowering drugs may also reduce postprandial inflammation. Rosiglitazone and metformin have been shown to increase levels of the antioxidant enzyme paraoxonase (PON)-1 and decrease postprandial levels of MCP-1 [82,83]. Treatment of patients with type 2 diabetes mellitus with nateglinide, a glucose-lowering drug belonging to the meglitinides class of drugs, significantly reduced postprandial oxidative stress and increased endothelial function [51]. Infusion of glucagon-like peptide 1 during hyperglycemia significantly reduced oxidative stress, inflammation and endothelial dysfunction in patients with type 1 diabetes mellitus [84]. To the best of our knowledge, no studies have compared the effect of lipid-lowering versus glucose-lowering therapy on postprandial inflammation in patients with diabetes mellitus. The effects of different dietary and pharmaceutical interventions are summarized in Table 1.1.

**Table 1.1** Overview of interventions effective in reducing postprandial inflammation

| | Intervention | Compound | Effect | Reference |
|---|---|---|---|---|
| **Diet** | Strawberries | Polyphenols | ↓oxLDL | [63] |
| | Black raspberries | Polyphenols | ↓IL-6 | [64] |
| | Orange juice | Flavonoids | ↓ROS, ↓LPS | [66] |
| | Tomatoes | Carotenoids | ↓oxLDL, ↓IL-6 | [67] |
| | Red wine | Quercetin<br>A-tocopherol succinate | ↓NF-κB | [68] |
| | Extra virgin olive oil | MUFA | ↓NF-κB, ↓MCP-1, ↓MMP-9 | [69,70] |
| | Avocado | MUFA | ↓IL-6, ↓NF-κB | [71] |
| **Weight loss** | | | ↓s-ICAM-1, ↓MCP-1, ↓hs-CRP, ↓IL-6 | [75,76] |
| **Drugs** | Statins | Simvastatin | ↓C3 | [77] |
| | | Atorvastatin | ↓C3 | [78] |
| | | Pitavastatin | ↓urinary isoprostane | [79] |
| | Fibrates | Fenofibrate | ↓TNF-α, ↓IL-1β, ↓IL-6, ↓MCP-1 ↓MIP-1α | [80,81] |
| | Glucose-lowering drugs | Rosiglitazone | ↑PON-1, ↓MCP-1 | [82,83] |
| | | Metformin | ↑PON-1, ↓MCP-1 | [83] |
| | | Nateglinide | ↓MDA | [51] |
| | | GLP-1 | ↓sICAM-1, ↓IL-6, ↓nitrotyrosine, ↓serum isoprostane | [84] |

C3: complement component 3; hs-CRP: high-sensitivity C-reactive protein; IL-1β: interleukin-1 beta; IL-6: interleukin-6; LPS: lipopolysaccharide; MCP-1: monocyte chemotactic protein-1; MDA: malondialdehyde; MIP-1α: macrophage inflammatory protein-1 alpha; MMP-9: matrix metalloproteinase 9; NF-κB: nuclear factor kappaB; oxLDL: oxidized low-density lipoprotein; PON-1: paraoxonase 1; ROS: reactive oxygen species; s-ICAM-1: soluble intercellular adhesion molecule 1; TNF-α: tumor necrosis factor alpha

## VITAMIN D: EVIDENCE FOR A ROLE IN ATHEROSCLEROSIS

Vitamin D is a fat-soluble vitamin, which we can obtain from sunlight exposure, diet and dietary supplements [85]. Vitamin D2, or ergocalciferol, is formed by ultraviolet irradiation of ergosterol from yeast [85]. Vitamin D3, or colecalciferol, is formed through the ultraviolet irradiation of 7-dehydrocholesterol from lanolin [85]. Once vitamin D enters the human body, it is bound to vitamin D binding protein and transported to the liver, where it is converted to its major circulating form, 25-hydroxyvitamin D [86]. In the kidneys, 25-hydroxyvitamin D is metabolized to its active form, 1,25-dihydroxyvitamin D, by the enzyme 25-hydroxyvitamin 1-alpha-hydroxylase [85,86]. This conversion is regulated by levels of parathyroid hormone, calcium and phosphorus [85].

The importance of vitamin D in bone homeostasis has been acknowledged for many years [87]. Lately, it has become evident that vitamin D also plays a role in CVD and in mortality. Low vitamin D is associated with increased risk of death due to cardiovascular disease, cancer and all-cause mortality [88,89]. Evidence even suggests that supplementation of vitamin D3 reduces all-cause mortality in adults [89].

Although it is evident that most tissues and cells in the body, including endothelial cells [90] and inflammatory cells [91-93], have a vitamin D receptor, much is still unknown about the precise mechanisms involved in the modulation of atherosclerosis by vitamin D. Possibly, these beneficial effects may partly be due to an effect of vitamin D on postprandial inflammation. A pilot study by our group indicates that vitamin D3 supplementation may reduce postprandial leukocyte activation [94], but these results need to be confirmed in a randomized controlled trial.

## BLOOD CELL-BOUND APOLIPOPROTEIN B AND ATHEROSCLEROSIS

Current measurement of apo B focusses on the lipoproteins present in plasma or serum. However, our research group has developed a method to measure apo B present on the surface of circulating blood cells by flow cytometry, and demonstrated that apo B can be detected on the surface of erythrocytes, monocytes and neutrophil granulocytes [26,95,96]. A high level of apo B bound to circulating erythrocytes (ery-apoB) was associated with a decreased prevalence of clinical and subclinical atherosclerosis [95,96]. We speculate that this inverse association may be due to protection of the vessel wall from interaction with atherogenic apo B-containing lipoproteins, and that erythrocytes transport these particles from the circulation to the liver, for uptake and elimination, resulting in erythrocyte-mediated reverse cholesterol transport [97]. There is strong evidence that the binding of apo B to blood cells is mediated by the complement receptor 1 (CR1),

and we have demonstrated *in vitro* and *ex vivo* that both the classical and alternative complement pathway are involved [98].

More research into the phenomenon of cell-bound apo B is needed. For instance, it is still unknown why some subjects have high ery-apoB, while others have very low or undetectable ery-apoB, and what determinants influence the level of ery-apoB. In addition, while cross-sectional data point at a protective effect of ery-apoB, prospective data are lacking. Since leukocytes play an important role in atherogenesis, and apo B has been detected on the surface of circulating leukocytes [26], the relation between leukocyte-bound apo B and atherosclerosis needs to be investigated. Finally, the expression of CR1 on erythrocytes is tightly regulated by polymorphisms in the CR1 gene [99]. Since CR1 is very likely involved in the binding of apo B-containing lipoproteins to erythrocytes, and ery-apoB may protect against atherosclerosis, the role of CR1 polymorphisms in future CVD needs to be established.

## GOALS OF THIS THESIS

In Part 1 of this thesis, we aim to further investigate postprandial inflammation. We will review the value of postprandial lipid measurements, as opposed to fasting samples, in the prediction cardiovascular risk (Chapter 2). We will investigate the role of postprandial glucose and lipids in leukocyte activation (Chapters 3 and 4). We will investigate whether vitamin D may inhibit postprandial inflammation (Chapter 5), and assess whether leukocyte activation may predict future cardiovascular events (Chapter 6).

In Part 2, we aim to further investigate blood cell-bound apo B. We will investigate the clinical determinants of ery-apoB (Chapter 7), assess the value of ery-apoB in the prediction of future cardiovascular events (Chapter 8), investigate whether leukocyte-bound apo B is related to cardiovascular disease (Chapter 9), and whether CR1-polymorphisms, the receptor likely being involved in binding of apo B to erythrocytes, are related to future cardiovascular events (Chapter 10).

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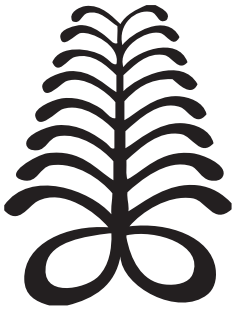

# Chapter 2

## The use of the nonfasting lipid profile for lipid-lowering therapy in clinical practice-point of view

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Atherosclerosis. 2014;234(2):473-5

## ABSTRACT

Current guidelines for the management of dyslipidaemias recommend measuring lipid profiles in the fasting state. The primary lipid targets are traditionally plasma total cholesterol and low-density lipoprotein-cholesterol (LDL-C) levels. However, triglycerides, apolipoprotein (apo) B and non-high-density lipoprotein-cholesterol (non-HDL-C) are also suitable parameters to assess cardiovascular risk and to guide lipid-lowering therapy. The advantage of the use of these variables is that they can be used in both the fasting and nonfasting state. In most cases, postprandial lipid profiles in combination with apo B are as useful as fasting lipid profiles for the differentiation between familial lipid disorders, such as heterozygous familial hypercholesterolemia, familial combined hyperlipidemia and familial hypertriglyceridemia. This article will address the interpretation, applications and limitations of a nonfasting lipid profile for daily clinical practice.

## INTRODUCTION

The current guideline for the management of dyslipidaemias, issued by the European Society of Cardiology (ESC) and the European Atherosclerosis Society (EAS), recommends the use of total cholesterol (TC) and low density lipoprotein-cholesterol (LDL-C) as the primary targets of lipid-lowering therapy [1]. According to this guideline, these measurements should be carried out after a 12 hour fast. LDL-C is calculated with the use of the Friedewald formula, which contains TC, high density lipoprotein-cholesterol (HDL-C) and triglycerides (LDL-C = TC – HDL-C – (fasting triglycerides / 2.2), in the case of mmol/L). Fasting triglycerides are believed to be more appropriate for the calculation of LDL-C, since triglycerides rise postprandially and are influenced by many dietary factors. Several studies suggest that certain lipid parameters may also be used in the nonfasting state for cardiovascular risk management, including triglycerides [2–5], apolipoprotein (apo) B and non-HDL-C. In contrast to triglycerides, apo B and non-HDL-C levels are not influenced by the prandial situation [6–8]. In Denmark, standard lipid measurements are performed nonfasting nowadays [7]. A nonfasting lipid measurement allows patients to eat normally before blood sampling and allows physicians to determine the lipid profile at any random moment. This will also contribute to patient adherence when long-term follow-up after initiating lipid lowering therapy is necessary, and may prevent long waiting times for venipunctures in the early morning.

## THE INFLUENCE OF THE NONFASTING STATE ON LDL-C

Since triglyceride levels rise on average 0.5 mmol/L in women and 1.0 mmol/L in men, the calculated LDL-C decreases during the day, with the risk of underestimation when measured in the nonfasting state [9]. Recently, it has been suggested that LDL-C levels are 0.3–0.6 mmol/L lower when measured two hours postprandially than fasting measurements [7]. Furthermore, nonfasting LDL-C levels may be less sensitive to predict cardiovascular disease (CVD) [4], even if a direct method of measurement is used [10]. Finally, it has been suggested that the concentration of LDL-C is falsely low in the nonfasting state due to hemodilution. Recently, a cross-sectional study with 209,180 subjects demonstrated that fasting affected LDL-C subclass levels only slightly [11]. In this study, the variation of LDL-C between fasting and nonfasting samples was up to 10%. Taken together, we suggest that the use of LDL-C in the nonfasting state is debatable, since LDL-C is slightly lowered in the nonfasting state.

## NONFASTING TRIGLYCERIDES AS PREDICTORS OF CARDIOVASCULAR RISK

Recent studies have demonstrated the direct association between triglycerides and CVD [5,12]. However, the relationship between triglycerides and CVD may be caused by its close relationship between triglycerides and atherogenic remnant cholesterol levels [5].

Large epidemiological studies have established the power to predict CVD by the use of nonfasting triglycerides. The Copenhagen City Heart Study has demonstrated that elevated nonfasting triglycerides are associated with an increased risk of myocardial infarction, ischemic heart disease and death in men and women [5]. However, when adjusted for several covariates, including diabetes mellitus, body mass index and high-sensitivity C-reactive protein, nonfasting triglycerides lost their predictive value of future cardiovascular events in women, but not in men. A meta-analysis of 29 prospective studies has shown that fasting and nonfasting triglycerides were similarly predictive of fatal and nonfatal coronary events [13]. Results from the Women's Health Study even suggest that triglycerides measured two to four hours postprandially might be a better predictor of CVD than fasting triglycerides [2,4]. Each 1 standard deviation increase in nonfasting triglycerides was associated with a hazard ratio (HR) of 1.17 (95% confidence interval 1.04–1.31), while the HR for fasting triglycerides was not significant [4]. The HR for a cardiovascular event for subjects with triglycerides >1.9 mmol/L (171 mg/dL) was 1.98 (95% confidence interval 1.21–3.25). These data suggest that nonfasting triglycerides can be used for cardiovascular risk prediction.

The measurement of triglycerides is complicated by its high intra-individual variability, on average 22.5% [9]. The absolute intra-individual variability is higher in hypertriglyceridemic patients than in patients with normal triglycerides. It has been suggested that the variability of fasting triglycerides is lower than that of nonfasting triglycerides. However, we have shown that the variability of triglycerides increases only slightly during the day, in contrast to current believes [9]. In women and in patients with hypertriglyceridemia, the variability does not even differ between the fasting and nonfasting state [9].

Therefore, we suggest that the risk of false positive or false negative hypertriglyceridemia is equally present in the fasting and nonfasting state with regard to the intra-individual variability. However, since triglycerides rise during the day, triglyceridemia may be falsely overestimated when reference values for fasting triglycerides are used [9,11].

## NON-HDL-C AND APO B AS ALTERNATIVE FOR LDL-C IN THE NONFASTING STATE



In patients receiving lipid-lowering therapy, the risk of cardiovascular events often remains increased despite lowering of LDL-C, due to inadequate lowering of cholesterol in triglyceride-rich lipoproteins [14]. This has been called 'residual risk'. Non-HDL-C and apo B can be considered as alternative parameters for LDL-C to guide lipid lowering therapy and CVD risk assessment more accurately, since they take all atherogenic lipoproteins into account [15,16].

Non-HDL-C represents the cholesterol content of all atherogenic lipoproteins, including remnant lipoproteins, and it is calculated by subtracting HDL-C from TC. Apo B reflects the number of atherogenic lipoproteins, since each atherogenic lipoprotein contains a single apo B molecule. The measurement of apo B has been validated and standardized, resulting in a low coefficient of variation between different laboratories in samples with low as well as high levels of apo B [17]. Prospective studies and meta-analyses have consistently demonstrated that non-HDL-C and apo B are at least as good as [4,8,18,19] or superior to [6,14,15] LDL-C in the prediction of CVD during lipid-lowering therapy. Another advantage of non-HDL-C and apo B over LDL-C is the fact that these measurements are not influenced by the prandial state and can therefore be measured nonfasting [20]. It has to be noted that results from a large prospective study indicated that, despite small changes in their concentrations, the predictive value of apo B and non-HDL-C decreased when these markers were measured in the nonfasting state [4].

The 2011 ESC/EAS guideline recommends the use of non-HDL-C as secondary target of lipid-lowering therapy in patients with hypertriglyceridemia or type 2 diabetes mellitus (T2DM), since in contrast to LDL-C, non-HDL-C reflects the atherogenic burden of circulating remnant cholesterol [1,21]. The treatment target for non-HDL-C is 0.8 mmol/L (30 mg/dL) higher than the respective target for LDL-C [1]. The treatment target for non-HDL-C recommended by the ESC/EAS is 3.3 mmol/L (130 mg/dL) for patients with high cardiovascular risk.

Besides non-HDL-C, apo B may also be an adequate parameter for initial cardiovascular risk assessment [16]. The goal for apo B recommended by the ESC/EAS guideline is 1.0 g/L for patients with increased cardiovascular risk and 0.8 g/L for patients with severely increased risk [1]. Table 2.1 gives an overview of suggested cut-off risk points for the nonfasting lipid profile, based on the ESC/EAS guideline [1] and results from the Women's Health Study [2].

**Table 2.1** Suggested cut-off risk points for lipid-lowering therapy according to cardiovascular risk independent from postprandial state

| Cardiovascular risk | Non-HDL-C | | Apo B | | Triglycerides | |
|---|---|---|---|---|---|---|
| | mmol/L | mg/dL | g/L | mg/dL | mmol/L | mg/dL |
| Moderate risk | < 3.8 | < 145 | | | | |
| High risk | < 3.3 | < 130 | < 1.0 | < 100 | | |
| Very high risk | < 2.6 | < 100 | < 0.8 | < 80 | <1.93 | <171 |

Note: these recommendations are based on the guidelines published by the EAS/EAS [1] and results of the Women's Health Study [2].

We suggest that the potential inaccuracy of LDL-C in the nonfasting state is not a limiting factor when using nonfasting lipid profiles, since non-HDL-C and apo B can be used as alternatives for LDL-C.

## NONFASTING APO B AND TRIGLYCERIDES IN THE DIAGNOSIS OF PRIMARY HYPERLIPIDEMIAS

Mild hypercholesterolemia, hypertriglyceridemia or a combination of both are frequently caused by a polygenic predisposition combined with inadequate lifestyle. Nevertheless, it is important to detect primary lipid disorders when present. The three most frequent primary hyperlipidemias are familial hypercholesterolemia (FH), familial combined hyperlipidemia (FCH) and familial hypertriglyceridemia (FHTG). All three diseases are characterized by a different pathogenesis and lipid profile. FH and FCH are both associated with a strongly increased cardiovascular risk, as opposed to FHTG [22,23]. Patients with FHTG have an increased risk of T2DM and pancreatitis [24].

FCH, FH and FHTG can be differentiated by family history, physical examination and, in most cases, by a nonfasting lipid profile. Apo B and triglycerides are the most important parameters of the lipid profile to distinguish FHTG from FH and FCH [25,26]. In FHTG, apo B is normal, in contrast to FH and FCH [25,27], and triglycerides are strongly elevated, whether triglycerides are measured fasting or nonfasting [28]. Determining a nonfasting lipid profile will often be sufficient for differentiation between FH and FCH. Both diseases are accompanied by elevated apo B and LDL-C, but in FH, triglycerides are usually normal, whereas in FCH, triglycerides are usually elevated [29]. The value of apo B to distinguish FCH from FHTG is also underlined by the authors of the guideline for evaluation and treatment of hypertriglyceridemia, provided by the Endocrine Society [30]. The Nijmegen group has provided a nomogram for the diagnosis of FCH. This nomogram uses apo B, triglycerides and TC values [31]. However, one of the risks of using a nonfasting lipid profile is to get "falsely" elevated triglycerides in the case of FH, which

can make differentiation between FH and FCH difficult. In this case, when both apo B and triglycerides are elevated in the nonfasting state, we suggest to consider a second lipid profile in the fasting state to adequately distinguish FH from FCH. It should be noted that differentiation between FCH and FH can be difficult even when using fasting lipid profiles, because FCH can be present if triglycerides are normal or almost normal, and FH can be accompanied by elevated triglycerides due to the genetic heterogeneity of the primary lipid disorders [30]. Here we have focused on classic genetic types of hyperlipidemia, although most cases of hyperlipidemia are polygenic.

## CONCLUSION

In most cases, a nonfasting lipid profile is adequate for cardiovascular risk assessment, evaluation of lipid lowering therapy and for the differentiation between primary lipid disorders. The measurement of nonfasting lipid profiles has several advantages: patients do not need to fast and clinicians can have the lipid profile determined at any random time of day. Non-HDL-C and apo B are good alternatives for LDL-C and are not influenced by the nonfasting state.

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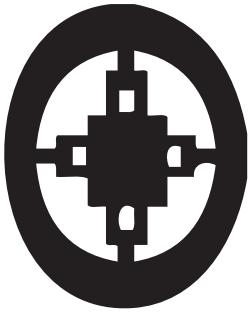
# Chapter 3



## Glucose-dependent leukocyte activation in patients with type 2 diabetes mellitus, familial combined hyperlipidemia and healthy controls

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Metabolism. 2015;64(2):213-7

## ABSTRACT

**Background:** Leukocyte activation has been associated with vascular complications in type 2 diabetes mellitus (T2DM). Hyperglycemia may be involved in this leukocyte activation. Our aim was to investigate the role of elevated glucose concentrations on leukocyte activation in patients with a wide range of insulin sensitivity.

**Methods:** Leukocyte activation was determined after ingestion of 75 gram glucose in subjects with T2DM, familial combined hyperlipidemia (FCH) and healthy controls. Leukocyte activation markers were measured by flow cytometry. Postprandial changes were calculated as the area under the curve (AUC), and the incremental area under the curve corrected for baseline values (dAUC).

**Results:** 51 subjects (20 T2DM, 17 FCH and 14 controls) were included. Fasting neutrophil CD66b expression and CD66b-AUC were respectively 36% and 39% higher in T2DM patients than in controls ($p=0.004$ and $p=0.003$). Fasting neutrophil CD66b expression correlated positively with glucose-AUC (Spearman's rho 0.481, $p<0.001$) and HbA1c (rho 0.433, $p=0.002$). Although fasting monocyte CD11b expression was not significantly different between subjects, monocyte CD11b-AUC was 26% higher in T2DM than in controls ($p=0.006$). Similar trends were observed for FCH patients. Monocyte CD11b-dAUC correlated positively with glucose-AUC (rho 0.322, $p=0.022$) and HbA1c (rho 0.319, $p=0.023$).

**Conclusions:** These data suggest that both acute and chronic hyperglycemia, associated with insulin resistance as seen in T2DM and FCH, are involved in the increased fasting and postprandial leukocyte activation observed in these conditions.

## INTRODUCTION

Inflammation is important in the development of diabetic complications. Increased leukocyte activation has been established in patients with insulin resistance and type 2 diabetes mellitus (T2DM) [1,2]. This leukocyte activation has been linked to microvascular diabetic complications and atherosclerosis [3–5]. Activated leukocytes are able to adhere to the intact endothelium, and can migrate to the subendothelial space, where the development of atherosclerosis starts [6].

Leukocyte activation can be determined by quantitation of cell surface integrins like CD11b and CD66b. CD11b, or Mac-1, is involved in early adhesion of monocytes and neutrophils to the endothelium. CD66b, or CEACAM8, is a marker of neutrophil degranulation [7].

Postprandial studies have shown that leukocytes are activated by lipids [8–10]. In addition, several studies have demonstrated the effect of glucose on leukocyte activation. An *in vivo* study showed a rapid increase in monocyte CD11b expression following the ingestion of glucose in T2DM patients and in healthy controls [11]. In a different study, administration of glucose during 15 days induced leukocyte activation in non-diabetic volunteers [12].

However, little is known about leukocyte activation in non-diabetic insulin resistant conditions. The aim of this study was to investigate the role of acute and chronic glycemia in leukocyte activation in patients with a wide range of insulin sensitivity, such as T2DM and familial combined hyperlipidemia (FCH) [13] and in healthy controls.

## MATERIALS AND METHODS

Subjects visiting our Department of Vascular Medicine, who met the diagnostic criteria for T2DM or FCH, were asked to undergo an oral glucose tolerance test (OGTT). Healthy volunteers were recruited by advertisement. T2DM was defined using the diagnostic criteria of the World Health Organization [14]. FCH was defined as familial hyperlipidemia with a dominant inheritance pattern, elevated plasma apolipoprotein B concentrations (>1.2 g/L) and elevated fasting triglyceride levels (>1.7 mmol/L) [13]. Exclusion criteria were the presence of inflammatory disorders, a plasma C-reactive protein level above 10 mg/L and disorders of kidney, liver and thyroid function.

In order to investigate the effect of statins on leukocyte activation, a second group of subjects was selected. The study design of this statin withdrawal substudy has been published elsewhere [15].

The studies were approved by The Institutional Review Board of the Sint Franciscus Gasthuis in Rotterdam and the Regional Independent Medical Ethical Committee at the

Maasstad Hospital in Rotterdam (registered at clinicaltrials.gov under clinical trial numbers NCT02130505 and NCT0634906). All participants gave written informed consent.

Blood samples were obtained fasting, and 1 and 2 hours after ingestion of 75 grams of oral anhydrous glucose. Blood was drawn from a peripheral vein of the forearm. For leukocyte activation markers, blood samples were obtained in sodium EDTA (2 mg/mL).

All clinical chemistry measurements were performed as described previously, according to standard procedures in our laboratory [10]. The cell surface expression of CD11b and CD66b on monocytes and neutrophils was determined by flow cytometry, as described previously [10]. The fluorescent intensity of each cell was expressed as the mean fluorescent intensity (MFI), given in arbitrary units (au).

Data are given as mean ± SEM in the text, table and figure. The total area under the curve (AUC) was calculated by the trapezoidal rule using Graphpad Prism version 5.0 (LA, USA). The incremental integrated AUC (dAUC) was calculated after correction for baseline values. Differences were tested by analysis of variance (ANOVA), with Fisher's least significant difference (LSD) test as post-hoc analysis, or Chi-Square test for dichotomous variables. Correlation analysis was carried out using Spearman correlation statistics. P-values <0.05 (2-tailed) were considered statistically significant.

## RESULTS

A total of 51 subjects (20 T2DM patients, 17 FCH patients and 14 healthy volunteers) underwent an OGTT. Their baseline characteristics are listed in Table 3.1.

**Table 3.1** General characteristics, fasting biochemical markers, use of medication, and fasting and postprandial glucose and leukocyte activation markers in T2DM patients, FCH patients and healthy controls

| | T2DM (n=20) | FCH (n=17) | Controls (n=14) | P-value |
|---|---|---|---|---|
| Male/female (number) | 12/8 | 10/7 | 5/9 | 0.316 |
| Smoking, n (% smokers) | 5 (25) | 3 (18) | 0 (0) | 0.138 |
| Age (years) | 56.35 ± 1.33* | 53.88 ± 1.51 | 50.07 ± 1.16 | 0.010 |
| Body mass index (kg/m$^2$) | 31.29 ± 1.32*$^{\dagger}$ | 27.84 ± 0.74 | 26.01 ± 1.25 | 0.007 |
| Waist circumference (m) | 1.14 ± 0.04**$^{\dagger}$ | 1.02 ± 0.02 | 0.93 ± 0.04 | <0.001 |
| Systolic blood pressure (mmHg) | 128 ± 3* | 127 ± 4$^{\ddagger}$ | 115 ± 2 | 0.009 |
| Diastolic blood pressure (mmHg) | 73 ± 2 | 78 ± 2 | 73 ± 2 | 0.069 |
| HbA1c (%) | 7.93 ± 0.26**$^{\dagger\dagger}$ | 5.60 ± 0.07 | 5.26 ± 0.08 | <0.001 |
| Triglycerides (mmol/L) | 1.88 ± 0.25* | 2.65 ± 0.47$^{\ddagger}$ | 0.94 ± 0.09 | 0.003 |
| Total cholesterol (mmol/L) | 5.12 ± 0.33 | 5.44 ± 0.32 | 5.09 ± 0.16 | 0.661 |

**Table 3.1** General characteristics, fasting biochemical markers, use of medication, and fasting and post-prandial glucose and leukocyte activation markers in T2DM patients, FCH patients and healthy controls (continued)

| | T2DM (n=20) | FCH (n=17) | Controls (n=14) | P-value |
|---|---|---|---|---|
| LDL cholesterol (mmol/L) | 2.93 ± 0.25 | 3.06 ± 0.31 | 3.28 ± 0.17 | 0.624 |
| HDL cholesterol (mmol/L) | 1.22 ± 0.44 | 1.12 ± 0.06 | 1.38 ± 0.10 | 0.160 |
| Apolipoprotein B (g/L) | 1.11 ± 0.10 | 1.13 ± 0.09 | 0.94 ± 0.05 | 0.306 |
| Apolipoprotein A-I (g/L) | 1.45 ± 0.07 | 1.36 ± 0.04 | 1.47 ± 0.07 | 0.447 |
| C-reactive protein (mg/L) | 3.1 ± 0.8 | 6.0 ± 3.3 | 1.7 ± 0.3 | 0.335 |
| Leukocyte counts ($10^9$ cells/L) | 7.78 ± 0.51 | 7.29 ± 0.50 | 6.23 ± 0.41 | 0.098 |
| Neutrophil counts ($10^9$ cells/L) | 4.25 ± 0.38 | 4.19 ± 0.41 | 3.59 ± 0.29 | 0.431 |
| Monocyte counts ($10^9$ cells/L) | 0.60 ± 0.04 | 0.56 ± 0.05 | 0.52 ± 0.05 | 0.480 |
| Lymphocyte counts ($10^9$ cells/L) | 2.62 ± 0.21* | 2.32 ± 0.16 | 1.89 ± 0.14 | 0.029 |
| Use of statins, n (%) | 14 (70) | 12 (71) | 0 (0) | <0.001 |
| Use of ezetimibe, n (%) | 2 (10) | 8 (47) | 0 (0) | 0.002 |
| Use of fibrates, n (%) | 2 (10) | 3 (18) | 0 (0) | 0.259 |
| Use of acetylsalicylic acid, n (%) | 4 (20) | 5 (29) | 0 (0) | 0.096 |
| Use of beta blockers, n (%) | 7 (35) | 3 (18) | 0 (0) | 0.040 |
| Use of diuretics, n (%) | 8 (40) | 2 (12) | 0 (0) | 0.009 |
| Use of ACE-inhibitors, n (%) | 4 (20) | 0 (0) | 0 (0) | 0.035 |
| Use of angiotensin II receptor antagonists, n (%) | 8 (40) | 6 (35) | 0 (0) | 0.025 |
| Use of calcium channel antagonists, n (%) | 4 (20) | 2 (12) | 0 (0) | 0.205 |
| Glucose (mmol/L) Fasting | 9.80 ± 0.72 **†† | 5.61 ± 0.11 | 4.93 ± 0.12 | <0.001 |
| AUC | 34.01 ± 1.33**†† | 14.72 ± 0.95‡ | 9.68 ± 0.38 | <0.001 |
| dAUC | 14.19 ± 0.77**†† | 3.58 ± 0.81‡ | -0.19 ± 0.27 | <0.001 |
| Neutrophil CD66b (au) Fasting | 9.07 ± 0.61*† | 7.12 ± 0.38 | 6.67 ± 0.59 | 0.006 |
| AUC | 17.94 ± 1.22*† | 14.50 ± 0.84 | 12.88 ± 1.27 | 0.008 |
| dAUC | -0.19 ± 0.36 | 0.26 ± 0.28 | -0.47 ± 0.29 | 0.324 |
| Monocyte CD11b (au) Fasting | 40.31 ±1.64 | 38.19 ± 2.38 | 37.21 ± 2.07 | 0.540 |
| AUC | 77.61 ± 4.38* | 71.39 ± 3.47 | 61.84 ± 2.73 | 0.022 |
| dAUC | -3.02 ± 2.31* | -4.99 ± 2.46‡ | -12.59 ± 2.19 | 0.021 |
| Neutrophil CD11b (au) Fasting | 34.69 ± 2.44 | 32.65 ± 2.28 | 29.90 ± 1.67 | 0.348 |
| AUC | 69.60 ± 4.46 | 68.09 ± 4.44 | 55.93 ± 3.39 | 0.073 |
| dAUC | 0.22 ± 2.15 | 2.80 ± 1.73 | -3.87 ± 2.99 | 0.152 |

Data are given as mean ± SEM or as number (percentage). P-value for difference between groups (one-way ANOVA for continuous variables with LSD as post-hoc test and chi-square test for discrete variables).

*: P<0.05 T2DM patients vs controls; **: P<0.001 T2DM patients vs controls; †: P<0.05 T2DM patients vs FCH patients; ††: P<0.001 T2DM patients vs FCH patients; ‡: P<0.05 FCH patients vs controls; ‡‡: P<0.001 FCH patients vs controls

AUC: Area Under the Curve; dAUC: delta Area Under the Curve

T2DM patients had a higher fasting plasma glucose level than FCH patients and healthy controls. Glucose levels increased postprandially in T2DM patients. In FCH patients, glucose increased only slightly. In healthy controls, glucose levels were not different from fasting measurements at 1 and 2 hours postprandially (Figure 3.1A and Table 3.1).

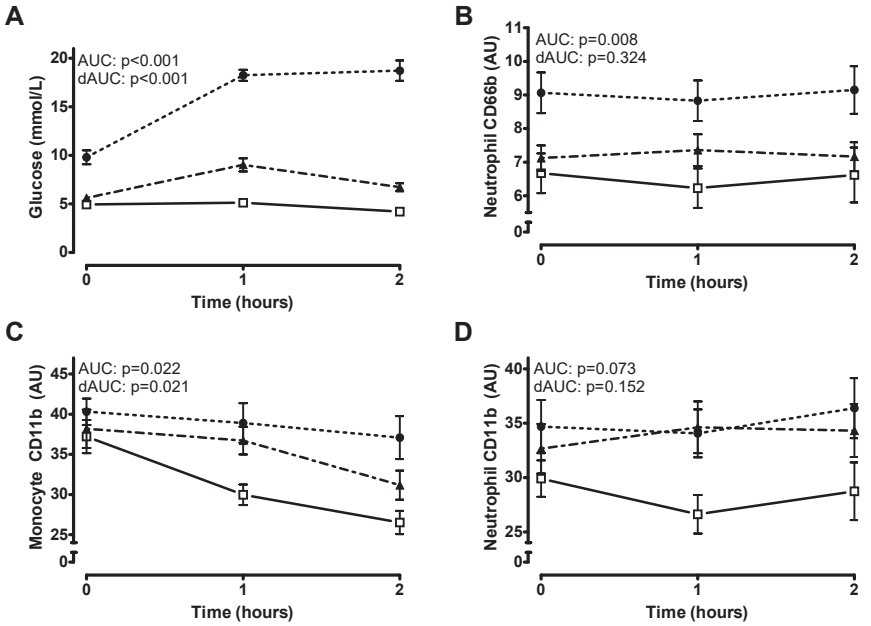


**Figure 3.1.** Glucose and leukocyte activation after OGTT in subjects with different levels of insulin resistance

Mean (error bars denote SEM) changes in glucose (A), expression of neutrophil CD66b (B), monocyte CD11b (C) and neutrophil CD11b (D) during OGTT in T2DM patients (closed circle), FCH patients (closed triangle) and healthy controls (open square).

Fasting neutrophil CD66b expression in T2DM was 36% higher than in controls and 27% higher than in FCH (p=0.006, Figure 3.1B). Fasting CD11b expression on monocytes or neutrophils was not different between the three groups (Figures 3.1C and 3.1D).

Postprandial leukocyte activation was highest in T2DM patients. Monocyte CD11b expression decreased postprandially in all groups, but the decrease was smallest in T2DM patients. In this group, monocyte CD11b-AUC was 26% higher than in controls (p=0.022, Table 3.1). Neutrophil CD66b-AUC in T2DM was 39% higher than in controls and 24% higher than in FCH (p=0.008).

For the total group, fasting neutrophil CD66b expression correlated positively with glucose-AUC (Spearman's rho: 0.481, p<0.001) and HbA1c (rho: 0.433, p=0.002). Post-

prandial changes in monocyte CD11b, reflected by the dAUC, correlated positively with glucose-AUC (rho 0.322, p=0.022) and HbA1c (rho 0.319, p=0.023). No correlations were found between fasting monocyte CD11b expression, or neutrophil CD66b-dAUC, with measurements of acute or chronic glycemia.

In a separate statin withdrawal cohort, 54 subjects were included. Their baseline characteristics have been published elsewhere [15]. The statins used were simvastatin (n=21), atorvastatin (n=6), rosuvastatin (n=22) and pravastatin (n=6). Discontinuation of statins for 6 weeks did not result in significant changes in fasting leukocyte activation. Monocyte CD11b expression was 30.53 ± 1.03 au before and 28.59 ± 0.81 au after statin withdrawal (p=0.108). Neutrophil CD11b expression was 43.81 ± 1.64 au before and 43.83 ± 1.69 au after statin withdrawal (p=0.910). Neutrophil CD66b expression was 6.14 ± 0.19 au before and 6.06 ± 0.21 au after statin withdrawal (p=0.977).

## DISCUSSION

To the best of our knowledge, this is the first study describing a correlation between acute and chronic hyperglycemia and postprandial leukocyte activation changes in subjects with a wide range of insulin sensitivity. The relevance of these observations lies in the fact that activated leukocytes contribute to the development of atherosclerosis [6] and microvascular diabetic complications [3,4].

Previous studies on the effect of glucose on leukocyte activation have shown conflicting results. In an earlier *in vivo* study by our group, baseline levels of CD11b and CD66b were higher in T2DM patients than in controls [1]. Others found higher fasting monocyte CD14 and CD18 expression in female T2DM patients compared to controls [2]. However, similar levels of leukocyte activation in T2DM patients and controls have also been reported [11].

We observed a surprisingly high fasting neutrophil CD66b expression in T2DM patients compared with healthy controls. This is in line with previous studies [1,16].

In contrast to our study, Sampson and co-workers showed a slight increase in monocyte CD11b two hours after an OGTT in T2DM patients and in controls [11]. At this point, we have no clear explanation for this discrepancy.

Our results indicate that in patients with FCH, who are known to be insulin-resistant [13], fasting and postprandial levels of leukocyte activation are intermediate, compared to T2DM patients and controls. Although there was no acute postprandial increase in the expression of activation markers, the observed correlation between postprandial glucose response and fasting as well as postprandial leukocyte activation, indicates an acute effect of glucose. In addition, the correlation between leukocyte activation and HbA1c suggests that also chronic hyperglycemia may be responsible for the increased

activation of leukocytes in patients with reduced insulin sensitivity. Therefore, acute and chronic hyperglycemia may be involved in increased fasting and postprandial leukocyte activation.

A limitation of this study is that only associations between glycemia and inflammation were studied. In order to establish a causal relationship, the effect of glucose-lowering interventions on leukocyte activation needs to be evaluated in future studies. One of the possible confounding factors may have been the use of drugs by T2DM and FCH patients. Especially statins have been proposed to have anti-inflammatory effects [17,18]. Results of our statin withdrawal cohort demonstrated that the expression of CD11b and CD66b is not affected by the use of statins. The role of ezetimibe and diuretics on leukocyte activation in humans is unknown [19]. Beta-blockers and angiotensin II AT1 receptor blockers have favorable effects on the adhesiveness of leukocytes to the endothelium [20].

In conclusion, acute and chronic hyperglycemia due to insulin resistance as seen in T2DM and FCH are associated with increased leukocyte activation.

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# Chapter 4

## *In vivo* evidence for chylomicrons as mediators of postprandial inflammation

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Atherosclerosis. 2015;243(2):540-5

## ABSTRACT

**Background:** The postprandial situation is a pro-inflammatory condition most likely linked to the development of atherosclerosis. We evaluated the relationship between apolipoprotein (apo) B48 and fasting and postprandial leukocyte activation markers.

**Methods:** Leukocyte activation markers and apo B48 were determined in 80 subjects with and without coronary artery disease (CAD). Twelve healthy subjects underwent an oral fat loading test (up to 8 hours).

**Results:** Fasting apo B48 was significantly higher in patients with CAD (n=47, 8.1 ± 5.2 mg/L) than in subjects without CAD (n=33, 5.9 ± 3.9 mg/L, p=0.022). Fasting apo B48 and triglycerides correlated positively with fasting monocyte CD11b and neutrophil CD66b expression. Plasma apo B48 and leukocyte activation markers increased after an oral fat load. Postprandial apo B48 correlated positively with postprandial monocyte CD11b (Spearman's rho: 0.615, p=0.033). No correlations were found between fasting or postprandial triglycerides and postprandial leukocyte activation markers. We observed no correlations between postprandial apo B48 and postprandial neutrophil CD11b or CD66b expression.

**Conclusion:** This study suggests that chylomicron remnants may be responsible for postprandial leukocyte activation in the circulation. The postprandial chylomicron response may be a stronger mediator of postprandial inflammation than postprandial triglyceridemia.

## INTRODUCTION



The postprandial situation is a pro-inflammatory condition most likely linked to the development of atherosclerosis. Postprandial lipemia activates circulating leukocytes, as has been demonstrated *in vitro* and *in vivo* [1–3]. Activated leukocytes can adhere to the intact endothelium and migrate to the subendothelial space, where the development of atherosclerosis is initiated [4].

Both, increased leukocyte activation and postprandial triglycerides have been linked to the presence of coronary artery disease (CAD) and peripheral artery disease [5–10]. The measurement of postprandial lipemia is time-consuming, requiring postprandial measurements during several hours after an oral fat loading test [11]. It has been suggested that fasting apolipoprotein (apo) B48 may be used as a surrogate marker for postprandial lipemia [11,12].

Diet-ingested lipids are transported to the circulation in chylomicrons. In the circulation, triglycerides present in the chylomicrons are rapidly hydrolyzed by the enzyme lipoprotein lipase, and chylomicron remnants are formed [13]. Each chylomicron carries a single apo B48 molecule on its surface [14]. Since apo B48 is synthesized exclusively in the intestine, the level of apo B48 represents the total amount of circulating chylomicrons and their remnants. Chylomicron remnants can migrate into the arterial wall, where they can induce foam cell formation without the need of prior oxidation or other modifications [15,16]. Although the infiltration rate into arterial tissue is approximately 10 times lower than that of low-density lipoprotein (LDL) particles, their efflux rate is also approximately 20 times lower [17]. Thus, chylomicron remnants are more easily retained in the subendothelial space than LDL particles. It has been postulated that chylomicron remnants are mainly involved in the early stages of atherogenesis, and fasting apo B48 levels have been associated with the development of atherosclerosis [18–21]. Carotid intima media thickness, a marker of subclinical atherosclerosis, was positively associated with fasting apo B48 [19]. In addition, fasting apo B48 was higher in patients with peripheral artery disease [20], and predicted the risk of coronary events independent of LDL cholesterol [21].

Chylomicrons may not only be directly involved in the development of atherosclerosis, but also indirectly, by stimulating inflammation following interaction with circulating leukocytes [1,2]. The aim of this study was to evaluate the relationship between apo B48 and leukocyte activation markers in the fasting and the postprandial situation.

## MATERIALS AND METHODS

### Participants

In order to assess the relationship between fasting apo B48 and markers of leukocyte activation, subjects who were scheduled to undergo a diagnostic coronary angiography were included. The design of this case-control study has been described extensively elsewhere [22]. Exclusion criteria were the presence of inflammatory disorders, plasma C-reactive protein above 10 mg/L, and disorders of kidney, liver and thyroid function. To investigate the role of postprandial apo B48 on leukocyte activation, a second group of healthy volunteers underwent an oral fat loading test. The design of this postprandial study has been described elsewhere [23]. The Institutional Review Board of the Sint Franciscus Gasthuis Rotterdam and the regional independent medical ethics committee of the Maasstad Hospital Rotterdam approved both studies. All participants gave written informed consent.

### Study design

For the case-control study, anthropometric measures, the use of medication and cardiovascular history were recorded on the day of the angiography. Shortly before coronary angiography, fasting venous blood was obtained from a peripheral vein of the forearm. Blood samples were collected in tubes containing EDTA (1 mg/mL) and kept on ice until processed for determination of leukocyte activation markers. Coronary angiography images were scored by an independent cardiologist.

For the postprandial study, volunteers visited the hospital after an overnight fast. A fasting venous blood sample was drawn. After venipuncture, volunteers ingested fresh cream in a concentration of 50 grams of fat per square meter body surface. Blood samples were obtained at 2-hourly intervals, for up to 8 hours after fat ingestion.

### Analytical methods

Parameters for glucose, C-reactive protein, total cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides were determined using Synchron LX-20 analyzers (Beckman Coulter, Brea, CA, USA) according to standard procedures in our laboratory. LDL cholesterol values were calculated using the Friedewald formula. Apo A-I and B were determined by rate nephelometry using an IMMAGE analyzer (Beckman Coulter). Blood cell counts were determined using LH750 analyzers (Beckman Coulter, Miami, FL, USA). The leukocyte differentiation was determined as a five-part differentiation on the same instruments. Apo B48 was measured using a high-sensitivity commercial ELISA (Shibayagi Co Ltd, Ishihara, Japan) [24]. Two plates with 4 replicates per plate of a low value internal quality serum control (mean 2.96 mg/L) were run. The mean intra-assay

coefficient of variation (CV) at this low concentration was 8.7%, the total CV for both assays was 10.3%. The inter-assay CV was (Geometric substract) 5.5%.

### Leukocyte activation markers



Blood samples for the measurement of leukocyte activation markers were collected in EDTA and were determined by flow cytometry on the same day. The expression of leukocyte activation markers on the cell surface was determined using fluorescent labeled monoclonal antibodies (Beckman Coulter). Antibodies against CD66b were labeled with fluorescein isothiocynate (FITC). Antibodies against CD11b were labeled with phycoerythrin (PE). Antibodies against CD45 labeled with PE-Texas Red (ECD) were used to differentiate leukocytes from erythrocytes and platelets. Whole blood was added to a combination of CD66b-FITC, CD11b-PE and CD45-ECD antibodies. Cells were incubated for 15 minutes in the dark at room temperature. In parallel, blood was incubated with FITC- and PE-conjugated mouse IgG1 as isotype control to correct for nonspecific binding. Erythrocytes were lysed by adding ice-cold isotonic erythrocyte lysing solution (NH4Cl 0.19 M; KHCO3 0.01 M; Na2EDTA.2H2O 0.12 M, pH 7.2) for 15 minutes. For the cross-sectional study, a Coulter Epics XL-MCL flow cytometer with a 488 nm Argon ion laser and EXPO 32 software were used for measurement and analysis. For the postprandial study, a Navios flow cytometer (Beckman Coulter) was used for measurement and Kaluza software version 1.2 (Beckman Coulter) was used for analysis. The fluorescent intensity of each cell was expressed as the mean fluorescent intensity, given in arbitrary units (au). Lymphocytes, monocytes and granulocytes were identified based on their side scatter and the level of CD45 on their cell surface.

### Statistical analysis

Data are given as mean ± SD in the text, tables and figures. Baseline differences between the groups were tested with Independent Samples T Test for normally distributed continuous variables and with Mann-Whitney U Test for continuous variables with skewed distributions (apo B48, triglycerides, monocyte CD11b, neutrophil CD11b and CD66b). Differences between fasting and postprandial values were tested with Wilcoxon Signed Rank Test. Correlation analysis was carried out using Spearman correlation statistics. The total area under the curve (AUC) and the AUC corrected for baseline values (dAUC) were calculated by the trapezoidal rule using Graphpad Prism version 5.0 (LA, USA). Statistical analysis was carried out with PASW statistics version 22.0 (IBM SPSS Statistics, New York, United States). P-values <0.05 (2-tailed) were considered statistically significant.

## RESULTS

### Baseline characteristics

A total of 80 patients participated in the cross-sectional study. Their baseline characteristics are listed in Table 4.1. The CAD group consisted of older patients and included more males. They had higher body mass index, systolic blood pressure and fasting triglycerides. Plasma apo A-I, LDL cholesterol and HDL cholesterol were lower than in subjects without CAD. Fasting apo B48 was significantly higher in patients with CAD (8.1 ± 5.2 mg/L) than in subjects without CAD (5.9 ± 3.9 mg/L, p=0.022). Data suggested a trend for higher expression of leukocyte activation markers in patients with CAD. The use of medication by all subjects, and of those with and without CAD separately, are

**Table 4.1** Baseline characteristics of participants (n=80) in the cross-sectional study

| | Total group (n=80) | CAD+ (n=47) | CAD- (n=33) | P-value |
|---|---|---|---|---|
| Age (years) | 60 ± 15 | 66 ± 11 | 51 ± 16 | <0.001 |
| Male gender, n (%) | 46 (58%) | 33 (70%) | 13 (40%) | 0.011 |
| Smoking, n (%) | 12 (15%) | 5 (11%) | 7 (21%) | 0.218 |
| Diabetes mellitus, n (%) | 18 (23%) | 14 (30%) | 4 (12%) | 0.101 |
| Body mass index (kg/m$^2$) | 26.8 ± 4.8 | 27.8 ± 4.2 | 25.4 ± 5.3 | 0.033 |
| Waist circumference (meters) | 1.07 ± 0.12 | 1.09 ± 0.11 | 1.00 ± 0.13 | 0.055 |
| Carotid intima media thickness (mm) | 0.719 ± 0.108 | 0.728 ± 0.100 | 0.690 ± 0.134 | 0.466 |
| Systolic blood pressure (mmHg) | 139 ± 23 | 147 ± 22 | 127 ± 21 | <0.001 |
| Diastolic blood pressure (mmHg) | 79 ± 12 | 80 ± 9 | 78 ± 16 | 0.636 |
| Glucose (mmol/L) | 6.4 ± 1.8 | 6.6 ± 1.7 | 6.1 ± 1.9 | 0.164 |
| Apolipoprotein A-I (g/L) | 1.47 ± 0.37 | 1.34 ± 0.33 | 1.66 ± 0.34 | <0.001 |
| Apolipoprotein B (g/L) | 0.94 ± 0.29 | 0.93 ± 0.28 | 0.96 ± 0.30 | 0.620 |
| Apolipoprotein B48 (mg/L) | 7.2 ± 4.8 | 8.1 ± 5.2 | 5.9 ± 3.9 | 0.022 |
| Triglycerides (mmol/L) | 1.58 ± 1.07 | 1.86 ± 1.19 | 1.18 ± 0.69 | 0.001 |
| Total cholesterol (mmol/L) | 4.8 ± 1.1 | 4.7 ± 1.1 | 5.1 ± 1.0 | 0.087 |
| HDL cholesterol (mmol/L) | 1.3 ± 0.4 | 1.2 ± 0.3 | 1.5 ± 0.4 | 0.002 |
| LDL cholesterol (mmol/L) | 2.8 ± 1.0 | 2.6 ± 1.0 | 3.1 ± 0.8 | 0.020 |
| C-reactive protein (mg/L) | 2 ± 2 | 3 ± 2 | 2 ± 2 | 0.555 |
| Complement C3 (g/L) | 1.20 ± 0.25 | 1.23 ± 0.21 | 1.17 ± 0.30 | 0.293 |
| Leukocyte count (10$^9$ cells/L) | 7.0 ± 1.8 | 7.1 ± 1.7 | 6.8 ± 1.9 | 0.430 |
| Lymphocyte count (10$^9$ cells/L) | 1.9 ± 0.6 | 2.0 ± 0.6 | 1.9 ± 0.6 | 0.355 |
| Monocyte count (10$^9$ cells/L) | 0.6 ± 0.2 | 0.6 ± 0.2 | 0.5 ± 0.2 | 0.057 |
| Neutrophil count (10$^9$ cells/L) | 4.3 ± 1.4 | 4.3 ± 1.3 | 4.3 ± 1.6 | 0.942 |
| Monocyte CD11b (au) | 30.5 ± 11.8 | 32.1 ± 9.6 | 28.2 ± 14.3 | 0.054 |
| Neutrophil CD11b (au) | 30.2 ± 11.3 | 31.5 ± 11.5 | 28.3 ± 10.7 | 0.263 |
| Neutrophil CD66b (au) | 6.59 ± 2.63 | 7.44 ± 2.44 | 5.39 ± 2.46 | <0.001 |

listed in Table 4.2. The use of statins, acetylsalicylic acid and calcium channel antagonists was significantly higher in the CAD group.

**Table 4.2** Use of medication by participants (n=80) in the cross-sectional study

| | Total group (n=80) | CAD+ (n=47) | CAD- (n=33) | P-value |
|---|---|---|---|---|
| Use of statins, n (%) | 47 (59%) | 38 (81%) | 9 (27%) | <0.001 |
| Use of acetylsalicylic acid, n (%) | 43 (59%) | 36 (77%) | 7 (21%) | <0.001 |
| Use of beta blockers, n (%) | 37 (46%) | 26 (55%) | 11 (33%) | 0.069 |
| Use of diuretics, n (%) | 23 (29%) | 15 (32%) | 8 (24%) | 0.616 |
| Use of ACE-inhibitors, n (%) | 22 (28%) | 13 (28%) | 9 (27%) | 1.000 |
| Use of angiotensin II receptor antagonists, n (%) | 16 (20%) | 12 (26%) | 4 (12%) | 0.166 |
| Use of calcium channel antagonists, n (%) | 22 (28%) | 20 (36%) | 5 (13%) | 0.011 |

## Fasting apo B48 and triglycerides in relation to fasting leukocyte activation (Figure 4.1)

There was a strong positive correlation between fasting apo B48 and fasting triglycerides (Spearman rho: 0.708, p<0.001). Weak but significant positive correlations were found between fasting apo B48 and fasting monocyte CD11b expression (rho: 0.261, p=0.019, Figure 4.1A), and between fasting apo B48 and fasting neutrophil CD66b expression (rho:

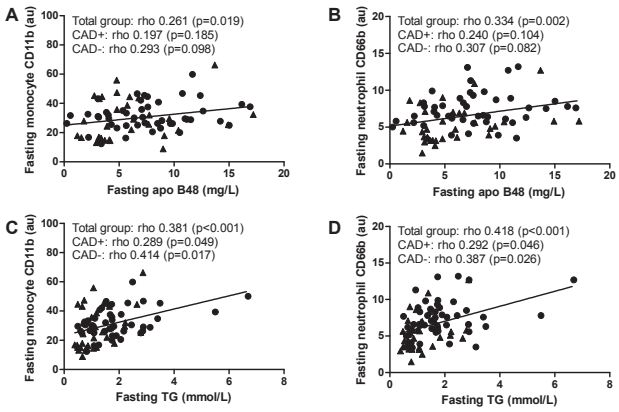


**Figure 4.1.** Fasting apo B48 and triglycerides in relation to fasting leukocyte activation

Correlation graphs between fasting lipid parameters and fasting leukocyte activation in patients with CAD (n=47, circles) and patients without CAD (n=33, triangles). Fasting apo B48 in relation to fasting monocyte CD11b expression (A). Fasting apo B48 in relation to fasting neutrophil CD66b expression (B). Fasting triglycerides in relation to fasting monocyte CD11b expression (C). Fasting triglycerides in relation to fasting neutrophil CD66b expression (D).

0.334, p=0.002, Figure 4.1B). Triglycerides correlated positively with fasting monocyte CD11b (rho: 0.381, p<0.001, Figure 4.1C) and neutrophil CD66b expression (rho: 0.418, p<0.001, Figure 4.1D). After exclusion of two outliers with triglycerides >5 mmol/L, the associations with monocyte CD11b and neutrophil CD66b remained significant (rho: 0.347, p=0.002 and rho: 0.389, p<0.001, respectively). When CAD+ and CAD- subjects were analyzed separately, the association between fasting triglycerides and leukocyte activation remained significant in both groups. For apo B48, significance was lost in the CAD+ group, while there was a trend for an association in the CAD- group. Plasma total apo B correlated positively with fasting neutrophil CD66b (rho: 0.243, p=0.030) but not with monocyte CD11b expression (data not shown). No correlations were found between these leukocyte activation markers and LDL cholesterol.

**Fasting and postprandial apo B48 and triglycerides in relation to postprandial leukocyte activation (Figures 4.2 and 4.3)**

Twelve healthy volunteers participated in the postprandial study. Plasma apo B48 increased after an oral fat load, with the maximal increase after 2 hours, from 3.6 ± 1.9 mg/L to 6.4 ± 3.1 mg/L (p=0.002, Figure 4.2A). Plasma triglycerides increased from 0.71 ± 0.24 mmol/L to 1.62 ± 0.70 mmol/L after 4 hours (p=0.002, Figure 4.2B). Monocyte CD11b expression tended to increase after 4 hours, from 14.8 ± 2.3 au to 16.5 ± 2.2 au (p=0.06, Figure 4.2C). Neutrophil CD66b expression increased after 4 hours, from 3.63 ± 1.10 au to 4.16 ± 1.22 au (p=0.019, Figure 4.2D).

Fasting apo B48 and the total postprandial apo B48 area under the curve correlated positively with postprandial monocyte CD11b area under the curve corrected for baseline values (rho: 0.595, p=0.041 and rho: 0.615, p=0.033, respectively, Figures 4.3A and 4.3B). The correlation between fasting apo B48 and postprandial monocyte CD11b was lost after exclusion of one outlier with high fasting apo B48 (rho: 0.474, p=0.121). No correlations were found between fasting or postprandial apo B48 and neutrophil CD11b or CD66b (data not shown). No correlations were found between fasting or postprandial triglycerides and postprandial leukocyte activation markers (Figures 4.3C and 4.3D).

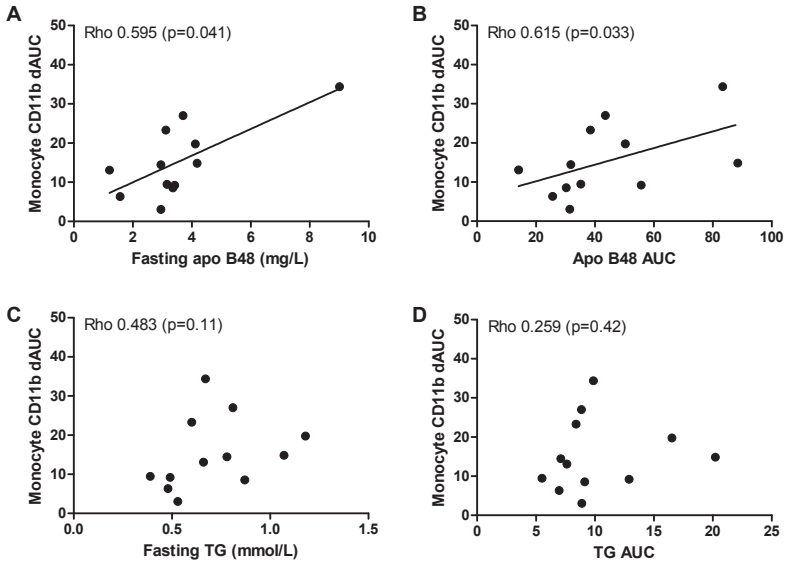


**Figure 4.3.** Fasting and postprandial apo B48 and triglycerides in relation to postprandial leukocyte activation

Fasting apo B48 in relation to postprandial monocyte CD11b (A). Postprandial apo B48 in relation to postprandial monocyte CD11b (B). Fasting triglycerides in relation to postprandial monocyte CD11b (C). Postprandial triglycerides in relation to postprandial monocyte CD11b (D).

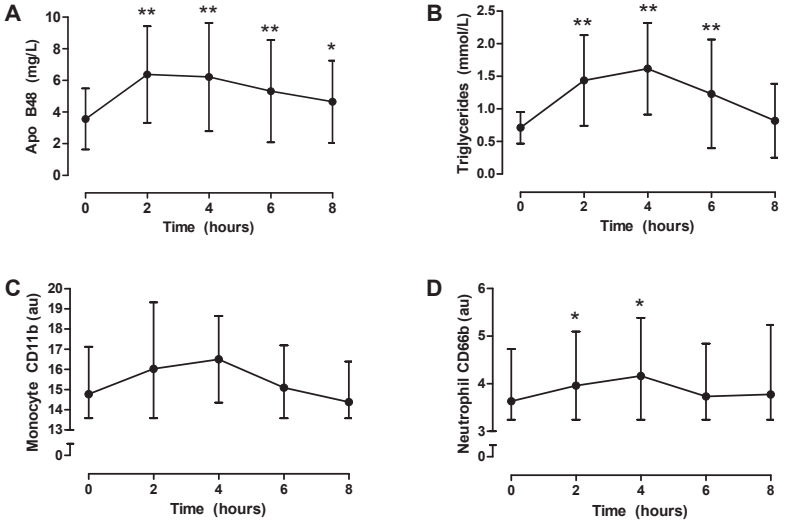


**Figure 4.2.** Postprandial response during an oral fat loading test in 12 healthy volunteers

Postprandial apo B48 (A), postprandial triglycerides (B), postprandial monocyte CD11b (C) and postprandial neutrophil CD66b (D). Data are given as mean ± SD. * p<0.05 compared to t=0; ** p<0.01 compared to t=0

## DISCUSSION

To the best of our knowledge, this is the first study describing a relationship between chylomicron remnants and postprandial leukocyte activation. Interestingly, this relationship was not found for plasma triglycerides. These data suggest that postprandial chylomicrons and their remnants may induce leukocyte activation in the circulation. The importance of these observations lies in the fact that both leukocyte activation and postprandial lipemia have been linked to the presence and the development of atherosclerosis [6,8–11,25–27].

In the present study, fasting apo B48 and triglycerides were positively associated with fasting leukocyte activation. The observed correlations were stronger for fasting triglycerides than for apo B48. We have previously demonstrated *ex vivo* that incubation of human monocytes in lipid emulsions in the hypertriglyceridemic range resulted in an increased expression of CD11b on monocytes and neutrophils [1]. Collectively, these data suggest that in the fasting situation and *ex vivo*, both the total triglyceride concentration and chylomicrons and their remnants may induce leukocyte activation. It should be noted however, that the association between fasting apo B48 and leukocyte activation was weakened when CAD+ and CAD- patients were analyzed separately, especially in the CAD+ group. This could be due to the relatively small number of subjects included, but it could also indicate that in patients with CAD, other factors than chylomicron remnants may be involved in fasting leukocyte activation, such as CRP. Although CRP levels did not significantly differ between both groups, we did not measure hsCRP.

Several studies have shown that postprandial lipemia induces leukocyte activation [1–3]. *In vivo*, ingestion of an oral fat load by healthy volunteers resulted in increased expression of several activation markers on monocytes and neutrophils [1–3,23]. Our data suggest that this postprandial leukocyte activation in the circulation may not per se be the result of increased triglycerides postprandially, but rather an effect of circulating chylomicrons and their remnants. According to current concepts, chylomicron remnants induce atherosclerosis by migrating to the subendothelial space, where they induce leukocyte activation and foam cell formation [16]. We propose that in addition, chylomicron remnants may contribute to atherogenesis by activating leukocytes in the circulation. Thus chylomicron remnants may facilitate the adhesion of these activated leukocytes to the intact endothelium. This theory is supported by an *in vitro* study, in which chylomicron remnant-like particles induced monocyte activation and enhanced monocyte migration towards the chemokine monocyte chemoattractant protein-1 [28]. Furthermore, also postprandial neutrophils show increased migration to chemokines [29]. It should be noted that the observed correlation between fasting apo B48 and postprandial monocyte CD11b expression seemed to be depending on one outlier. However,

the correlation between postprandial monocyte CD11b with postprandial apo B48 was stronger than with fasting apo B48, and this is consistent with our hypothesis.

The observed association between leukocyte activation and postprandial chylomicrons may be the result of binding and uptake of these lipoproteins by leukocytes via the apo B48-receptor. A postprandial study in healthy volunteers has demonstrated that circulating monocytes accumulate postprandial lipids, while simultaneously apo B48-receptor and CD11b mRNA expression increased [30]. This apo B48-receptor and CD11b transcriptional activity decreased in the late postprandial phase, paralleling the decrease of apo B48-containing lipoproteins [30]. Another candidate receptor for the binding and uptake of chylomicrons is the LDL related protein-1. It has been shown that monocytes take up triglyceride-rich lipoproteins via this receptor, resulting in increased CD11b expression [2]. In addition, we have recently demonstrated that the complement receptor 1 may be involved in the binding of lipoproteins by circulating leukocytes [31].

A possible confounding factor in our study may have been the higher use of statins by CAD patients, since it has been suggested that statins may have anti-inflammatory effects [32,33]. We have previously investigated the effect of statin use on fasting leukocyte activation. Six weeks of statin discontinuation in 54 hyperlipidemic subjects did not result in significant changes in fasting expression of monocyte CD11b, neutrophil CD11b or neutrophil CD66b [34]. Moreover, we have previously shown that the postprandial changes of these markers are not influenced by statin use in patients with CAD and combined hyperlipidemia [29]. Therefore, we believe that statin use did not influence the results of the current study.

Whether the observed relationship between postprandial chylomicron remnants and leukocyte activation in healthy subjects is relevant in CAD patients, which are known to have increased postprandial lipemia [11,27], remains to be investigated. Although the pattern of postprandial expression of leukocyte activation markers in CAD patients [29] does not seem to be very different from that observed in healthy controls [23], no studies have directly compared the postprandial inflammatory response in patients with and without CAD. Another limitation is that this is a clinical study, and that the described association between chylomicron remnants and inflammation does not necessarily demonstrate causality. Further mechanistic studies are needed in order to investigate the causality of the association.

In conclusion, our *in vivo* study suggests that chylomicron remnants may be responsible for postprandial leukocyte activation in the circulation. The postprandial chylomicron response may be a stronger mediator of postprandial inflammation than postprandial triglyceridemia.

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# Chapter 5

## Effect of a single dose vitamin D3 on postprandial arterial stiffness and inflammation in vitamin D deficient women: a double-blind randomized controlled trial

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J Clin Endocrinol Metab. 2016:jc20163394

## ABSTRACT

**Context:** Cholecalciferol (vitamin D3) may improve vascular function and reduce inflammation, potentially providing an explanation for the proposed cardiovascular protection by vitamin D.

**Objective:** We investigated whether cholecalciferol supplementation reduces postprandial arterial dysfunction and inflammation.

**Design:** This was a randomized 1:1, double blind trial.

**Setting:** The study was conducted in the Diabetes and Vascular Center of the Franciscus Gasthuis, Rotterdam, The Netherlands.

**Patients:** Twenty-four healthy premenopausal, overweight or obese, vitamin D deficient women.

**Interventions:** A single high (300 000 IU) or low dose (75 000 IU) cholecalciferol.

**Main outcome measures:** The effect of low and high dose cholecalciferol on postprandial leukocyte activation markers, pulse wave velocity (PWV) and augmentation index (AIx) during an oral fat loading test, expressed as area under the curve (AUC).

**Results:** High and low dose supplementation increased vitamin D by 163 ± 134%, $p<0.001$ and 66 ± 59%, $p<0.001$, respectively. Monocyte CD11b-AUC slightly increased after low but not high dose (+6 ± 2%, $p=0.012$ and +4 ± 1%, $p=0.339$ respectively). There were no significant effects on postprandial PWV or AIx by high or low dose vitamin D. Fasting complement component 3 (C3) levels decreased by 5.9% ($p=0.004$) in the high dose, and by 4.0% ($p=0.018$) in the low dose group.

**Conclusions:** A single dose of vitamin D does not seem to reduce arterial stiffness and leukocyte activation in overweight, vitamin D deficient women. Fasting C3 may decrease by vitamin D. Possibly, higher vitamin D concentrations may be needed to decrease inflammation and improve vascular function in overweight or obese vitamin D deficient women.

## INTRODUCTION

Postprandial lipemia, associated with acute inflammatory changes [1], negatively affects endothelial function [2] and potentially promotes atherosclerosis [3]. Postprandial lipemia is characterized by the secretion of intestinal chylomicrons and hepatic very low-density lipoproteins (VLDL) into the circulation. Chylomicrons transport dietary fat-soluble vitamins (A, D, E and K) to tissues in the body. Recently, vitamin D has received major interest in relation to cardiovascular disease, and vitamin D deficiency has been linked to increased cardiovascular mortality [4]. The proposed effects of vitamin D are numerous, and include modulation of vascular smooth muscle cell proliferation, cytokine production and monocyte and macrophage function [5]. Vitamin D supplementation has been associated with improvement of endothelial function [6], decreased cytokine production [7] and reduction of oxidative stress [8].

Leukocyte activation has been linked to atherosclerosis [9]. Activated neutrophils and monocytes express integrins, such as CD11b and CD66b on their cell surface, facilitating the binding of these leukocytes to the intact endothelium [10]. Arterial stiffness is associated with atherogenesis and reflects vascular health [11]. In a recent pilot study by our group, a single cholecalciferol dose of 100 000 IU reduced arterial stiffness and postprandial leukocyte activation in healthy, lean subjects [12]. Interestingly, the effects of cholecalciferol on leukocyte activation were solely observed in the females with relatively high vitamin D levels at baseline, although vascular function also improved in males.

The aim of the current double-blind randomized study was to further explore the effect of different doses of vitamin D3 on postprandial inflammation and arterial stiffness, in order to enhance our insight into the mechanisms behind the association between vitamin D deficiency and atherosclerosis. We hypothesized that vitamin D3 may reduce postprandial inflammation in premenopausal women and therefore improve arterial elasticity.

## MATERIALS AND METHODS

### Subjects and study design

The study was designed as a 1:1 randomized, double-blind trial comparing high (300 000 IU) and low (75 000 IU) dose vitamin D3 supplementation. The Institutional Review Board of the Franciscus Gasthuis Rotterdam and the regional independent medical ethics committee of the Maasstad Hospital Rotterdam approved the study. All participants gave written informed consent. The study was registered at Clinicaltrials.gov under clinical trial number NCT01967459.

Healthy overweight or obese women, who are known to have low vitamin D levels, were recruited by advertisement. Inclusion criteria were an age above 18 years, a premenopausal status, a body mass index (BMI) of 25 kg/m$^2$ or above and vitamin D deficiency, defined by a 25-hydroxyvitamin D (25OHD) below 50 nmol/L. Exclusion criteria were the use of any kind of medication except for oral contraceptives, smoking, pregnancy, participation in a clinical study less than 6 months before inclusion and the use of vitamin supplements.

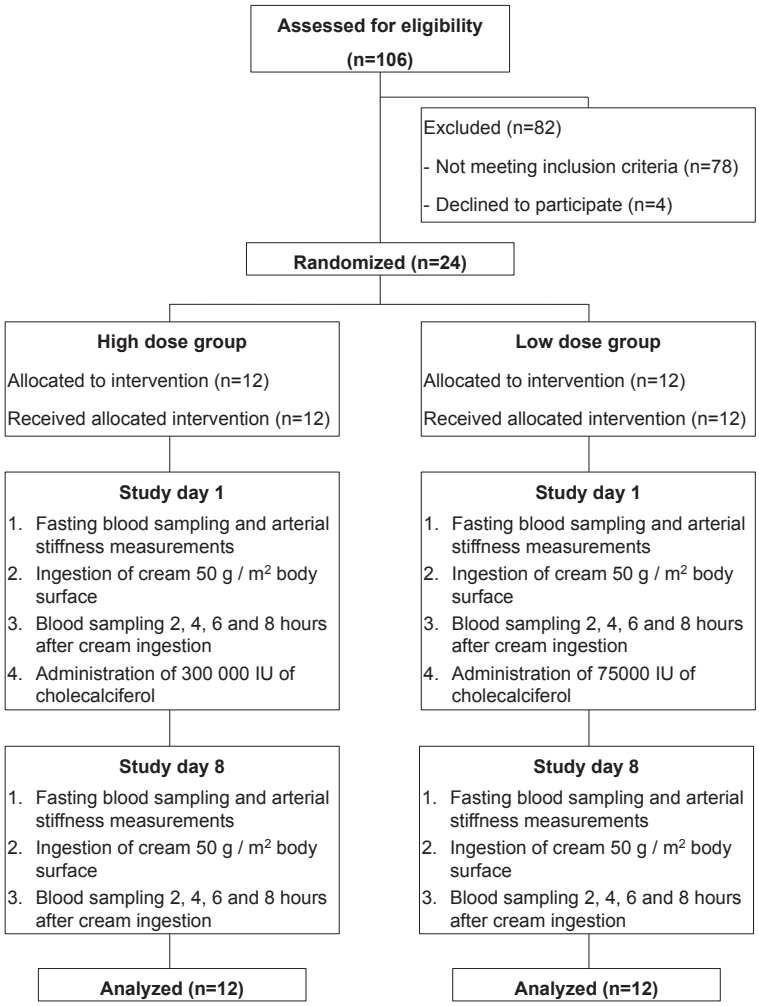


**Figure 5.1.** Study flowchart

Volunteers interested in study participation were screened for eligibility. During screening, anthropometric parameters and the medical and family history were recorded. The level of 25OHD was determined in a nonfasting blood sample.

All subjects visited the hospital after a 10-hour overnight fast. A fasting blood sample was obtained and baseline arterial stiffness measurements were performed after 5 to 10 minutes of rest. Fresh cream was ingested, in a concentration of 50 grams of fat per square meter body surface. Blood samples and arterial stiffness measurements were obtained at 2-hourly intervals, for up to 8 hours after fat ingestion. During this time, subjects were asked to rest, and they were not allowed to eat or drink, except for water. At the end of the first oral fat loading test (OFLT), volunteers received a single high or low dose of cholecalciferol. The OFLT was repeated after 7 days. After the second OFLT, no cholecalciferol was administered. An overview of the study design is given in Figure 5.1.

### Outcome measures

The endpoint of the study was the differential effect of 75 000 IU low dose versus 300 000 IU high dose vitamin D3 on postprandial leukocyte activation markers CD11b (primary endpoint), CD66b, CD35 and CD36, and vascular function, measured by pulse wave velocity (PWV) and augmentation index (AIx) (secondary endpoints).

### Pulse wave velocity (PWV) and augmentation index (AIx)

PWV and AIx were measured and calculated with the SphygmoCor Electronics Module MM3 (AtCor Medical, West Ryde, Australia) and SphygmoCor CvMS Software Suite version 8.0 (AtCor Medical, West Ryde, Australia). PWV is considered the gold standard to measure arterial stiffness as a marker for endothelial function [11]. The velocity of the pulse wave increases when the aortic stiffness increases. The PWV was measured using a noninvasive tonometry at the carotid and femoral artery. The distance between the carotid and femoral artery was measured with a measuring tape. The AIx, adjusted to a heart rate of 75 beats per minute (AIx@75), represents both macrovascular and microvascular function. The radial artery was used to measure the AIx. Both the PWV and AIx were measured in duplicate at each time interval.

### Analytical methods

Laboratory measurements were carried out according to standard procedures in our laboratory [13–15]. Parameters for glucose, C-reactive protein, total cholesterol, high-density lipoprotein (HDL)-cholesterol, triglycerides and PTH were determined using Synchron DxC analyzers (Beckman Coulter, Brea, CA, USA). LDL-cholesterol values were calculated using the Friedewald formula. Apolipoproteins (apo) AI and B were determined by rate nephelometry using an IMMAGE analyzer (Beckman Coulter). Blood cell counts were

determined using DxH800 analyzers (Beckman Coulter). The leukocyte differentiation was determined as a five-part differentiation on the same instruments. Vitamin D status was determined by measuring 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3 on serum, using a direct competitive chemiluminescence immunoassay (Liaison, DiaSorin, Saluggia, Italy). Serum complement component 3 (C3) was measured by nephelometry using an IMMAGE instrument (Beckman Coulter).

Blood samples for the measurement of leukocyte activation markers were collected in EDTA, and the flow cytometric measurement protocol was commenced within 30 minutes, as described previously [15]. The expression of leukocyte activation markers on the cell surface was determined using fluorescent labeled monoclonal antibodies (Beckman Coulter). Antibodies against CD66b and CD36 were labeled with fluorescein isothiocynate (FITC). Antibodies against CD11b and CD35 were labeled with phycoerythrin (PE). Antibodies against CD45 labeled with PE-Texas Red (ECD) were used to differentiate leukocytes from erythrocytes and platelets. Whole blood was added to a combination of CD66b-FITC, CD11b-PE and CD45-ECD antibodies, or CD36-FITC, CD35-PE and CD45-ECD antibodies. Cells were incubated for 15 minutes in the dark at room temperature. In parallel, blood was incubated with FITC- and PE-conjugated mouse IgG1 as isotype control to correct for nonspecific binding. Erythrocytes were lysed by adding isotonic erythrocyte lysing solution (NH4Cl 0.19 M; KHCO3 0.01 M; Na2EDTA.2H2O 0.12 M, pH 7.2) for 15 minutes. A Navios flow cytometer (Beckman Coulter) was used for measurement and Kaluza software version 1.3 (Beckman Coulter) was used for analysis. Lymphocytes, monocytes and neutrophil granulocytes were identified based on their side scatter and the level of CD45 on their cell surface. The fluorescent intensity of each cell was expressed as the mean fluorescent intensity in arbitrary units (au).

### Sample size calculation

Sample size calculation was based on the results of a pilot study performed by our group [14]. In the female participants of that study, postprandial monocyte CD11b expression was 132.3 ± 6.2 MFI*h before and 118.4 ± 14.2 MFI*h after receiving cholecalciferol in a dose of 100 000 IU. To demonstrate a difference of 13.85 ± 9.52 MFI*h in monocyte CD11b expression between subjects receiving low and high dose cholecalciferol, at least 2 x 11 subjects had to be included (type I error 0.05, two sided, type II error 0.20, randomization ratio 1:1). We included 2 x 12 subjects to adjust for possible dropout.

### Statistics

Data are given as mean ± standard deviation for normally distributed variables, and as median (interquartile range) for variables with skewed distribution (CRP, C3 and PWV). Differences within groups before and after vitamin D3 supplementation, for the high and low dose group separately and for pooled data for both groups, were determined by

paired Student's t-test or Wilcoxon matched pairs signed ranks test, where appropriate. Differences between groups were tested by independent Student's t-test or Mann-Whitney U test, where appropriate. The total area under the curve (AUC) was calculated by the trapezoidal rule using Graphpad Prism version 5.0 (LA, USA). Statistical analysis was carried out with PASW statistics version 22.0 (IBM SPSS Statistics, New York, United States). P-values <0.05 (2-tailed) were considered statistically significant.



## RESULTS

### Baseline characteristics

A total of 106 women responded to the advertisement. At first screening, 62 did not meet the inclusion criteria, due to use of medication, established vitamin D sufficiency or comorbidity. The remaining 44 women were invited for screening. Fifteen women were not vitamin D deficient and were therefore excluded. Four women decided not to participate in the study, and one was excluded due to a BMI below 25. The remaining 24 women were included and randomized to the high dose (n=12) or the low dose vitamin D3 group (n=12). An overview of participant recruitment and randomization is given in Figure 5.1. The baseline characteristics of the study participants, for the total group and for the high and low dose groups separately, are listed in Table 5.1. The groups were comparable in baseline characteristics, except for a higher leukocyte count in the high dose vitamin D3 group, due to a higher neutrophil count.

**Table 5.1** Baseline characteristics of study participants (n=24)

| | Total (n=24) | High dose (n=12) | Low dose (n=12) | P-value |
|---|---|---|---|---|
| Age (years) | 28 ± 9 | 27 ± 7 | 29 ± 10 | 0.60 |
| BMI (kg/m$^2$) | 32.1 ± 4.0 | 33.0 ± 3.5 | 31.2 ± 4.4 | 0.28 |
| SBP (mmHg) | 119 ± 12 | 116 ± 13 | 121 ± 10 | 0.28 |
| DPB (mmHg) | 74 ± 9 | 73 ± 10 | 74 ± 9 | 0.70 |
| Waist (cm) | 99 ± 11 | 101 ± 9 | 98 ± 13 | 0.50 |
| PWV (m/s) | 6.6 (6.0-7.3) | 6.7 (6.1-7.3) | 6.1 (6.0-7.3) | 0.95 |
| AIx (%) | 5.0 ± 12.4 | 5.8 ± 9.8 | 4.2 ± 15.0 | 0.76 |
| Glucose (mmol/l) | 5.2 ± 0.4 | 5.2 ± 0.4 | 5.2 ± 0.5 | >0.99 |
| 25OHD (nmol/l) | 27.1 ± 13.8 | 26.8 ± 12.4 | 27.3 ± 15.6 | 0.94 |
| PTH (pmol/l) | 6.2 ± 2.8 | 6.2 ± 2.8 | 6.2 ± 2.9 | 0.98 |
| TC (mmol/l) | 5.2 ± 0.9 | 5.3 ± 1.2 | 5.1 ± 0.7 | 0.61 |
| HDL-C (mmol/l) | 1.4 ± 0.4 | 1.4 ± 0.4 | 1.5 ± 0.3 | 0.24 |
| LDL-C (mmol/l) | 3.2 ± 0.9 | 3.4 ± 0.9 | 3.1 ± 0.8 | 0.47 |

**Table 5.1** Baseline characteristics of study participants (n=24) (continued)

| | Total (n=24) | High dose (n=12) | Low dose (n=12) | P-value |
|---|---|---|---|---|
| TG (mmol/l) | 1.00 (0.88-1.44) | 1.25 (0.91-1.84) | 0.99 (0.66-1.09) | 0.090 |
| Apo A-I (g/l) | 1.60± 0.32 | 1.61 ± 0.36 | 1.60 ± 0.30 | 0.95 |
| Apo B (g/l) | 1.14 ± 0.26 | 1.17 ± 0.28 | 1.12 ± 0.26 | 0.68 |
| CRP (mg/l) | 4.0 (2.0-7.8) | 6.0 (3.0-8.0) | 3.0 (2.0-6.3) | 0.21 |
| C3 (g/l) | 1.30 (1.16-1.47) | 1.35 (1.26-1.53) | 1.17 (1.09-1.36) | 0.15 |
| Leuko count ($10^9$/l) | 7.3 ± 1.8 | 8.1 ± 2.0 | 6.6 ± 1.2 | 0.031 |
| Mono count ($10^9$/l) | 0.5 ± 0.2 | 0.5 ± 0.1 | 0.5 ± 0.2 | 0.72 |
| Neutro count ($10^9$/l) | 3.9 ± 1.3 | 4.4 ± 1.4 | 3.4 ± 0.9 | 0.040 |

Data are given as mean ± standard deviation or median (interquartile range). BMI: body mass index; SBP: systolic blood pressure; DPB: diastolic blood pressure; AIx: augmentation index; PWV: pulse wave velocity; 25OHD: 25-hydroxyvitamin D; PTH: parathyroid hormone; TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; TG: triglycerides; Apo: apolipoprotein; CRP: C-reactive protein; C3: complement component 3; leuko count: leukocyte count; mono count: monocyte count; neutro count: neutrophil count.
P-value for difference between high and low dose vitamin D3 group.

### Effect of vitamin D3 supplementation on fasting measurements

The effects of vitamin D3 on fasting parameters are listed in Table 5.2. Both high and low dose vitamin D3 supplementation resulted in a significant increase in serum 25OHD, although the increase in the high dose group was larger. PTH levels decreased in the high dose group only. Fasting plasma C3 levels decreased by 5.9% (p=0.004) in the high dose group, and by 4.0% (p=0.018) in the low dose group. Serum CRP, leukocyte counts, plasma triglycerides and the measurements of arterial stiffness did not change significantly after vitamin D3 supplementation. The expression of fasting leukocyte activation markers was not significantly influenced by vitamin D3 supplementation, except for a higher expression of CD35 on monocytes after supplementation for the total group.

**Table 5.2** Fasting measurements before (baseline) and after supplementation (vitamin D3)

| | Total (n=24) | | High dose (n=12) | | Low dose (n=12) | | P-value | P-value |
|---|---|---|---|---|---|---|---|---|
| | Baseline | Vitamin D3 | Baseline | Vitamin D3 | Baseline | Vitamin D3 | Baseline Between groups[#] | After Vitamin D between groups[†] |
| 25OHD (nmol/l) | 27.1 ± 13.8 | 57.9 ± 24.0** | 26.8 ± 12.4 | 70.5 ± 25.4** | 27.3 ± 15.6 | 45.2 ± 14.6** | 0.940 | 0.002 |
| PTH (pmol/l) | 6.2 ± 2.8 | 5.9 ± 2.5 | 6.2 ± 2.8 | 5.4 ± 2.5** | 6.2 ± 2.9 | 6.5 ± 2.6 | 0.977 | 0.007 |
| CRP (mg/l) | 4.0 (2.0-8.0) | 5.0 (2.0-10.0) | 6.0 (3.0-8.0) | 6.0 (2.8-10.8) | 3.0 (2.0-7.0) | 3.0 (2.0-8.0) | 0.198 | 0.443 |
| C3 (g/l) | 1.30 (1.16-1.47) | 1.21 (1.08-1.34)** | 1.35 (1.26-1.53) | 1.27 (1.17-1.37)** | 1.24 (1.11-1.36) | 1.19 (1.05-1.31)* | 0.160 | 0.319 |
| Leuko count ($10^9$/l) | 7.3 ± 1.8 | 7.3 ± 1.7 | 8.1 ± 2.0 | 7.9 ± 1.8 | 6.6 ± 1.2 | 6.7 ± 1.4 | 0.031 | 0.426 |
| Mono count ($10^9$/l) | 0.5 ± 0.2 | 0.5 ± 0.2 | 0.5 ± 0.1 | 0.5 ± 0.1 | 0.5 ± 0.2 | 0.5 ± 0.2 | 0.718 | 0.836 |
| Neutro count ($10^9$/l) | 3.9 ± 1.3 | 3.9 ± 1.2 | 4.4 ± 1.4 | 4.3 ± 1.3 | 3.4 ± 0.9 | 3.4 ± 0.9 | 0.040 | 0.676 |
| TG (mmol/l) | 1.00 (0.88-1.44) | 0.96 (0.83-1.59) | 1.25 (0.91-1.84) | 1.13 (0.85-1.73) | 0.99 (0.66-1.09) | 0.93 (0.81-1.26) | 0.128 | 0.977 |
| TC (mmol/l) | 5.2 ± 0.9 | 5.1 ± 0.9 | 5.3 ± 1.2 | 5.2 ± 1.2 | 5.1 ± 0.7 | 4.9 ± 0.6* | 0.610 | 0.443 |
| HDL-C (mmol/l) | 1.4 ± 0.4 | 1.4 ± 0.3 | 1.4 ± 0.4 | 1.4 ± 0.3 | 1.5 ± 0.3 | 1.5 ± 0.3 | 0.242 | 0.323 |
| LDL-C (mmol/l) | 3.2 ± 0.9 | 3.1 ± 0.9 | 3.4 ± 0.9 | 3.3 ± 1.1 | 3.1 ± 0.8 | 2.9 ± 0.6 | 0.468 | 0.739 |
| Apo A-I (g/l) | 1.60 ± 0.32 | 1.56 ± 0.27 | 1.61 ± 0.36 | 1.60 ± 0.29 | 1.60 ± 0.30 | 1.52 ± 0.26* | 0.946 | 0.440 |
| Apo B (g/l) | 1.14 ± 0.26 | 1.10 ± 0.26* | 1.17 ± 0.28 | 1.15 ± 0.29 | 1.12 ± 0.26 | 1.05 ± 0.22** | 0.679 | 0.211 |
| PWV (m/s) | 6.6 ( 6.0- 7.3) | 6.1 (5.9-7.1) | 6.7 (6.1-7.3) | 6.7 (6.0-7.3) | 6.1 (6.0-7.3) | 6.0 (5.9-7.0) | 0.525 | 0.487 |
| AIx (%) | 5.0 ± 12.4 | 4.9 ± 12.1 | 5.8 ± 9.8 | 5.7 ± 9.6 | 4.2 ± 15.0 | 4.2 ± 14.5 | 0.761 | 0.988 |
| Mono CD11b (MFI) | 17.9 ± 4.0 | 19.4 ± 5.0 | 17.0 ± 3.2 | 18.8 ± 6.4 | 18.8 ± 4.6 | 20.1 ± 3.3 | 0.266 | 0.723 |
| Neutro CD11b (MFI) | 26.6 ± 7.1 | 28.3 ± 8.1 | 26.6 ± 7.1 | 27.8 ± 7.2 | 26.6 ± 7.5 | 28.8 ± 9.2 | 0.983 | 0.702 |
| Neutro CD66b (MFI) | 6.1 ± 2.1 | 6.5 ± 2.2 | 6.1 ± 2.4 | 6.5 ± 2.4 | 6.2 ± 1.8 | 6.4 ± 2.1 | 0.901 | 0.733 |
| Mono CD36 (MFI) | 52.0 ± 25.1 | 56.5 ± 26.6 | 42.6 ± 20.8 | 50.8 ± 26.7 | 61.4 ± 26.2 | 62.2 ± 26.4 | 0.064 | 0.456 |
| Mono CD35 (MFI) | 4.7 ± 1.9 | 5.3 ± 2.2* | 4.4 ± 1.7 | 5.3 ± 2.2 | 5.0 ± 2.1 | 5.2 ± 2.3 | 0.468 | 0.155 |
| Neutro CD35 (MFI) | 7.9 ± 3.4 | 8.5 ± 3.9 | 7.8 ± 3.2 | 8.1 ± 3.1 | 8.1 ± 3.6 | 8.9 ± 4.7 | 0.823 | 0.689 |

Data are given as median (interquartile range) or mean ± standard deviation

*P<0.05 for difference between before (control) and after vitamin D3 supplementation (vitamin D3) (paired Student's t-test or Wilcoxon matched pairs signed ranks test)

**P<0.01 for difference between before (control) and after vitamin D3 supplementation (vitamin D3) (paired Student's t-test or Wilcoxon matched pairs signed ranks test)

[#]P-value testing the difference between the high and low dose group before supplementation (independent Student's t-test or Mann-Whitney U test)

[†]P-value testing the difference (intrapersonal change, after vs. before supplementation) between the high and low dose group (independent Student's t-test or Mann-Whitney U test)

### Effect of vitamin D3 supplementation on postprandial lipids and inflammation

Postprandial plasma triglycerides, arterial stiffness and leukocyte activation before and after vitamin D3 supplementation are listed in Table 5.3.

Vitamin D3 supplementation in low or high dose did not alter the postprandial plasma triglyceride response, which was comparable between both groups (Figure 5.2A and Table 5.3). Vitamin D3 did not influence postprandial PWV or AIx (Table 5.3). Postprandial arterial stiffness was similar in both groups before vitamin D3 supplementation, and the change in PWV and AIx was comparable between the high and low dose vitamin D3 supplementation groups (Figure 5.2B and 5.2C and Table 5.3).

**Table 5.3** Postprandial measurements before (baseline) and after supplementation (vitamin D3)

| | Total (n=24) | | High dose (n=12) | | |
|---|---|---|---|---|---|
| | Baseline | Vitamin D3 | Baseline | Vitamin D3 | Change |
| TG-AUC (mmol/l*h) | 11.56 (9.49 - 17.27) | 12.44 (9.55 - 18.53) | 13.60 (11.10 - 19.79) | 13.43 (10.00 - 19.28) | 0.33 (-1.28 - 2.28) |
| PWV-AUC (m/s*h) | 51.8 (47.4 - 58.2) | 49.4 (47.9 - 57.9) | 54.7 (50.3 - 58.9) | 54.5 (49.0 - 60.3) | -0.5 (-3.2 - 3.1) |
| AIx-AUC (%*h) | 213.7 ± 85.6 | 212.2 ± 85.0 | 215.2 ± 73.9 | 223.8 ± 78.1 | 8.7 ± 22.1 |
| C3-AUC (g/l*h) | 9.65 (9.16 - 10.70) | 9.78 (8.86 - 10.51) | 11.09 (9.79 - 11.81) | 10.63 (9.74 - 11.67) | -0.10 (-0.70 - 0.11) |
| mCD11b-AUC (MFI*h) | 147.3 ± 27.8 | 154.8 ± 31.5* | 140.3 ± 24.5 | 145.5 ± 32.7 | 5.2 ± 18.1 |
| nCD11b-AUC (MFI*h) | 224.0 ± 46.9 | 227.6 ± 53.3 | 230.6 ± 37.6 | 226.7 ± 35.6 | -4.0 ± 19.1 |
| nCD66b-AUC (MFI*h) | 50.3 ± 16.8 | 50.7 ± 16.7 | 51.7 ± 20.0 | 51.6 ± 18.8 | -0.1 ± 3.5 |
| mCD36-AUC (MFI*h) | 407.5 ± 172.0 | 448.9 ± 208.3 | 327.7 ± 136.1 | 395.2 ± 208.2 | 67.4 ± 138.6 |
| mCD35-AUC (MFI*h) | 40.1 ± 16.7 | 40.7 ± 16.0 | 40.1 ± 16.4 | 40.5 ± 16.3 | 0.5 ± 6.3 |
| nCD35-AUC (MFI*h) | 68.4 ± 28.3 | 66.5 ± 28.4 | 70.7 ± 27.1 | 65.7 ± 26.0 | -5.0 ± 7.9 |

In the total group, vitamin D3 supplementation resulted in a slight increase in postprandial monocyte CD11b expression (AUC 147.3 ± 27.8 MFI*h before and 154.8 ± 31.5 after supplementation, p=0.021, Table 5.3). The change in monocyte CD11b expression did not differ between the high and low dose groups (Figure 5.3A and Table 5.3).

The expression of CD11b, CD66b and CD35 on neutrophils and CD36 and CD35 on monocytes did not change after vitamin D3 supplementation in the total group (Table 5.3). Before vitamin D3, the postprandial monocyte CD36 expression was lower in the high dose group than in the low dose group (327.7 ± 136.1 and 487.3 ± 171.5 MFI*h, respectively, p=0.019). The expression of the other leukocyte activation markers did not differ between both groups before supplementation. There was no differential effect of high or low dose vitamin D3 supplementation on any of the investigated leukocyte activation markers (Figures 5.3B-F and Table 5.3). Postprandial C3 levels did not change, and were unaffected by vitamin D3 supplementation.

| | Low dose (n=12) | | P-value | P-value |
|---|---|---|---|---|
| Baseline | Vitamin D3 | Change | Baseline Between groups[#] | After Vitamin D between groups[†] |
| 10.05 (8.86 - 15.05) | 11.86 (8.20 - 14.10) | -0.49 (-1.85 - 2.24) | 0.114 | 0.235 |
| 49.2 (46.7 - 55.2) | 48.7 (45.7 - 57.0) | -0.7 (-4.7 - 1.7) | 0.288 | 0.566 |
| 212.3 ± 99.3 | 200.6 ± 93.4 | -11.7 ± 33.9 | 0.936 | 0.096 |
| 9.65 (9.16 - 10.70) | 9.78 (8.86 - 10.51) | -0.25 (-0.95 - 0.08) | 0.143 | 0.590 |
| 154.2 ± 30.2 | 164.1 ± 28.6 | 9.9 ± 11.4 | 0.229 | 0.456 |
| 217.4 ± 55.5 | 228.6 ± 68.4 | 11.2 ± 26.2 | 0.501 | 0.120 |
| 49.0 ± 13.5 | 49.8 ± 15.0 | 0.8 ± 5.0 | 0.709 | 0.632 |
| 487.3 ± 171.5 | 502.6 ± 202.8 | 15.3 ± 119.5 | 0.019 | 0.335 |
| 40.1 ± 17.7 | 40.9 ± 16.3 | 0.8 ± 5.5 | 0.993 | 0.891 |
| 66.2 ± 30.5 | 67.3 ± 31.8 | 1.1 ± 14.0 | 0.706 | 0.201 |

Area under the curve (AUC) as median (interquartile range) or mean ± standard deviation.
*P<0.05 for difference between before (control) and after vitamin D3 supplementation (vitamin D3) (paired Student's t-test or Wilcoxon matched pairs signed ranks test)
**P<0.01 for difference between before (control) and after vitamin D3 supplementation (vitamin D3) (paired Student's t-test or Wilcoxon matched pairs signed ranks test)
[#]P-value testing the difference between the high and low dose group before supplementation (independent Student's t-test or Mann-Whitney U test)
[†]P-value testing the difference (intrapersonal change, after vs. before supplementation) between the high and low dose group (independent Student's t-test or Mann-Whitney U test)

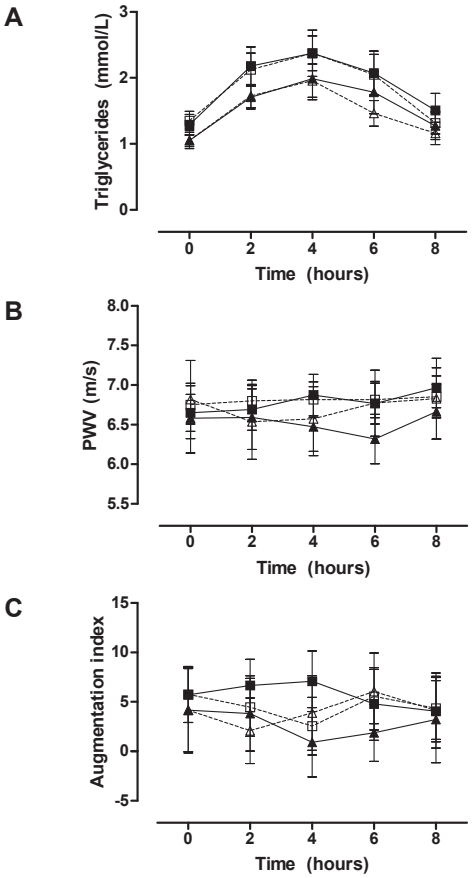


**Figure 5.2.** Triglycerides and arterial stiffness after an oral fat load

Mean (SEM) triglycerides (A), pulse wave velocity (B) and augmentation index (C) in the high dose group before (open square and dotted line) and after vitamin D3 supplementation (closed square and continuous line) and in the low dose group before (open triangle and dotted line) and after supplementation (closed triangle and continuous line) during an oral fat load.

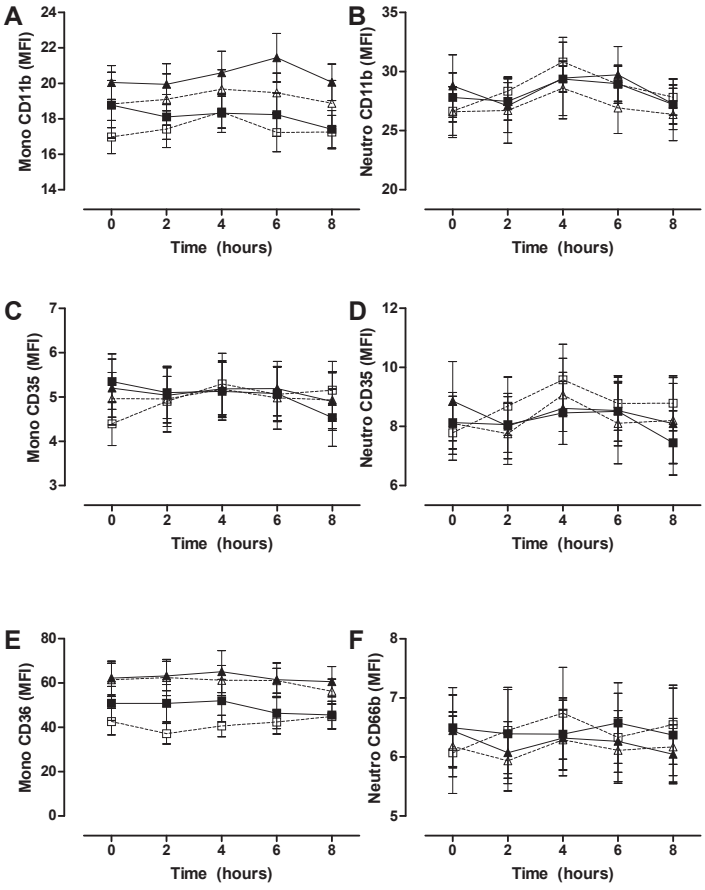


**Figure 5.3.** Leukocyte activation after an oral fat load

Mean (SEM) expression of monocyte CD11b (A), neutrophil CD11b (B), monocyte CD35 (C), neutrophil CD35 (D), monocyte CD36 (E) and neutrophil CD66b (F) in the high dose group before (open square and dotted line) and after vitamin D3 supplementation (closed square and continuous line) and in the low dose group before (open triangle and dotted line) and after supplementation (closed triangle and continuous line) during an oral fat load.

## DISCUSSION

In this paper, we describe the effect of a single high and low dose of vitamin D3 on postprandial arterial stiffness and leukocyte activation markers in healthy premenopausal overweight and obese women. Contrary to our hypothesis, a single dose of either 300 000 or 75 000 IU cholecalciferol did not reduce postprandial arterial stiffness or leukocyte activation.

Obesity is associated with increased arterial stiffness [16] and with vitamin D deficiency [17]. We exclusively included premenopausal overweight and obese women, who usually have vascular dysfunction and vitamin D deficiency. Therefore, vitamin D was expected to provide more pronounced results in this human model.

The present results are in contrast with our previous pilot study [12]. Several differences in participant characteristics may explain this discrepancy. Firstly, participants in the current study were on average 8 years younger than the female participants of the pilot study. Secondly, the volunteers in the present study were either overweight or obese, with an average BMI of 32 kg/m$^2$, in contrast to the lean female participants in the previous study. Thirdly, while the increase in vitamin D after supplementation was comparable (in both studies vitamin D increased by approximately 30 nmol/l), vitamin D levels after supplementation were approximately twice as high in the pilot study (109 nmol/l versus 58 nmol/l in the present study). We cannot exclude that higher vitamin D levels must be reached to obtain beneficial vascular and anti-inflammatory effects as found earlier [12]. This assumption is underlined by a comparison of several randomized-controlled trials investigating the effect of vitamin D3 supplementation on vascular function and inflammation, where trials observing a positive effect [6,7,12,18,19] reported higher vitamin D levels after supplementation (range 80 to 380 nmol/L) than studies in which vitamin D had no beneficial effect [20–23] (range 70-110 nmol/L).

The lack of a vitamin D effect on the augmentation index may possibly be due to the low baseline values measured in the present study. The augmentation index is known to increase with age [24], and it has been suggested that the augmentation index decreases with increasing body mass index [25]. The volunteers in this study were relatively young and either overweight or obese.

The expression of all measured leukocyte activation markers was higher in the present study than reported earlier [12], most likely due to systemic inflammation in obesity [26]. We speculate that in this condition, higher vitamin D levels may be needed to achieve anti-inflammatory effects.

Although several cross-sectional studies have shown that vitamin D levels may be inversely associated with fasting arterial stiffness [27,28], studies on the effect of vitamin D supplementation on PWV and augmentation index have shown conflicting results. Some studies using high doses of cholecalciferol demonstrated a beneficial effect on arterial

stiffness after four months [19] and after one year [29], while other studies reported no effect [20,21]. Trials investigating inflammation after vitamin D supplementation report contrasting results as well. While some investigators reported a cytokine profile shift to an anti-inflammatory profile [7] and reduced oxidative stress, assessed by malondialdehyde, nitric oxide and protein carbonyl groups [8], others found no effect on CRP [20,21], interleukin-6 or tumor necrosis factor-alpha levels [20]. To the best of our knowledge, besides the pilot study by our group [12], no other previous studies have investigated *postprandial* arterial stiffness and leukocyte activation after vitamin D administration.

Strikingly, in our overweight and obese female study participants, fasting C3 levels were comparable to the levels observed in familial combined hyperlipidemia and metabolic syndrome patients [30,31]. Increased C3 has been associated with metabolic syndrome [31] and cardiovascular disease [32]. Interestingly, both high and low dose vitamin D3 supplementation reduced fasting C3 levels. C3 is mainly produced in the liver [33], but also by adipose tissue [34]. Vitamin D has been shown to reduce adipose tissue cytokine secretion and inflammation [35]. Possibly, vitamin D may also reduce adipocyte C3 secretion. We speculate that the observed effect could be a transcription effect. It has been suggested that vitamin D downregulates the transcription of pro-inflammatory genes [36]. Whether this is also the case for C3 remains to be investigated. C3 is not only involved in the immune system, but it also has an endocrine effect as precursor of acylation-stimulating protein (ASP), a hormone produced by adipose tissue [37]. ASP increases fat storage by stimulating lipogenesis in adipocytes and by inhibiting lipolysis [37], and it stimulates glucose transport through increased translocation of glucose transporters [37]. Possibly, a decrease in C3 levels may lead to increased sensitivity for the C3/ASP effect on fatty acid metabolism in the adipose tissue. The concept of C3/ASP resistance has been proposed previously [30,34]. In healthy lean subjects, adipose tissue C3 and ASP production increases after a fatty meal, largely due to stimulation of adipocytes by chylomicrons [34], resulting in a postprandial increase of plasma C3 levels [30,31,38]. In subjects with a high level of apolipoprotein B-containing lipoproteins, binding of ASP to the ASP receptor on adipocytes may be impaired, which makes adipocytes less sensitive to the effects of ASP, resulting in enhanced ASP secretion [39]. It has indeed been demonstrated that fasting C3 levels are increased in subjects with cardiometabolic diseases, such as familial combined hyperlipidemia [30], the metabolic syndrome [31] and type 2 diabetes mellitus [38]. In these subjects, in contrast to lean healthy individuals, C3 levels do not (or only slightly) increase postprandially [30,31,38]. The increased C3 levels in obesity may be explained by C3/ASP resistance as well [40]. Indeed, C3 levels are higher in obese than in lean subjects [40], and C3 decreases after weight loss [40].

This study has several strengths. This is the first double-blind randomized trial to investigate the effect of a single dose of vitamin D3 on postprandial leukocyte activa-

tion and arterial elasticity. Furthermore, the study seemed to be well-powered, based on results of a pilot study, and there was no drop-out of subjects or loss of data.

However, several limitations apply as well. Firstly, we investigated a selected group of subjects, i.e. premenopausal vitamin D deficient overweight and obese women. Therefore, the results in this study population may not be representative for the general population. Secondly, the study sample was relatively small. While the number of included subjects was based on a sample size calculation based on our pilot study, a larger sample size may have resulted in a different outcome. Contrary to our pilot study, there was a mild but significant increase in postprandial monocyte CD11b expression after vitamin D supplementation. This slight increase may not be clinically relevant and is contra-intuitive, and may possibly be due to the relatively small sample size. Thirdly, no placebo group was included, which could have emphasized any potential effect of vitamin D. Finally, we studied the effect of a single dose of vitamin D3 with one week between measurements. We cannot exclude that a longer duration of supplementation and higher vitamin D levels may have had positive effects on postprandial arterial stiffness and inflammation, especially since 42% of the study subjects remained vitamin D deficient (<50 nmol/L) after supplementation.

In conclusion, this study suggests reduced fasting C3 levels after vitamin D3 supplementation, most likely by an effect on the adipose tissue, but it does not provide evidence for a short-term beneficial effect of a single dose of 75 000 or 300 000 vitamin D3 on arterial function or leukocyte activation in vitamin D deficient women. Possibly, higher vitamin D levels must be achieved for a positive effect on arterial elasticity and inflammation.

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# Chapter 6

## Coronary leukocyte activation in relation to progression of coronary artery disease

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Front Med. 2016;10(1):85-90

## ABSTRACT

**Background:** Leukocyte activation has been linked to atherogenesis, but there is little *in vivo* evidence for its role in the progression of atherosclerosis. We evaluated the predictive value of leukocyte activation markers in the coronary circulation for progression of coronary artery disease (CAD).

**Methods:** Monocyte and neutrophil CD11b, neutrophil CD66b expression and intracellular neutrophil myeloperoxidase (MPO) in the coronary arteries was determined by flow cytometry in patients undergoing coronary angiography. The primary outcome included fatal and nonfatal myocardial infarction or arterial vascular intervention due to unstable angina pectoris.

**Results:** In total 99 subjects were included, 70 had CAD at inclusion (26 patients had single-vessel disease, 18 patients had two-vessel disease and 26 patients had three-vessel disease). The median follow-up duration was 2242 days (interquartile range: 2142-2358). During follow-up, 13 patients (13%) developed progression of CAD. Monocyte CD11b, neutrophil CD11b and CD66b expression and intracellular MPO measured in blood obtained from the coronary arteries were not associated with the progression of CAD.

**Conclusion:** These data indicate that coronary monocyte CD11b, neutrophil CD11b and CD66b expression and intracellular MPO do not predict the risk of progression of CAD.

## INTRODUCTION

Classical risk factors used to predict cardiovascular risk include hyperlipidemia, hypertension, smoking, obesity and diabetes mellitus [1]. In the past decades, it has become evident that atherosclerosis is an inflammatory disease [2]. Therefore, attention has shifted to inflammatory markers as predictors of future cardiovascular events. Total leukocyte count, C-reactive protein (CRP) and the third component of complement (C3) are predictors of cardiovascular risk [3–5]. In this respect, besides leukocyte count, also the level of leukocyte activation may be an interesting marker. Leukocytes can become activated by several stimuli, such as lipids and glucose [6–10]. Activated neutrophils and monocytes express integrins on their cell surface, facilitating the binding of these leukocytes to the intact endothelium [11,12]. One of these integrins, CD11b, is present on the surface of activated monocytes and neutrophils and binds to intercellular adhesion molecule-1 on endothelial cells [11]. It has been demonstrated *in vitro* that increased expression of CD11b on monocytes and neutrophils is accompanied by enhanced leukocyte adhesion to the endothelium [13–15]. CD66b is a marker of neutrophil degranulation [16]. Myeloperoxidase (MPO) is an important enzyme in neutrophil host defense, as it inactivates bacterial toxins [17]. Activated neutrophils release MPO from their granules and therefore, intracellular MPO levels are a negative marker of neutrophil activation, with low levels representing increased activation [17]. When bound to the endothelium, activated leukocytes migrate to the subendothelial space. Monocytes bind modified lipoproteins and form foam cells, and the development of the atherosclerotic plaque is initiated [2].

Several lines of evidence suggest that increased leukocyte activation is linked to the presence of ischemic heart disease and peripheral artery disease [18–21]. The expression of monocyte and neutrophil CD11b is higher in patients with multiple risk factors for atherothrombosis than in those with none or only one risk factor [22]. Intracellular MPO is reduced in patients presenting with acute coronary syndromes [23]. Finally, increased leukocyte activation has been associated with the presence of microvascular diabetic complications [24,25].

We have previously described an inflammatory gradient of intracellular MPO in patients with stable coronary artery disease (CAD), with the highest level of neutrophil activation in the coronary arteries, suggesting focal inflammation in CAD [26]. In healthy controls, this gradient was observed as well, although levels of neutrophil activation were lower than in patients with CAD [26]. For monocyte CD11b and neutrophil CD11b and CD66b expression, this gradient was not present [26].

Although the current literature underlines an association between leukocyte activation and CAD, no studies have evaluated the use of leukocyte activation markers CD11b and CD66b and intracellular MPO as possible indicators of future coronary events. The

aim of the current study was to assess whether levels of leukocyte activation in the coronary circulation may help to predict which patients are at risk of progression of CAD, in patients undergoing elective coronary angiography (CAG).

## METHODS

### Study design and study population

The design of the study has been described extensively and published elsewhere [26]. The study was designed as a prospective follow-up study. Briefly, subjects who visited the outpatient clinic of the Department of Cardiology, Sint Franciscus Gasthuis, Rotterdam, The Netherlands, between July 2007 and September 2008 and who were scheduled to undergo a diagnostic CAG were invited to participate. Their indications for CAG were typical chest pain and a positive cycle ergometry.

Exclusion criteria were the presence of inflammatory disorders such as rheumatoid arthritis, systemic lupus erythematosus and infections, a plasma CRP level above 10 mg/L, and disorders of kidney, liver and thyroid function. The study was conducted according to the Declaration of Helsinki. The Institutional Review Board of the Sint Franciscus Gasthuis Rotterdam and the regional independent medical ethics committee of the Maasstad Hospital Rotterdam approved the study. The study was registered at clinicaltrials.gov under clinical trial number NCT02376738. All participants gave written informed consent.

On the day of the angiography, anthropometric measures, the use of medication and cardiovascular history were recorded. Shortly before CAG, venous blood was obtained from a peripheral vein of the forearm. During the angiography, blood samples were obtained from each coronary artery. In addition, blood was collected from the femoral artery and midway from the abdominal aorta. The first 2 mL were discarded to avoid contamination with contrast. Subsequently, blood samples were collected in tubes containing EDTA (1 mg/mL) and kept on ice until processed for determination of leukocyte activation markers. CAG images were scored by an independent cardiologist.

### Scoring of coronary events

The primary outcome was defined as progression of CAD, evaluated by an independent investigator at approximately six years follow-up. This endpoint included fatal and non-fatal myocardial infarction and any arterial vascular intervention that had not already been planned at the time of inclusion (e.g. coronary bypass, percutaneous coronary intervention, peripheral vascular surgery or angioplasty/stenting).

### Analytical methods

Parameters for renal and liver function, glucose, CRP, total cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides were determined using Synchron LX-20 analyzers (Beckman Coulter, Brea, CA, USA) according to standard procedures in our laboratory. Low-density lipoprotein (LDL) cholesterol values were calculated using the Friedewald formula. Apolipoproteins A-I and B were determined by rate nephelometry using an IMMAGE analyzer (Beckman Coulter). Blood cell counts were determined using LH750 analyzers (Beckman Coulter, Miami, FL, USA).

### Leukocyte activation markers

The expression of leukocyte activation markers on the cell surface was determined using fluorescent labeled monoclonal antibodies (Immunotech Coulter, Marseille, France). Antibodies for CD66b were labeled with fluorescein isothiocynate (FITC) and were diluted 10 times from their stock concentration. Antibodies for CD11b were labeled with phycoerythrin (PE) and were diluted 40 times. Antibodies for CD45 labeled with PE-Texas Red (ECD) were used in order to be able to differentiate leukocytes from erythrocytes and platelets. Twenty microliters of blood from an EDTA-anticoagulated blood sample were added to 2.5 µL of each CD66b-FITC, CD11b-PE and CD45-ECD. Cells were incubated for 15 minutes in the dark at room temperature. Erythrocytes were lysed by adding 300 µL of ice-cold isotonic erythrocyte lysing solution (NH4Cl 0.19 M; KHCO3 0.01 M; Na2EDTA.2H2O 0.12 M, pH 7.2) for 15 minutes.

Antibodies against MPO (Beckman Coulter) were FITC-conjugated. Membrane permeabilization was performed with Intraprep (Beckman Coulter). To each blood sample of 20 µL, 2.5 µL of titrated anti-MPO (2 times diluted) was added. Cells were incubated for 15 minutes in the dark at room temperature.

A Coulter Epics XL-MCL flow cytometer with a 488 nm Argon ion laser and EXPO 32 software were used for measurement and analysis. Fluorescent intensity of each cell was expressed as the mean fluorescent intensity (MFI), given in arbitrary units (au). Lymphocytes, monocytes and granulocytes were identified based on their side scatter and the level of CD45 on their cell surface.

Patients were considered to have high leukocyte activation if the level of monocyte CD11b, neutrophil CD11b or CD66b expression was above, or intracellular MPO was below, the median of the total group.

### Statistical analysis

Data are given as mean ± standard deviation (SD) for continuous variables with a normal distribution, and median (interquartile range (IQR)) for continuous variables with a skewed distribution (triglycerides, CRP, leukocyte count, C3, monocyte CD11b

expression, neutrophil CD11b and CD66b expression and MPO). Differences in leukocyte activation between groups were tested with the Mann Whitney-U test.

A Cox-regression analysis was performed to study the impact of leukocyte activation markers on the progression of CAD, using a two-step approach. First, a univariate analysis was performed with several known cardiovascular risk factors as factor, using progression of CAD as event, and time to event as the time to coronary event since CAG at inclusion. The presence of CAD at CAG at inclusion was found to predict progression of CAD, while age (above or below the median of 65 years) and gender did not. In the second step, leukocyte activation markers in the coronary arteries were added to the Cox-regression model, with the presence of CAD entered into the model and the individual markers entered as additional variables. A p-value <0.05 (two-sided) was regarded as statistically significant. All statistical analyses were performed using PASW statistics version 22.0 (IBM SPSS Statistics, New York, United States).

## RESULTS

### General characteristics

A total of 99 subjects were included. Their baseline characteristics are listed in Table 6.1. The median duration of follow-up was 2242 days (IQR: 2142-2358). At the time of inclusion, 37 (37%) patients had a history of CAD. In 70 patients (71%), CAD was established by CAG at inclusion, resulting in 33 new diagnoses of CAD: 26 patients had single-vessel disease, 18 patients had two-vessel disease and 26 patients had three-vessel disease. In the remaining 29 patients, no significant coronary stenosis was established by CAG, and none of these patients had a history of clinical CAD.

### Presence of CAD at inclusion predicts progression of CAD

During follow-up, 13 patients (13%) showed progression of CAD: 10 patients developed unstable angina pectoris requiring intervention (percutaneous coronary intervention or bypass surgery) and 3 patients developed fatal myocardial infarction. A comparison between the baseline characteristics of those with progression of CAD, and those remaining free of progression, is given in Table 6.1. All patients with progression of CAD had significant coronary stenosis by CAG at inclusion. In a univariate analysis, only the presence of CAD, established by CAG at inclusion, predicted the progression of CAD (Log Rank Test p=0.014).

**Table 6.1** Baseline characteristics of study participants (n=99)

| | Total group (n=99) | Progression CAD (n=13) | No progression CAD (n=86) | P-value |
|---|---|---|---|---|
| Age (years) | 65 ± 12 | 67 ± 11 | 64 ± 12 | 0.50 |
| Gender (% male) | 60 | 62 | 60 | >0.99 |
| CAD (%) | 71 | 100 | 66 | 0.009 |
| DM (%) | 28 | 15 | 30 | 0.34 |
| Smoking (%) | 14 | 15 | 14 | >0.99 |
| Statin use (%) | 68 | 92 | 64 | 0.06 |
| ASA use (%) | 67 | 92 | 63 | 0.06 |
| ACE-i use (%) | 33 | 23 | 35 | 0.54 |
| BMI (kg/m$^2$) | 27.6 ± 4.1 | 25.5 ± 4.6 | 27.9 ± 4.0 | 0.06 |
| SBP (mmHg) | 144 ± 22 | 154 ± 27 | 143 ± 21 | 0.09 |
| Glucose (mmol/L) | 6.8 ± 1.7 | 6.5 ± 1.3 | 6.8 ± 1.8 | 0.59 |
| LDL-c (mmol/L) | 2.8 ± 1.0 | 2.8 ± 1.2 | 2.8 ± 1.0 | 0.95 |
| HDL-c (mmol/L) | 1.3 ± 0.3 | 1.3 ± 0.3 | 1.2 ± 0.3 | 0.56 |
| Triglycerides (mmol/L) | 1.55 (1.07-2.19) | 1.46 (0.90-1.78) | 1.56 (1.08-2.25) | 0.43 |
| CRP (mg/L) | 2.0 (1.0-4.0) | 1.0 (1.0-5.0) | 2.0 (1.0-4.0) | 0.64 |
| Leukocyte count (10$^9$/L) | 7.1 (6.0-8.3) | 7.1 (5.6-7.7) | 7.1 (6.1-8.4) | 0.32 |
| Complement C3 (g/L) | 1.23 ± 0.24 | 1.28 ± 0.24 | 1.22 ± 0.24 | 0.39 |
| Mono CD11b LCA (au) | 31.8 (25.5-40.8) | 28.9 (21.0-42.4) | 32.2 (25.6-40.7) | 0.66 |
| Mono CD11b RCA (au) | 32.2 (26.5-41.8) | 29.8 (23.0-42.0) | 32.2 (26.7-41.7) | 0.56 |
| Neutro CD11b LCA (au) | 30.9 (24.3-38.6) | 29.0 (22.2-38.8) | 31.8 (24.4-38.6) | 0.51 |
| Neutro CD11b RCA (au) | 31.5 (25.6-37.6) | 23.7 (20.0-43.8) | 31.8 (26.2-37.6) | 0.26 |
| Neutro CD66b LCA (au) | 7.2 (6.0-9.0) | 7.6 (5.5-9.8) | 7.1 (6.1-8.9) | 0.70 |
| Neutro CD66b RCA (au) | 7.3 (5.8-9.3) | 7.7 (5.0-11.3) | 7.3 (5.8-9.0) | 0.72 |
| MPO LCA (au) | 162.5 (130.2-202.9) | 160.4 (136.4-169.4) | 169.6 (129.8-205.0) | 0.69 |
| MPO RCA (au) | 165.3 (130.1-194.7) | 166.9 (147.1-177.3) | 164.0 (129.3-196.5) | 0.99 |

Data are given as mean ± SD, median (IQR) or as percentage. P-value for difference between groups. ACE-i: angiotensin-converting enzyme inhibitor; ASA: acetylsalicylic acid; au: arbitrary units; BMI: body mass index; CAD: coronary artery disease; CRP: C-reactive protein; DM: diabetes mellitus; LCA: left coronary artery; mono: monocyte; neutro: neutrophil; RCA: right coronary artery; SBP: systolic blood pressure.

## Coronary leukocyte activation and risk of progression of CAD

In a Cox-regression analysis, none of the investigated leukocyte activation markers in the coronary circulation contributed significantly to the predictive power of progression of CAD in addition to CAD at inclusion (Table 6.2). Monocyte CD11b, neutrophil CD11b and CD66b and MPO in the other vascular regions (peripheral vein, femoral artery and abdominal aorta) did not increase the predictive power of progression of CAD either (data not shown).

**Table 6.2** Coronary leukocyte activation and risk of future CAD

| | HR (95% CI) | P-value |
|---|---|---|
| Monocyte CD11b in LCA | 0.97 (0.94 – 1.04) | 0.61 |
| Monocyte CD11b in RCA | 0.97 (0.90 – 1.04) | 0.42 |
| Neutrophil CD11b in LCA | 1.00 (0.94 – 1.06) | 0.87 |
| Neutrophil CD11b in RCA | 0.97 (0.90 – 1.04) | 0.38 |
| Neutrophil CD66b in LCA | 1.10 (0.86 – 1.34) | 0.45 |
| Neutrophil CD66b in RCA | 1.06 (0.82 – 1.37) | 0.65 |
| Myeloperoxidase in LCA | 1.00 (0.99 – 1.01) | 0.95 |
| Myeloperoxidase in RCA | 1.00 (0.99 – 1.01) | 0.70 |

Hazard ratios (HR) with 95% confidence interval (CI) for progression of coronary artery disease in patients per coronary leukocyte activation marker for each point increase in arbitrary units, as additional factor in a Cox Regression analysis with presence of CAD as covariate.

## DISCUSSION

This is the first prospective study investigating the value of coronary monocyte CD11b, neutrophil CD11b and CD66b expression and intracellular MPO in the prediction of future coronary events. In the present study, in patients scheduled for CAG due to typical chest pain or positive cycle ergometry, these leukocyte activation markers measured in the coronary circulation, or in other vascular regions, did not predict the risk of CAD progression.

We have previously published the baseline values of the investigated leukocyte activation markers in the coronary arteries in this study population [26]. While MPO was significantly lower in patients with CAD, the expression of CD11b and CD66b in the coronary circulation was similar in patients with and without CAD [26].

Several studies have pointed at a role for leukocyte activation in the development of CAD. Neutrophil infiltration has been associated with acute coronary syndromes, and autopsy studies have shown that ruptured plaques contained more neutral endopeptidase positive neutrophils than eroded plaques [27]. In survivors of non-ST elevation myocardial infarction, plasma MPO was associated with short-term risk (<30 days) of recurrent acute coronary syndrome and myocardial infarction, but this association was lost after 180 days [28]. In a large prospective study among patients without previous CAD, plasma MPO in peripheral blood was associated with increased risk of future coronary heart disease in men but not in women [29]. In addition, the level of neopterin, a marker of monocyte activation, in the peripheral circulation was an independent predictor of future cardiovascular events in patients with and without CAD, indicating the presence of systemic leukocyte activation in CAD [30,31].

The effect of reducing leukocyte activation on CAD has been investigated in animal studies. In rats, treatment with antibodies selectively blocking CD11b or CD18 reduced leukocyte adhesion to endothelial cells [13]. Treatment with anti-CD11b or CD18 reduced myocardial infarct size after regional myocardial ischemia in dogs [14,32]. However, studies on the effect of these blocking antibodies on myocardial infarction size or on risk of future coronary events in humans are lacking.

The lack of an association with the presently investigated leukocyte activation markers may possibly be explained by the nature of these markers. The integrins CD11b and CD66b can bind to various selectins on the endothelial surface [12–15,33]. Moreover, activated neutrophils transfer MPO to endothelial cells via the CD11b integrin, contributing further to endothelial cell activation and leukocyte adhesion [34]. Therefore, the measurement of these integrins on the leukocyte surface in blood samples may in fact be an underestimation of the true level of leukocyte activation, since activated leukocytes expressing these integrins, adhere to the endothelial surface and may be missed by blood sampling.

Some limitations of the present study are the relatively small number of patients included, the low number of reported coronary events, the relatively short duration of follow-up and the heterogeneity of the included patients. Our study group comprised patients with and without a history of CAD, and many of the study subjects already received intensive treatment for primary or secondary intervention purposes. All of the study subjects with progression already had significant coronary sclerosis at inclusion, and the number of patients without clinical coronary sclerosis at inclusion was low. Therefore, no conclusions can be drawn regarding the value of coronary leukocyte activation in the prediction of future CAD in healthy subjects. A strength of the present study is that this is the first study to investigate coronary leukocyte activation in the progression of CAD *in vivo* in humans.

In conclusion, expression of monocyte and neutrophil activation markers CD11b and CD66b, and intracellular MPO in the coronary arteries was not associated with the risk of progression of CAD.

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# Part 2

## Blood cell-bound apolipoprotein B-containing lipoproteins may provide protection against atherosclerosis

# Chapter 7

## Clinical determinants of systemic erythrocyte-bound apolipoprotein B

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## ABSTRACT

**Background:** Apolipoprotein (apo) B bound to circulating erythrocytes (ery-apoB) has been associated with less atherosclerosis. We evaluated the clinical characteristics of subjects with different levels of ery-apoB.

**Methods:** Ery-apoB was measured by flow cytometry in 427 subjects, expressed as mean fluorescent intensity in arbitrary units. Metabolic syndrome criteria were scored.

**Results:** Ery-apoB was inversely associated with CRP (Spearman's rho -0.169, p=0.001) and complement C3 (rho -0.162, p=0.001), and was lower in patients with hypertriglyceridemia (p=0.029), hypertension (p=0.031), abdominal obesity (p=0.007) and low HDL-C (p=0.05). Ery-apoB decreased with increasing metabolic syndrome criteria number (p=0.025). Median ery-apoB was higher in subjects with the wildtype His1208Arg complement receptor 1 (CR1) polymorphism (n=82) than in the mutated allele (n=42) (1.1 (IQR 0.4-2.0) versus 0.6 (IQR 0.2-1.5), p=0.036). In multivariate analysis, markers of inflammation, cardiometabolic factors, ABO blood group and CR1 polymorphism significantly predicted ery-apoB (adjusted $R^2$=0.35, p<0.001). Without ABO blood group, the $R^2$ decreased to adjusted $R^2$ =0.16, p=0.004.

**Conclusions:** Apo B bound to circulating erythrocytes is associated with several characteristics of the metabolic syndrome, inflammation, ABO blood group and CR1-polymorphism. These data may help to better understand the mechanisms involved in the binding of atherogenic lipoproteins to erythrocytes and its clinical relevance.

## INTRODUCTION

Apolipoprotein (apo) B is the structural protein present on the surface of all atherogenic lipoproteins, including very low-density lipoprotein, low-density lipoprotein (LDL), intermediate-density lipoprotein, chylomicrons and their respective remnants [1,2]. Plasma apo B is a sensitive marker of cardiovascular risk, and recent reports indicate that it may even be a better indicator of cardiovascular risk than LDL-cholesterol [3,4]. The migration of apo B-containing lipoproteins and their remnants to the subendothelial space is a major contributor to the development of atherosclerosis. Once in the intima, these lipoproteins can be taken up by macrophages, either in their native form [5] or after (oxidative) modification [6]. This macrophage-lipoprotein interaction may result in the formation of foam cells, causing an inflammatory response which further contributes to the development of atherosclerosis [7].

Apo B-containing lipoproteins are not only present in plasma or serum, but it has been postulated that there is also a 'marginated pool' of lipoproteins attached to the surface of several types of cells, such as endothelial cells [8]. In addition, already in the 1980's it was shown that human erythrocytes bind LDL particles [9]. Our group has confirmed the presence of apo B-containing lipoproteins on circulating erythrocytes (ery-apoB) *in vivo*, using fluorescent labeled antibodies [10,11]. Interestingly, ery-apoB was inversely associated with the presence of clinical and subclinical atherosclerosis [10,11].

We have previously shown that ery-apoB was higher in subjects with blood group O, than those with blood group A, B or AB [11]. However, much is still unknown about the mechanisms involved in the binding of apo B-containing lipoproteins to erythrocytes. In the present study, we aimed to further explore the determinants of ery-apoB in an outpatient setting and to determine their impact on ery-apoB.

## MATERIALS AND METHODS

### Study design and participants

Subjects were recruited from the Department of Cardiology and the Diabetes and Vascular Center of the Franciscus Gasthuis in Rotterdam between 2009 and 2013 [10,11]. In addition, a group of healthy volunteers was recruited among hospital employees. Subjects were included if they were 18 years or older, and if they had no disorders of kidney, liver and thyroid function. The study was approved by the independent Regional Medical Ethics Committee, Maasstad Hospital Rotterdam, the Netherlands. All subjects gave informed consent. Anthropometric measurements, clinical history and use of medication were recorded. Carotid intima media thickness (cIMT) was measured with the ART-LAB (Esaote, Italy) [10].

In order to investigate the effect of the prandial state on ery-apoB, measurements were carried out in healthy volunteers before and after an oral fat loading test in a separate cohort [12]. Healthy volunteers received an oral fat load using fresh cream in a dose of 50 grams of fat per square meter body surface. Blood samples were drawn fasting and 4 and 8 hours after the fat load.

### Laboratory measurements

Parameters for glucose, C-reactive protein (CRP), total cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides were determined using Synchron LX-20 or DxC analyzers (Beckman Coulter, Brea, CA, USA), according to standard procedures in our laboratory. Low-density lipoprotein (LDL) cholesterol values were calculated using the Friedewald formula. Complement component 3 (C3) and serum apolipoproteins A1 an B were measured by nephelometry using an IMMAGE instrument (Beckman Coulter). The ABO blood group was determined by standard procedures using agglutination techniques (Galileo Echo; Immucor Gamma, Heppignies, Belgium).

### Measurement of ery-apoB

The procedure of the measurement of ery-apoB has been described in detail previously [10,11]. In short, a nonfasting blood sample was obtained from a peripheral vein of the forearm. The presence of apo B on the erythrocyte surface was determined using a polyclonal goat antibody directed against human apo B (catalogue number AB742, Millipore, Billerica, MA, USA), with a rabbit-anti-goat antibody conjugated with fluorescein isothiocynate (FITC) (Nordic Immunological Laboratories, Tilburg, the Netherlands) as secondary antibody. As a control for background staining, in parallel a sample was stained with FITC-labeled rabbit-anti-goat, but without the apo B antibody. Ery-apoB was expressed as mean fluorescent intensity in arbitrary units (au) of the signal obtained with the apo B antibody, minus the background signal.

### Complement Receptor 1 single nucleotide polymorphism selection and genotyping

Genomic DNA was extracted from leukocytes obtained from EDTA-treated whole blood by the standard procedure. The most studied three single nucleotide polymorphisms of the CR1 gene are His1208Arg (rs2274567), intron 27 HindIII (rs11118133) and Pro1827Arg (rs3811381). Moreover, these three polymorphisms are in strong linkage disequilibrium.

His1208Arg was analyzed by restriction fragment length polymorphism technique. Primers were designed with the Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) as following; forward 5'-TTC ACA TTG GAT AGC CAG AGC-3'and reverse 5'- CCA GAG GTT TAA TCT CCC TGG A-3' and where used to amplify a 682bp fragment. Amplifications were performed in a final volume of 23 µl containing 10 ng of

DNA template using the AmpliTaq Gold 360 Master Mix (Applied Biosystems, Madrid, Spain). A Verity Thermal Cycler (Applied Biosystems, Madrid, Spain) was used for the PCR. PCR conditions were as follows: 5 min at 95˚C; 40 cycles at 95˚C for 30 s, 57˚C for 30 s and 72˚C for 30 s; and, finally, 72˚C for 7 min. Rsa I restriction enzyme (New England Biolabs, Ipswich, UK) was used for digestion of the amplification product. Hydrolysis of the PCR product with the restriction enzyme was conducted for one hour at 37ºC. The RFLP product containing segments with 520bp and 162bp was identified as wild type (WT) alleles, product containing segments with 520bp, 458bp, 162bp and 62bp was identified as heterozygote and product containing segments with 458bp, 162bp and 62bp was identified as recessive homozygote. RFLP products were analyzed by a 12% polyacrylamide gel electrophoresis.

### Scoring of the metabolic syndrome

The presence of the metabolic syndrome was determined by using the adapted National Cholesterol Education Program (NCEP) criteria [13]. Since no fasting measurements were available in our study population, nonfasting measurements were used. Metabolic syndrome was defined as the presence of at least 3 of the following 5 criteria: abdominal obesity (defined as a waist circumference >102 cm for men and >88 cm for women), hypertriglyceridemia (defined as nonfasting triglycerides >2.5 mmol/L or the use of fibrates), low HDL-C (defined as HDL-C <1.0 mmol/L for men and <1.3 mmol/L for women or the use of lipid-lowering drugs), hypertension (defined as office systolic blood pressure >130 mmHg, diastolic blood pressure >85 or the use of antihypertensive drugs due to a history of hypertension) and insulin resistance (defined as nonfasting glucose >11 mmol/L or the use of glucose lowering drugs).

### Statistics

For normally distributed continuous variables, data are given as mean ± standard deviation in the text, tables and figures. The distribution of ery-apoB, triglycerides, CRP and C3 was skewed. Therefore, these variables were logarithmically transformed before analysis, and data are given as median (interquartile range (IQR)). For clarity, the non-transformed data are shown in tables and figures. Differences between groups were tested with the Independent Samples T test for continuous variables and Chi-square test for binary variables. Differences in ery-apoB in subjects with different numbers of metabolic syndrome criteria were tested with one-way analysis of variance (ANOVA). Differences between fasting and postprandial ery-apoB levels were tested with repeated measures ANOVA with Dunnett's Multiple Comparison Test as post hoc analysis. Correlation analysis was carried out using Spearman correlation statistics.

A multiple regression analysis was carried out with logarithmically transformed ery-apoB as dependent variable, and a history of coronary artery disease, cIMT, HDL-

cholesterol, waist circumference, systolic and diastolic blood pressure, ABO blood group and logarithmically transformed triglycerides, CRP and complement C3 as independent variables, using the enter method. The multiple regression analysis was then repeated without ABO blood group as independent variable.

Statistical analysis was carried out with PASW statistics version 18.0 (IBM SPSS Statistics, New York, United States). P-values below 0.05 (2-tailed) were considered statistically significant.

## RESULTS

### Baseline characteristics (Table 7.1)

A total of 427 patients were included, their baseline characteristics are listed in Table 7.1. Median ery-apoB was 0.9 (IQR 0.4 – 1.6). The distribution of ery-apoB values ranged from 0.0 to 5.5 au and was skewed, with skewness being 1.29 and kurtosis 2.19. The variance of ery-apoB values was 0.74 au.

**Table 7.1** Baseline characteristics of study participants (n=417). For total group, and for those with metabolic syndrome (MetS+) and without MetS (Mets-)

| | Total (n=417) | MetS+ (n=165) | MetS- (n=252) | P-value |
|---|---|---|---|---|
| Age (years) | 58 ± 12 | 62 ± 9 | 56 ± 13 | <0.001 |
| Male gender, n (%) | 229 (54) | 103 (60) | 126 (50) | 0.048 |
| History of CAD, n (%) | 183 (43) | 122 (71) | 61 (24) | <0.001 |
| DM, n (%) | 73 (17) | 70 (41) | 3 (1) | <0.001 |
| Smoking, n (%) | 76 (18) | 33 (19) | 43 (17) | 0.608 |
| BMI (kg/m$^2$) | 27.1 ± 4.7 | 30.0 ± 4.7 | 25.2 ± 3.5 | <0.001 |
| Waist (cm) | 98 ± 14 | 108 ± 13 | 91 ± 11 | <0.001 |
| Systolic BP (mmHg) | 132 ± 18 | 139 ± 19 | 128 ± 16 | <0.001 |
| Use of statin, n (%) | 218 (51) | 150 (87) | 68 (27) | <0.001 |
| Use of ACE-I, n (%) | 106 (25) | 73 (42) | 33 (13) | <0.001 |
| Use of ASA, n (%) | 182 (43) | 109 (63) | 73 (29) | <0.001 |
| Glucose (mmol/L) | 6.2 ± 2.3 | 7.4 ± 2.9 | 5.5 ± 1.3 | <0.001 |
| TG (mmol/L) | 1.29 (0.88 - 1.94) | 1.77 (1.15 - 2.68) | 1.12 (0.77 - 1.58) | <0.001 |
| LDL-C (mmol/L) | 2.8 ± 1.0 | 2.3 ± 0.8 | 3.1 ± 1.0 | <0.001 |
| HDL-C (mmol/L) | 1.39 ± 0.41 | 1.24 ± 0.35 | 1.50 ± 0.41 | <0.001 |
| Apo A-I (g/L) | 1.55 ± 0.31 | 1.47 ± 0.29 | 1.60 ± 0.31 | <0.001 |
| Apo B (g/L) | 0.93 ± 0.27 | 0.87 ± 0.24 | 0.96 ± 0.29 | 0.001 |
| CRP (mg/L) | 2.0 (1.0 - 3.0) | 2.0 (1.0 - 4.0) | 1.0 (1.0 - 3.0) | <0.001 |
| C3 (g/L) | 1.2 (1.0 - 1.4) | 1.3 (1.2 - 1.5) | 1.1 (1.0 - 1.3) | <0.001 |
| cIMT (mm) | 0.648 ± 0.143 | 0.697 ± 0.129 | 0.616 ± 0.142 | <0.001 |
| Ery-apoB (au) | 0.9 (0.4 - 1.6) | 0.7 (0.4 - 1.3) | 0.9 (0.5 - 1.8) | 0.002 |

ASA: acetylsalicylic acid; ACE-i: angiotensin-converting enzyme inhibitor; BMI: body mass index; BP: blood pressure; CAD: coronary artery disease; DM: diabetes mellitus; TG: triglycerides.

## Ery-apoB in relation to inflammation and cardiometabolic factors (Figures 7.1-3)

Ery-apoB was inversely associated with CRP (Spearman's rho -0.169, p=0.001, Figure 7.1A) and with C3 (rho -0.162, p=0.001, Figure 7.1B). Ery-apoB was lower in patients with hypertriglyceridemia (p=0.029, Figure 7.2A) and tended to be lower in patients with low HDL-C (p=0.05, Figure 7.2B). Ery-apoB was lower in patients with hypertension (p=0.031, Figure 7.2C) and in subjects with abdominal obesity (p=0.007, Figure 7.2D). Median ery-apoB decreased with increasing number of metabolic syndrome criteria (p=0.025, Figure 7.3). Ery-apoB was similar in men (0.9, IQR 0.4-1.4) and women (0.9, IQR 0.4-1.8; p=0.43). Ery-apoB did not correlate with age (rho -0.067, p=0.18) or with plasma apo B (rho -0.071, p=0.15).

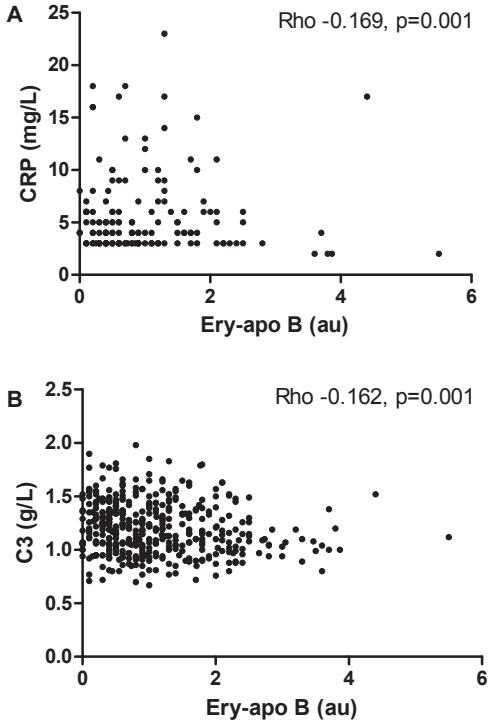


**Figure 7.1.** Ery-apoB in relation to markers of inflammation
Ery-apoB is inversely correlated with CRP (Spearman's rho -0.172, p=0.001) (A) and with complement C3 (Spearman's rho -0.165, p=0.001) (B).

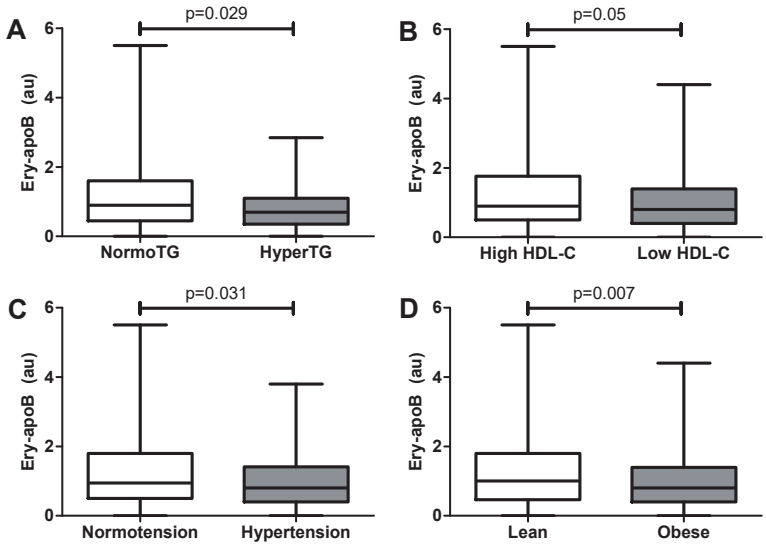


**Figure 7.2.** Ery-apoB in relation to several cardiometabolic factors

Ery-apoB is lower in patients with hypertriglyceridemia (nonfasting triglycerides >2.5 mmol/L or the use of fibrates) (A), ery-apoB tends to be lower in subjects with low HDL-C (HDL-C <1.0 mmol/L for men and <1.3 mmol/L for women or the use of lipid-lowering drugs) (B), ery-apoB is lower in patients with hypertension (office systolic blood pressure >130 mm/Hg, diastolic blood pressure >85 or the use of antihypertensive drugs due to a history of hypertension) (C) and ery-apo is lower in subjects with abdominal obesity (waist circumference >102 cm for men and >88 cm for women) (D). Data are given as median with IQR, with the whiskers depicting minimal and maximal values.

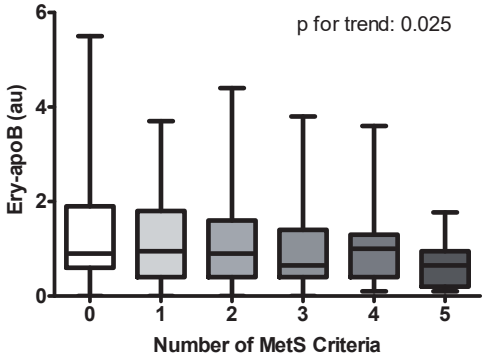


**Figure 7.3.** Ery-apoB and metabolic syndrome

Ery-apoB decreases with increasing number of adapted NCEP criteria for metabolic syndrome (MetS). Data are given as median with IQR, with the whiskers depicting minimal and maximal values.

### Ery-apoB in relation to ABO blood group

Data on ABO blood group was available in 135 subjects. Ery-apoB was almost threefold higher in subjects with blood group O (n=65) than in those with a non-O blood group (n=70) (1.3 (IQR 0.8-2.1) versus 0.5 (IQR 0.1-1.1), p<0.001).

### Ery-apoB in relation to CR1 polymorphism

The His1208Arg CR1 polymorphism was determined in 124 subjects. The minor allele frequency of His1208Arg was 0.18 and the genotype distribution was in Hardy-Weinberg equilibrium. Median ery-apoB was significantly higher in subjects with the wildtype polymorphism (n=82) than in those with a mutated allele (n=42) (1.1 (IQR 0.4-2.0) versus 0.6 (IQR 0.2-1.5), p=0.036, Figure 7.4).



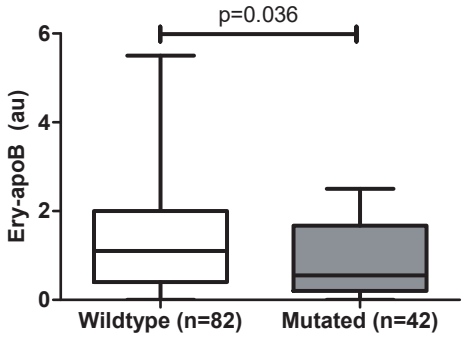


**Figure 7.4.** Ery-apoB and CR1 gene polymorphism
Ery-apoB is significantly lower in those with the minor His1208Arg CR1 allele (heterozygous or homozygous) than those with the wildtype allele.

### Fasting and postprandial ery-apoB levels

To explore the effect of the prandial state on ery-apoB, 12 healthy volunteers underwent an oral fat loading test. Median ery-apoB was 1.1 (0.4-1.9) fasting, 1.2 (0.5-1.8) 4 hours after fat ingestion and 1.4 (0.6-1.9) 8 hours after fat ingestion (p=0.50).

### Multiple regression analysis

In a multiple regression analysis, with ery-apoB as dependent variable and a history of coronary artery disease, cIMT, triglycerides, HDL-cholesterol, CRP, complement C3, waist circumference, systolic and diastolic blood pressure, CR1 polymorphism and ABO blood group as independent variables, the entered variables combined significantly predicted ery-apoB: adjusted $R^2 = 0.35$, p<0.001. When the multiple regression analysis was carried

out without ABO blood group as independent variable, the strength of the regression decreased, to adjusted $R^2$ = 0.16, p=0.004. Without both ABO blood group and CR1 polymorphism, the explained variance decreased even further, to adjusted $R^2$ = 0.049, p=0.002.

## DISCUSSION

The binding of atherogenic apo B-containing lipoproteins to erythrocytes in the circulation may represent a protective mechanism against atherosclerosis. In the current paper we describe that ery-apoB is associated with several cardiometabolic factors, such as abdominal obesity, hypertriglyceridemia, hypertension and inflammation, all of them closely associated to the metabolic syndrome. Ery-apoB decreased with increasing number of criteria for the metabolic syndrome. Furthermore, ery-apoB was associated with CR1 polymorphisms.

As we have postulated before [14], the binding of apo B-containing lipoproteins to circulating erythrocytes may protect the vessel wall from interaction with these lipoproteins, and contribute to clearance of these particles from the circulation. The concept of the removal of harmful particles from the circulation by erythrocytes is not new. Erythrocytes bind immune complexes, which they can transfer to phagocytic cells in the liver and spleen [15]. This process is known as 'immune adherence' [16]. Immune adherence is dependent on the complement system, and the major receptor on the erythrocyte membrane involved is the complement receptor 1 (CR1) [16]. A similar mechanism may be involved in the phenomenon of ery-apoB, in which atherogenic lipoproteins bind to erythrocytes. These apo B-containing lipoproteins may then be detached from the erythrocyte in the liver.

Our group is not the first to demonstrate the binding of lipoproteins to human erythrocytes. Already in the 1980's Hui et al. described the binding of LDL to red blood cells [9]. Recently, Hung et al. demonstrated in mice that transfusion of cholesterol-labeled erythrocytes resulted in delivery of cholesterol to the feces [17]. This study supports the hypothesis of reverse cholesterol transport by erythrocytes, which in fact shows many similarities to the apo B-mediated immune adherence to erythrocytes proposed by our group. The authors postulated a working model, in which lipoprotein-free cholesterol is transferred to erythrocytes in the circulation, and then carried to the liver. Whether this binding of lipoproteins to red blood cells contributes to reverse cholesterol transport in humans needs to be investigated.

In the current study, there was no relation between ery-apoB and plasma apo B, as we have published previously [10,11]. Furthermore, ery-apoB did not change in the postprandial state in healthy subjects. In addition, we have previously shown that dis-

continuation of statin use during six weeks did not affect ery-apoB [11]. This indicates that the metabolism of apo B-containing lipoproteins bound to circulating blood cells differs from that of atherogenic lipoproteins in plasma.

The binding mechanism of atherogenic lipoproteins to circulating erythrocytes is slowly being elucidated. Since erythrocytes do not express classical lipoprotein receptors, such as the LDL-receptor [18], other mechanisms must be involved. We have recently shown *in vitro* and *ex vivo* in humans that CR1, the receptor involved in the transport of immune complexes by erythrocytes, is also involved in the binding of lipoproteins to blood cells, and that this binding is mediated by the classical and the alternative pathways of complement activation [19]. Results from the current study underline the involvement of CR1 in apo B-binding to erythrocytes. Ery-apoB was significantly higher in subjects with the wildtype variant of the His1208Arg CR1 polymorphism, which is associated with higher expression of CR1 on erythrocytes [20]. In a multivariate analysis, CR1 polymorphisms contributed to 11% of the explained variance in ery-apoB.

Results from our multiple linear regression analysis indicate that the level of ery-apoB is determined by many different factors. The set of 11 variables tested here only explained 35% of the variation in ery-apoB. Therefore, other yet still unidentified factors must be involved as well. Interestingly, the variable with the strongest association with ery-apoB was the O blood group. In this extended dataset, in line with our previous findings [11], ery-apoB was almost threefold higher in those with blood group O, as opposed to subjects with a non-O blood group. In this paper, we focused on the additional value of ABO blood group in the prediction of ery-apoB. Result from our multiple regression analysis indicate that approximately 19% of the explained variance in ery-apoB is due to ABO blood group. Currently, we cannot explain this association. It is tempting to speculate that the presence of A and B antigens on the erythrocyte surface may interfere with the binding of lipoproteins to the cell, but mechanistic studies are warranted. Subjects with a non-O blood group are known to have a higher cardiovascular risk than those with blood group O [21,22]. Previous reports have suggested that this increased risk may be attributed to differences in plasma Von Willebrand factor and factor VIII levels [22–24]. Interestingly, differences in ery-apoB may partly explain the increased cardiovascular risk in these subjects as well.

A limitation of the present study is the cross-sectional design. Correlation analyses do not prove causality, and therefore, mechanistic and interventional studies are needed to further investigate the impact of inflammation, metabolic syndrome, CR1 polymorphism and ABO blood group on ery-apoB. In addition, our results need to be confirmed in an independent cohort. Finally, prospective data on the value of ery-apoB in the prediction of cardiovascular risk are lacking. A prospective study by our research group investigating ery-apoB in relation to cardiovascular risk is ongoing.

Before ery-apoB can be used as a potential novel cardiovascular risk marker, the measurement of ery-apoB should be standardized and validated. Quantification of the number of apo B molecules on erythrocyte surface is needed. Previous experiments with $^{125}$I-labeled LDL have indicated that human erythrocytes have approximately 200 binding sites for LDL particles [9]. In addition, it needs to be established what type of lipoproteins are present on the erythrocyte surface. The polyclonal antibody used for the ery-apoB measurement recognizes both apo B48 and apo B100. Therefore, we cannot distinguish between apo B100-containing LDL, very low-density lipoprotein and intermediate-density lipoprotein particles, and apo B48-containing chylomicrons and their remnants. Unpublished results from our group, with antibodies exclusively directed against apo B48 [25], and against apo B100 [26], indicate that the apo B isoform detected with the present measurement technique is predominantly apo B100 and not apo B48.

In conclusion, apo B bound to circulating erythrocytes is associated with several characteristics of the metabolic syndrome, inflammation, CR1 polymorphism and ABO blood group. These data may give clues into the mechanisms involved in the binding of atherogenic lipoproteins to erythrocytes, and may shed new light on lipoprotein metabolism in humans. Ery-apoB could potentially serve as a novel cardiovascular risk marker or pharmacological target.

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# Chapter 8

## Erythrocyte-bound apolipoprotein B predicts mortality and cardiovascular events in patients with high cardiovascular risk: results from a 5-year follow-up study

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Submitted

## ABSTRACT

**Background:** The binding of apolipoprotein (apo) B-containing lipoproteins to circulating erythrocytes (ery-apoB) has been associated with a decreased prevalence of atherosclerosis. In this study, we evaluated ery-apoB as a possible prognostic factor in cardiovascular events and all-cause mortality, in a prospective cohort study.

**Methods:** Ery-apoB was measured by flow cytometry in subjects with and without cardiovascular disease (CVD). The primary endpoint was the cardiovascular event rate. Secondary endpoints were all-cause mortality and the combined endpoint of all-cause mortality and cardiovascular events (any event rate). A Cox-regression analysis with a univariate and multivariate analysis and Kaplan Meier survival analysis was performed.

**Results:** Follow-up data were available of 384 subjects. Subjects were divided according to high (>2.0 au, n=60), intermediate (0.2-2.0 au, n=274) or low (<0.2 au, n=50) ery-apoB. Median follow-up was 1767 days (IQR 1564–2001). In univariate analysis, low ery-apoB was associated with increased all-cause mortality (HR 9.9 (1.2-79.0), p=0.031) and any event rate (HR 3.4 (95% CI 1.3-8.7), p=0.012). In a Cox regression analysis, only a history of CVD was significantly associated with any event rate (HR 3.6 (1.6-8.0), p=0.002), while low ery-apoB showed a trend (HR 2.4 (0.9-6.4), p=0.07). In a subgroup analysis, in subjects with a history of CVD, ery-apoB was significantly associated with all-cause mortality (Log Rank p=0.021) and any event rate (Log Rank p=0.009).

**Conclusion:** Low ery-apoB is associated with increased mortality and cardiovascular risk, especially in patients with a prior history of CVD. These subjects may benefit from more aggressive secondary prevention treatment.

## INTRODUCTION

Cardiovascular disease (CVD) is one of the major causes of mortality and morbidity in the general population. The most important contributor to CVD is atherosclerosis [1]. Lipids and fibrous elements accumulate in the large arteries and narrow the vessel lumen [1]. This is accompanied by an ongoing inflammatory response [1–3]. All atherogenic lipoproteins, including low density lipoprotein (LDL), intermediate density lipoprotein (IDL), very low density lipoprotein (VLDL), chylomicrons and their remnants, have a single apolipoprotein (apo) B molecule as their structural protein [4–6]. Apo B remains with the lipoprotein particle from the moment of formation in the intestine or liver until catabolization and cellular uptake [7]. Apo B yields the best current estimate of the total number of circulating atherogenic particles, representing the atherogenic burden caused by atherogenic lipoproteins [8]. Serum apo B has proved to be a strong predictor of CVD [9].

Studies on apo B as a predictor of CVD have always focused on apo B present in plasma or serum. Our research group has demonstrated that apo B is also present on circulating erythrocytes [2,10,11]. In two cross-sectional studies, a low level of erythrocyte-bound apo B (ery-apoB) was associated with an increased prevalence of CVD and with an increased carotid intima media thickness [10,11]. This may indicate that a high adherence of apo B to erythrocytes reflects a protective situation against atherosclerosis, whereas low ery-apoB seems atherogenic. An explanation for this phenomenon may be that the binding of atherogenic apo B-containing lipoproteins to blood cells prevents their interaction with the endothelium [2]. In addition, high ery-apoB may indicate the presence of an alternative blood-cell mediated lipoprotein transport system in the circulation [2,11].

However, a limitation of previous studies investigating ery-apoB in relation to CVD is their cross-sectional design [10,11]. The aim of the present study was to examine the role of ery-apoB as a possible prognostic factor in all-cause mortality and cardiovascular events, in a prospective cohort study.

## MATERIALS AND METHODS

### Study design and subjects

The study design was a prospective follow-up study of patients and healthy volunteers. Subjects older than 18 years, who visited the outpatient clinics of the Diabetes and Vascular Center and the Department of Cardiology of the Franciscus Gasthuis, Rotterdam, the Netherlands, and the healthy volunteers, were included between July 2009 and February 2013 [10,11]. Exclusion criteria for both patients and healthy controls were the use of any experimental medication or drugs and the use of more than two units of

alcohol per day. The use of statins was not an exclusion criterion, because these drugs do not affect ery-apoB levels [11]. The study was approved by the independent Regional Medical Ethics Committee, Maasstad Hospital Rotterdam, the Netherlands. All study participants gave written informed consent. At baseline, subjects were divided according to low ery-apoB (<0.2 au), intermediate ery-apoB (0.2-2.0 au) and high ery-apoB (>2.0 au), as has been defined previously [11].

### Analytical methods

Anthropometric parameters and cardiovascular history were recorded. Laboratory measurements were carried out according to standard procedures in our laboratory for clinical chemistry [10,11]. Glucose, C-reactive protein (CRP), total cholesterol, high-density lipoprotein cholesterol (HDL-C) and triglycerides were determined using Synchron LX-20 or DxC analyzers (Beckman Coulter, Brea, CA, USA). Low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald formula. Complement component 3 (C3) and serum apo B were measured by nephelometry using an IMMAGE instrument (Beckman Coulter). Blood cell counts were determined using LH750 or DxH800 analyzers (Beckman Coulter).

### Measurement of erythrocyte-bound apo B

The method of the *in vivo* measurement of ery-apoB has been described in detail previously [10–13]. In short, a nonfasting blood sample was obtained from a peripheral vein of the forearm. The presence of apo B on the erythrocyte surface was determined using a polyclonal goat antibody directed against human apo B (catalogue number AB742, Millipore, Billerica, MA, USA), with a rabbit-anti-goat antibody conjugated with fluorescein isothiocynate (FITC) (Nordic Immunological Laboratories, Tilburg, the Netherlands) as secondary antibody. As a control for background staining, a sample was stained in parallel with FITC-labeled rabbit-anti-goat, but without the apo B antibody. Ery-apoB was expressed as mean fluorescent intensity (MFI) in arbitrary units (au) of the signal obtained with the apo B antibody, minus the background signal. The intra-individual coefficient of variation for the measurement of ery-apoB is 9.4% (unpublished data).

### Primary and secondary outcomes

Follow-up data were derived from the digital hospital information system and/or from questionnaires by telephone. If a study participant had experienced any event, the type of event was verified by review of medical records with the participant's permission. The primary outcome was the cardiovascular event rate. The secondary endpoints were all-cause mortality and the combined endpoint of all-cause mortality and cardiovascular events (any event rate). The endpoints were evaluated by an independent investigator at approximately 5 years follow-up. A cardiovascular event was defined as fatal or

nonfatal myocardial infarction, any arterial vascular intervention that had not already been planned at the time of inclusion (e.g. coronary bypass, percutaneous coronary intervention, peripheral vascular surgery or angioplasty/stenting), ischemic stroke, transient ischemic attack, amaurosis fugax, transient global amnesia or hospitalization due to unstable angina pectoris.

### Statistics

For normally distributed continuous variables, data are given as mean ± standard deviation (SD) in the text, tables and figure. The distributions of ery-apoB, triglycerides, CRP and complement C3 were skewed. Therefore, these variables were logarithmically transformed before analysis. For sake of clarity, the non-transformed data are shown in tables and figure, and data are given as median (interquartile range (IQR)). Differences between the low, intermediate and high ery-apoB groups were tested with one-way analysis of variance (ANOVA) for continuous variables, with the Bonferroni test as post-hoc analysis, or with Pearson's chi-square test for dichotomous variables.



A Cox-regression analysis was performed to study the impact of ery-apoB and other covariates on mortality, cardiovascular events and any event, using a two-step approach. First, the impact of ery-apoB and other variables related to cardiovascular risk on all-cause mortality, cardiovascular events and any event was assessed in a univariate survival analysis for each outcome measure separately, using the outcome measure as event, and time to outcome measure as the time to event since inclusion. Significance was assessed with the Log Rank test, a significant effect being $p<0.10$. In the second step, only the significant variables from the univariate analysis differing significantly between with patients with high, intermediate and low ery-apoB, i.e. a history of CVD, the presence of the metabolic syndrome and HDL-C levels, were added as covariates to a Cox-regression model. A p-value <0.05 (two-sided) was regarded as statistically significant. All statistical analyses were performed using PASW statistics version 22.0 (IBM SPSS Statistics, New York, United States). The analyses were performed in the total group, and in the subgroups of subjects without prior CVD and patients with a prior history of CVD.

## RESULTS

### Baseline characteristics

The total cohort consisted of 392 subjects. In 8 subjects, no baseline ery-apoB value was present due to technical difficulties during measurement. Therefore, analyses were performed on 384 subjects. Subjects were divided according to high (>2.0 au, n=60), intermediate (0.2-2.0 au, n=274) or low (<0.2 au, n=50) ery-apoB levels. The median

**Table 8.1** Baseline characteristics of study participants (n=384)

| | Total group (n=384) | Low ery-apoB (n=50) | Intermediate ery-apoB (n=274) | High ery-apoB (n=60) | p-value |
|---|---|---|---|---|---|
| Age (years) | 59 ± 12 | 61 ± 13 | 59 ± 12 | 57 ± 11 | 0.154 |
| Gender (% male) | 57 | 58 | 60 | 43 | 0.056 |
| Waist (cm) | 98.8 ± 14.1 | 100 ± 14 | 100 ± 14 | 93 ± 12 | 0.004*† |
| SBP (mmHg) | 133 ± 18 | 135 ± 16 | 133 ± 19 | 134 ± 18 | 0.727 |
| CVD history (%) | 48 | 64 | 47 | 40 | 0.034*‡ |
| DM (%) | 20 | 26 | 19 | 17 | 0.428 |
| Metabolic Syndrome (%) | 44 | 58 | 44 | 30 | 0.013*‡ |
| ASA use (%) | 45 | 64 | 41 | 44 | 0.014† |
| Statin use (%) | 55 | 68 | 55 | 44 | 0.044*‡ |
| ACE-i use (%) | 27 | 36 | 27 | 19 | 0.013 |
| Glucose (mmol/L) | 6.3 ± 2.3 | 6.6 ± 2.2 | 6.3 ± 2.4 | 6.2 ± 2.2 | 0.634 |
| Triglycerides (mmol/L) | 1.31 (0.90 - 1.99) | 1.6 (0.9 - 2.3) | 1.4 (1.0- 2.0) | 1.0 (0.8 - 1.6) | 0.013*† |
| LDL-C (mmol/L) | 2.7 ± 1.0 | 2.6 ± 0.9 | 2.8 ± 1.1 | 2.7 ± 1.0 | 0.511 |
| Apo B (g/l) | 0.92 ± 0.27 | 0.90 ± 0.25 | 0.93 ± 0.28 | 0.87 ± 0.24 | 0.232 |
| HDL-C (mmol/L) | 1.4 ± 0.4 | 1.2 ± 0.3 | 1.4 ± 0.4 | 1.5 ± 0.5 | 0.018* |
| Leukocyte count ($10^9$/L) | 6.7 ± 1.9 | 7.4 ± 1.6 | 6.6 ± 2.0 | 6.3 ± 1.5 | 0.008*‡ |
| CRP (mg/L) | 2 (1 - 4) | 2 (1- 5) | 2 (1 - 4) | 1 (1 - 3) | 0.022*† |
| Complement C3 (g/L) | 1.20 (1.05 - 1.39) | 1.22 (1.07- 1.45) | 1.23 (1.06 - 1.39) | 1.10 (0.99 - 1.26) | 0.029* |
| Ery-apoB (au) | 0.90 (0.40 - 1.60) | 0.10 (0.10 - 0.20) | 0.85 (0.50 - 1.27) | 2.30 (2.10 - 2.85) | <0.001*†‡ |

Data are given as mean ± SD, median (IQR) or as percentage, p-value represents the difference across the low, intermediate and high groups, as described in the Materials and Methods section.

Post hoc analysis (Bonferroni or adjusted standardized residuals): *Low versus high ery-apoB *p<0.05*; †Intermediate versus high ery-apoB *p<0.05*; ‡Low versus intermediate ery-apoB *p<0.05*

ACE-i: angiotensin-converting enzyme inhibitor; ASA: acetylsalicylic acid; au: arbitrary units; CVD: cardiovascular disease; CRP: C-reactive protein; DM: diabetes mellitus; SBP: systolic blood pressure.

follow-up was 1767 days (IQR 1564 – 2001). Table 8.1 shows the baseline characteristics of the total group, and for each of the ery-apoB classes.

Compared to subjects with high ery-apoB, subjects with low ery-apoB more often had metabolic syndrome, lower HDL-C and higher C3 levels. Subjects with low or intermediate ery-apoB had higher waist circumference, triglycerides and CRP than those with high ery-apoB. Those with low ery-apoB more often had a history of CVD, used statins more often and had higher leukocyte counts than subjects with intermediate or high ery-apoB. Subjects with low ery-apoB more often used acetylsalicylic acid than those with intermediate ery-apoB.



**Mortality and cardiovascular events**

During follow-up, a cardiovascular event occurred in 9% (n=35): 13 subjects developed unstable angina pectoris requiring intervention (percutaneous coronary intervention or bypass surgery), 11 developed fatal or nonfatal myocardial infarction, 6 subjects suffered from nonfatal ischemic stroke, 3 had a transient ischemic attack, 1 subject had an episode of amaurosis fugax and 1 subject experienced transient global amnesia.

Death occurred in 7% (n=26) of the subjects: 3 died of fatal myocardial infarction, 2 of cancer, 1 of end-stage chronic obstructive pulmonary disease, 1 of pneumonia, and in 19 subjects the cause of death was not specified in the medical records.

A total of 14% (n=53) of the subjects reached the combined endpoint of death or cardiovascular event: 27 patients had a nonfatal cardiovascular event and were alive at the end of follow-up, 3 patients had a fatal cardiovascular event and 23 patients died of non-cardiovascular causes.

**Univariate survival analysis**

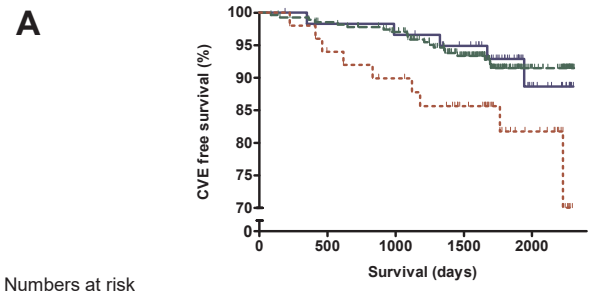
In univariate analysis, a history of CVD, age above 60 years, low HDL-C, hypertension, the presence of the metabolic syndrome and diabetes mellitus were associated with increased risk of any event (death or cardiovascular event). A history of CVD, age above 60 years, low HDL-C and diabetes mellitus were associated with increased risk of mortality. A history of CVD, age above 60 years, low HDL-C, hypertension and presence of the metabolic syndrome were associated with increased risk of future cardiovascular events. Gender, abdominal obesity and hypertriglyceridemia were neither associated with mortality nor with the cardiovascular event rate. Of these factors, the history of CVD, the presence of the metabolic syndrome and HDL-C levels differed significantly between patients with high, intermediate and low ery-apoB (Table 8.1).

**Table 8.2** Univariate survival analysis

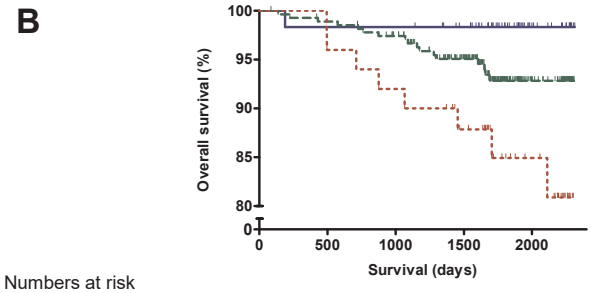
| | CVE | | Death | | Death/CVE | |
|---|---|---|---|---|---|---|
| | HR (95% CI) | *p-value* | HR (95% CI) | *p-value* | HR (95% CI) | *p-value* |
| History of CVD | 6.2 (2.5-15.2) | *<0.001* | 3.2 (1.4-7.7) | *0.008* | 4.6 (2.4-8.8) | <0.001 |
| Age above 60 years | 2.7 (1.1-4.7) | *0.028* | 8.2 (2.5-27.6) | *0.001* | 3.5 (1.9-6.5) | <0.001 |
| Low HDL-C | 2.8 (1.2-6.5) | *0.016* | 3.8 (1.3-11.0) | *0.014* | 3.2 (1.6-6.4) | 0.001 |
| Presence of hypertension | 5.2 (1.6-17.2) | *0.007* | 1.5 (0.6-3.8) | *0.355* | 2.9 (1.3-6.1) | 0.006 |
| Presence of metabolic syndrome | 2.1 (1.0-4.2) | *0.040* | 1.9 (0.9-4.2) | *0.102* | 2.1 (1.2-3.7) | 0.007 |
| Presence of diabetes mellitus | 1.4 (0.6-3.2) | *0.387* | 2.7 (1.2-5.9) | *0.015* | 2.1 (1.2-3.7) | 0.013 |
| Low ery-apoB | 2.4 (0.8-7.2) | *0.112* | 9.9 (1.2-79.0) | *0.031* | 3.4 (1.3-8.7) | 0.012 |
| Intermediate ery-apoB | 0.9 (0.3-2.5) | *0.860* | 4.1 (0.5-30.5) | *0.174* | 1.3 (0.6-3.1) | 0.572 |

The table shows the results of univariate Kaplan Meier analysis of the impact of several factors on risk of cardiovascular event (CVE), mortality or the combined endpoint of death or CVE as hazard ratio (HR) with 95% confidence interval (CI) for: history of cardiovascular disease (CVD as binary variable), age above 60 years (as binary variable), low HDL-C (as binary variable as outlined by NCEP criteria), presence of hypertension (as binary variable as outlined by NCEP criteria), presence of metabolic syndrome (as binary variable as outlined by NCEP criteria), presence of diabetes mellitus (as binary variable), low and intermediate ery-apoB represent the impact compared to subjects with high ery-apoB as reference.

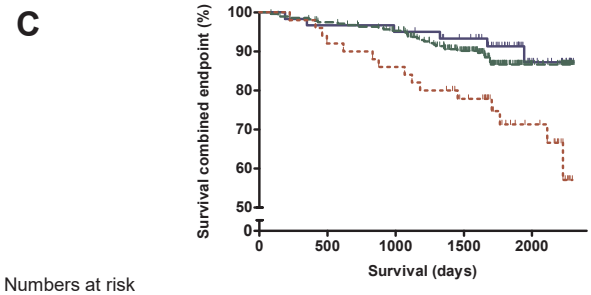
The Kaplan Meier survival curves for the three endpoints are shown in Figure 8.1. The cardiovascular event rate differed significantly between groups (Log Rank p=0.039, Figure 8.1A). However, the hazard ratio for a cardiovascular event was not significantly different between those with low and high ery-apoB (Table 8.2). All-cause mortality and the any event rate were significantly different between the three groups (Log Rank p=0.015 and p=0.002, respectively, Figure 8.1B and 8.1C). Subjects with low ery-apoB had significantly higher risk of death and any event than subjects with high ery-apoB (Table 8.2).




| Numbers at risk | | | | | |
|---|---|---|---|---|---|
| Low ery-apoB | 50 | 46 | 43 | 34 | 16 |
| Intermediate ery-apoB | 274 | 266 | 253 | 201 | 56 |
| High ery-apoB | 60 | 58 | 57 | 52 | 15 |
| p-value (Log Rank) | 0.039 | | | | |




| Numbers at risk | | | | | |
|---|---|---|---|---|---|
| Low ery-apoB | 50 | 48 | 46 | 39 | 21 |
| Intermediate ery-apoB | 274 | 269 | 258 | 210 | 58 |
| High ery-apoB | 60 | 59 | 59 | 54 | 16 |
| p-value (Log Rank) | 0.015 | | | | |




| Numbers at risk | | | | | |
|---|---|---|---|---|---|
| Low ery-apoB | 50 | 46 | 43 | 34 | 16 |
| Intermediate ery-apoB | 274 | 266 | 253 | 201 | 56 |
| High ery-apoB | 60 | 58 | 57 | 52 | 15 |
| p-value (Log Rank) | 0.002 | | | | |

**Figure 8.1.** Univariate survival analysis (Kaplan Meier curves) for cardiovascular event (CVE)-free survival (A), overall survival (B) and the combined endpoint of death or CVE (C), in subjects with high ery-apoB (continuous black line), intermediate ery-apoB (dotted dark grey line) and low ery-apoB (striped light grey line).

**Table 8.3** Baseline characteristics per subgroup with or without a history of prior CVD

| | CVD+ | | | | CVD- | | | |
|---|---|---|---|---|---|---|---|---|
| | Low ery-apoB (n=32) | Intermediate ery-apoB (n=129) | High ery-apoB (n=24) | *p-value* | Low ery-apoB (n=18) | Intermediate ery-apoB (n=145) | High ery-apoB (n=36) | p-value |
| Age (years) | 65 ± 11 | 62 ± 9 | 62 ± 8 | *0.317* | 55 ± 15 | 56 ± 13 | 54 ± 11 | 0.638 |
| Gender (% male) | 66 | 69 | 67 | *0.923* | 44 | 52 | 28 | 0.029 |
| Waist (cm) | 102.8 ± 14.2 | 103.5 ± 13.1 | 97.4 ± 13.6 | *0.135* | 95.1 ± 11.8 | 96.5 ± 14.4 | 90.3 ± 11.0 | 0.062 |
| SBP (mmHg) | 138 ± 17 | 137 ± 20 | 134 ± 14 | *0.749* | 129 ± 12 | 129 ± 16 | 134 ± 21 | 0.310 |
| DM (%) | 38 | 26 | 25 | *0.426* | 6 | 12 | 11 | 0.689 |
| MetS (%) | 75 | 67 | 50 | *0.134* | 28 | 23 | 17 | 0.589 |
| ASA use (%) | 81 | 78 | 92 | *0.148* | 33 | 9 | 11 | 0.010 |
| Statin use (%) | 88 | 88 | 92 | *0.556* | 33 | 24 | 11 | 0.127 |
| ACE-i use (%) | 56 | 47 | 42 | *0.570* | 0 | 9 | 3 | 0.197 |
| Glucose (mmol/L) | 7.0 ± 2.4 | 6.7 ± 2.7 | 7.1 ± 2.5 | *0.631* | 5.8 ± 1.3 | 6.0 ± 2.1 | 5.5 ± 1.7 | 0.436 |
| TG (mmol/L) | 1.77 (1.03-2.50) | 1.50 (1.05-2.20) | 1.17 (0.74-1.84) | *0.070* | 0.98 (0.74-1.88) | 1.26 (0.88-1.85) | 1.01 (0.75-1.54) | 0.108 |
| LDL-C (mmol/L) | 2.4 ± 0.6 | 2.3 ± 0.9 | 1.9 ± 0.6 | *0.042* | 3.0 ± 1.1 | 3.2 ± 1.1 | 3.2 ± 0.8 | 0.792 |
| Apo B (g/l) | 0.88 ± 0.19 | 0.85 ± 0.24 | 0.71 ±0.17 | *0.009*[*†] | 0.93 ± 0.34 | 1.01 ± 0.30 | 0.98 ± 0.22 | 0.518 |
| HDL-C (mmol/L) | 1.2 ± 0.3 | 1.3 ± 0.4 | 1.4 ± 0.4 | *0.071* | 1.4 ± 0.3 | 1.4 ± 0.5 | 1.5 ± 0.5 | 0.323 |
| Leuko (109/L) | 7.6 ± 1.5 | 6.7 ± 1.9 | 6.5 ±1.4 | *0.022*[‡] | 6.9 ± 1.6 | 6.6 ± 2.0 | 6.1 ± 1.5 | 0.312 |
| CRP (mg/L) | 3 (2-6) | 2 (1-4) | 1 (1-2) | *0.007*[*] | 1 (1-3) | 2 (1-3) | 1 ( 1-3) | 0.226 |
| C3 (g/L) | 1.35 (1.08-1.54) | 1.26 (1.13-1.39) | 1.18 (1.02-1.48) | *0.090* | 1.10 (0.99-1.34) | 1.18 (1.00-1.38) | 1.05 (0.97-1.19) | 0.105 |
| Ery-apoB (au) | 0.10 (0.10-0.20) | 0.90 (0.50-1.30) | 2.25 (2.10-2.85) | *<0.001*[*†‡] | 0.10 (0.00-0.20) | 0.83 (0.50-1.20) | 2.35 (2.10-2.96) | <0.001[*††π] |

Data are given as mean ± SD, median (IQR) or as percentage. Subjects were divided according to low ery-apoB (<0.2 au), intermediate ery-apoB (0.2-2.0 au) and high ery-apoB (>2.0 au), p-value for difference between groups within the subgroup of prior history of cardiovascular disease (CVD+) or without history of CVD (CVD-) (ANOVA for continuous variables and Chi-square test for binary variables).

Post hoc analysis within subgroup (Bonferroni): [*]Low versus high ery-apoB *p<0.05*; [†]Intermediate versus high ery-apoB *p<0.05*; [‡]Low versus intermediate ery-apoB *p<0.05*.

ACE-i: angiotensin-converting enzyme inhibitor; ASA: acetylsalicylic acid; au: arbitrary units; CVD: cardiovascular disease; CRP: C-reactive protein; DM: diabetes mellitus; SBP: systolic blood pressure.

In a subgroup analysis, in subjects with a history of CVD, ery-apoB was not significantly associated with cardiovascular event rate (Log Rank p=0.14). However, all-cause mortality (Log Rank p=0.021) and any event rate (Log Rank p=0.009) differed significantly between the three groups in subjects with prior CVD. The hazard ratios for subjects with low ery-apoB, compared to those with high ery-apoB, for mortality (HR 5.8 (0.7-46.7), p=0.098) and any event (HR 2.2 (0.8-6.2), p=0.13), were not significant.

In subjects without a history of CVD, ery-apoB was not associated with cardiovascular event rate, all-cause mortality or any event rate (HR 2.1 (0.1-33.3), p=0.60, HR 1.0 (0.0-1.9·106), p>0.99 and HR 2.1 (0.1-33.5), p=0.60; for low ery-apoB compared to high ery-apoB, respectively). The baseline characteristics of both subgroups are listed in Table 8.3.



### Cox regression analysis

A Cox regression analysis was carried out with ery-apoB, a history of CVD, metabolic syndrome and HDL-C as covariates for the any event rate (Table 8.4). In this model, a history of CVD was associated with an increased any event rate (HR 3.6 (95% CI 1.6-8.0), p=0.002). Low ery-apoB tended to be associated with an increased any event rate (HR 2.4 (95% CI 0.9-6.4), p=0.07). In contrast, HDL-C and the metabolic syndrome were not significantly associated with mortality or cardiovascular events in this multivariate analysis. No Cox regression analysis was carried out in the subgroups of subjects with or without prior CVD, due to the limited event rate.

**Table 8.4** Cox regression analysis

| | HR (95% CI) | p-value |
|---|---|---|
| History of CVD | 3.6 (1.6-8.0) | 0.002 |
| Low HDL-C | 1.3 (0.5-3.2) | 0.583 |
| Presence of metabolic syndrome | 1.0 (0.6 -2.0) | 0.883 |
| Low ery-apoB | 2.4 (0.9-6.4) | 0.070 |
| Intermediate ery-apoB | 1.1 (0.5-2.7) | 0.808 |

The table shows the results of the Cox regression analysis, with the hazard ratio (HR) and 95% confidence interval (CI) for the primary outcome measure (any event, i.e. all-cause mortality or cardiovascular event): history of CVD (as binary variable), HDL-C (as binary variable as outlined by NCEP criteria), presence of metabolic syndrome (as binary variable as outlined by NCEP criteria), low and intermediate ery-apoB represent the impact compared to subjects with high ery-apoB as reference.

## DISCUSSION

This is the first cohort study to report that the binding of apo B-containing lipoproteins to circulating erythrocytes is associated with all-cause mortality and future cardiovascular events. These results are in line with previous cross-sectional observations [10,11],

and suggest that the binding of apo B-containing lipoproteins to erythrocytes in the circulation may be a protective mechanism against atherosclerosis and mortality.

The first report about the phenomenon of LDL bound to the surface of erythrocytes dates back to the 1980s, when Hui and colleagues performed a study investigating chemically modified and native LDL exchanging cholesterol with erythrocytes, which lack the capacity of whole lipoprotein internalization [14,15]. They described that LDL could bind to freshly isolated human erythrocytes, and that this binding resulted in an altered morphology [16,17]. The binding of $^{125}$I-labeled LDL to erythrocytes, measured by spectrometry, appeared to be temperature-independent and concentration-dependent until a plateau was reached. About 200 LDL particles were associated with each erythrocyte at saturation [14].

Roughly thirty years later, our research group developed a flow cytometric method to measure blood cell-bound apo B [2,10–12]. We demonstrated an inverse relationship between ery-apoB and clinical and subclinical atherosclerosis [10,11] and we formulated a new hypothesis on the mechanism of action, proposing similarities with the process of 'immune adherence' [2].

Immune adherence is a well-documented phenomenon: erythrocytes bind immune complexes via the complement receptor 1 (CR1) and carry them from the circulation to the liver and spleen for elimination [18,19]. This mechanism may possibly be involved in elimination of atherogenic lipoproteins from the circulation as well, possibly through uptake by hepatocytes, followed by biliary excretion [2]. Mechanistic studies of the molecular mechanisms involved in the binding of apo B-containing lipoproteins to circulating erythrocytes are needed. The absence of a correlation between plasma apo B and ery-apoB, and the observation that plasma apo B is normal in subjects with low ery-apoB, suggests that the binding mechanism may be independent of lipid metabolism. Since erythrocytes do not carry classical lipoprotein receptors, such as the LDL-receptor or LRP-1 [13,14,16,20], a different receptor must be involved. CR1 is one of the candidates. We have shown *in vitro* that binding of apo B to human leukocytes and to Chinese hamster ovarian cells is, at least in part, mediated by CR1 [13]. This binding seems to be dependent on activation of the complement system. We demonstrated that the binding of native LDL to CR1 is mediated via the classical pathway, and the binding of modified LDL is mediated via both the classical and alternative pathways [13].

The observed association of high ery-apoB and lower cardiovascular event rate could be of clinical relevance. These results imply that ery-apoB may be a valuable additional parameter in the assessment of cardiovascular risk, especially in high-risk subjects. Moreover, if ery-apoB is indeed causally associated with cardiovascular risk, and ery-apoB is found to be modifiable, this would be an interesting target for future therapy. In the present study, ery-apoB did not seem to predict cardiovascular risk or mortality in subjects without a prior history of CVD. This could be due to lack of power, since

only 12 events occurred in this group. Therefore, no conclusions can be drawn on the additional value of ery-apoB in primary prevention strategies. Results of the subgroup analysis in patients with a cardiovascular history suggest that ery-apoB may indeed be a valuable marker in the identification of subjects with a very high risk of recurrent CVD or death. As long as we do not know the precise mechanism by which ery-apoB may protect against atherosclerosis or how to modulate ery-apoB, this measurement may help to select patients who may benefit from intensive secondary prevention strategies.

Despite aggressive statin treatment for secondary prevention, about one in five patients experiences a major cardiovascular event within five years [21]. This residual cardiovascular risk has been attributed to increased plasma apo B, age, body mass index, male gender and diabetes mellitus [22]. Perhaps low ery-apoB is partly involved in this residual cardiovascular risk as well, since most patients with CVD in our cohort were on statins or other lipid lowering drugs.



However, before ery-apoB can be regarded as a marker or a target for cardiovascular risk reduction strategies, certain conditions must be met. For instance, it needs to be established whether the measurement of ery-apoB has consequences for outcomes such as CVD and death when applied on top of the parameters currently used in common practice, requiring further validation studies. Furthermore, the current method for ery-apoB measurement is a time-consuming two-step staining procedure, which makes implementation on large scale difficult. Therefore, the development of a faster and easier measurement procedure is warranted. In addition, no longitudinal data on ery-apoB measurement are available. The average life span of an erythrocyte is 127 days [23]. We cannot exclude that ery-apoB may change over the course of several years, for instance under the influence of lifestyle changes. This is now being investigated by our group.

The main limitation of this study is the heterogeneity in classic cardiovascular risk factors between our groups at baseline. While age, blood pressure and diabetes mellitus did not significantly differ between groups, the higher prevalence of metabolic syndrome, a history of CVD and lower HDL-C in subjects with low ery-apoB may have been a confounder in our study. Results from the multivariate analysis showed that a history of CVD was the most powerful predictor of future cardiovascular events or death. However, in patients with a history of CVD, low ery-apoB was significantly associated with the risk of death or cardiovascular events. In addition, in this subgroup of patients with prior CVD, the prevalence of the metabolic syndrome and levels of HDL-C did not significantly differ between subjects with low, intermediate or high ery-apoB. These data indicate that the association between ery-apoB and the risk of mortality and CVD may be independent from such traditional risk factors as prior CVD, the metabolic syndrome and low HDL-C.

Another limitation is the relatively small number of study subjects and the number of events, despite a median follow-up of 4.8 years. Therefore, correction for other variables was difficult in this cohort. The results need to be confirmed in an independent cohort.

In conclusion, ery-apoB may be a novel cardiovascular risk marker in high-risk subjects. These subjects may benefit from more aggressive secondary prevention treatment.

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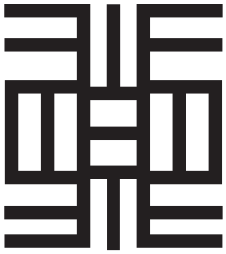
# Chapter 9



## Leukocyte-bound apolipoprotein B in the circulation is inversely associated with the presence of clinical and subclinical atherosclerosis

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Eur J Clin Invest 2016;46(8):690-7

## ABSTRACT

**Background:** Atherosclerosis is a pro-inflammatory condition, in which leukocyte activation plays an important role. The interaction between circulating leukocytes and apolipoprotein (apo) B-containing lipoproteins results in pro-inflammatory changes of these cells. We aimed to evaluate the relationship between apo B bound to circulating leukocytes and atherosclerosis.

**Methods:** Apo B on circulating leukocytes was measured by flow cytometry in subjects with and without cardiovascular disease (CVD), expressed as mean fluorescent intensity in arbitrary units (au). Carotid intima media thickness (cIMT) was measured using B-mode ultrasound. Data are given as median (interquartile range).

**Results:** 396 subjects were included, of whom 183 had a history of CVD. Compared to subjects without CVD, patients with CVD had lower apo B bound to neutrophils (12.7 au (9.8-16.2) and 14.2 au (10.1-17.5), respectively, p=0.038) and to monocytes (2.5 au (1.7-3.1) and 2.7 (1.9-3.6) au, respectively, p=0.025). No differences were found for lymphocyte-bound apo B. Neutrophil- and monocyte-bound apo B were inversely correlated with cIMT (Spearman's rho: -0.123, p=0.017 and -0.108, p=0.035, respectively). Both monocyte- and neutrophil-bound apo B were inversely associated to different factors related to the metabolic syndrome, such as body mass index, triglycerides and complement C3. There was a positive association between erythrocyte-bound apo B and apo B bound to each of the leukocyte classes, possibly reflecting a similar mechanism. Discontinuation of statins in 54 subjects did not influence leukocyte-bound apo B.

**Conclusion:** Unexpectedly, the presence of non-internalized apo B-containing lipoproteins on circulating neutrophil and monocyte membranes may represent a protective mechanism against atherosclerosis.

## INTRODUCTION

The development of atherosclerosis starts when leukocytes and endothelial cells are activated by different well-described stimuli, such as lipoproteins, oxidative stress and glucose [1–5]. According to classical concepts, circulating leukocytes can adhere to the intact endothelium and migrate to the subendothelial space [6]. Subendothelial monocytes mature into macrophages and take up modified lipoproteins, with the risk of foam cell formation [6]. Evidence also suggests a pathophysiological role for lymphocytes and neutrophils in atherogenesis [7,8]. It has been shown *in vivo* in mice that reduced leukocyte adhesion to the endothelium is associated with a reduced atherosclerotic plaque size [9]. These data underscore the importance of the interaction between leukocytes and the endothelium in the development of atherosclerosis.



All atherogenic lipoproteins carry a single apolipoprotein (apo) B molecule [10]. The level of plasma apo B is a strong predictor of cardiovascular risk [11,12]. However, not only plasma apo B, but also apo B-containing lipoproteins bound to circulating blood cells may be associated with cardiovascular disease (CVD). It has been demonstrated that circulating leukocytes interact with apo B-containing lipoproteins [2,13]. For example, these leukocytes internalize dietary fatty acids during the postprandial phase [2,13]. The interaction between leukocytes and lipids results in activation of these leukocytes [2,3,13,14].

We recently demonstrated that not only circulating leukocytes, but also circulating erythrocytes carry apo B-containing lipoproteins [15,16]. A higher level of erythrocyte-bound apo B was associated with a lower prevalence of CVD and with less subclinical atherosclerosis, assessed by measurement of the carotid intima media thickness (cIMT) [15,16].

The aim of the present study was to investigate the relationship between leukocyte-bound apo B-containing lipoproteins and the presence of atherosclerosis. We hypothesized that apo B bound to circulating leukocytes may be positively associated with the presence of clinical and subclinical atherosclerosis, due to the fact that these cells become increasingly activated by these lipoproteins [2,3,13,14].

## MATERIALS AND METHODS

### Subjects and study design

The study design was a cross-sectional study that has been described in detail previously [15,16]. Subjects aged 18 years or above, who visited the outpatient clinics of the Diabetes and Vascular Center or the Department of Cardiology of the Sint Franciscus Gasthuis in Rotterdam for cardiovascular risk assessment between July 2009 and Febru-

ary 2013 were included. In addition, a group of hospital employees were included as healthy volunteers. Exclusion criteria were the use of any experimental medication or drugs and the use of more than two units of alcohol per day. Anthropometric parameters and cardiovascular history were recorded. A history of CVD was defined as the presence of one or more of the following conditions at time of inclusion: ischemic stroke, transient ischemic attack, myocardial infarction, percutaneous coronary intervention due to stable or unstable coronary artery disease, angina pectoris based on clinical characteristics or coronary artery disease based on coronary angiography.

The cIMT was measured with the ART-lab (Esaote, Italy), as described previously [15], by trained and experienced sonographers. ART-lab is based on dedicated ultrasound signal processing, using radio frequency ultrasound signals for automatic detection of the vessel wall [17]. Ultrasound scans were performed with the patient lying in a supine position with the head resting comfortably and the neck slightly hyperextended and rotated in the opposite direction of the probe. The ultrasound images were obtained of the distal 1 cm of the far wall of each common carotid artery (CCA) using B-mode ultrasound, producing two echogenic lines. These lines represent the combined thickness of the intima and media layers of the arterial wall. Each CCA was imaged in three different projections: CCA right side 90-120-150 and CCA left side 210-240-270 degrees. The segments were measured semi-automated in triplicate. Laboratory measurements were carried out according to standard procedures in our laboratory for clinical chemistry [15,16].

In order to investigate the effect of statins on the binding of apo B-containing lipoproteins to leukocytes, a second group of subjects was selected. The design of this statin withdrawal substudy has been published elsewhere [16]. Briefly, subjects who were on statin therapy for primary or secondary prevention were invited to participate. These patients visited the outpatient clinic twice after an overnight fast. During the first visit, while on statins, a baseline lipid profile and neutrophil- and monocyte-bound apo B were determined. After the first visit, participants discontinued their statin use for 6 weeks. After 6 weeks, participants returned to the outpatient clinic, and a fasting lipid profile and leukocyte-bound apo B were determined again. The effect of the withdrawal of statins on the fasting lipid profile in these subjects has previously been published [16]. The effect of statin withdrawal on monocyte- and neutrophil-bound apo B levels has not been reported previously.

Both studies were approved by the independent Regional Medical Ethics Committee, Maasstad Hospital Rotterdam, the Netherlands. All study participants gave written informed consent. Reporting of the study conforms to STROBE statement along with references to STROBE statement and the broader EQUATOR guidelines [18].

### Measurement of leukocyte-bound apolipoprotein B

The method of the *in vivo* measurement of leukocyte-bound apo B has been described in detail previously [2,19]. Nonfasting blood samples were collected in tubes containing 5.4 mg K2 EDTA (Becton Dickinson, Plymouth, United Kingdom). We have previously shown that leukocyte-bound apo B is not influenced by the prandial state [2]. The staining procedure was started within one hour after venipuncture. One mL of blood was taken and erythrocytes were lysed by adding 10 mL of lysis solution (1.5 M ammonium chloride, 100 mM potassium hydrogen carbonate, 0.82 mM EDTA, pH 7.4), followed by 15 minutes of incubation. Samples were washed three times in PBS supplemented with 0.5% BSA (PBS-BSA). Leukocytes were resuspended in 1 mL of PBS-BSA. Twenty µL of leukocyte-suspension was added to tubes containing 2.5 µL of a 25-times diluted polyclonal goat antibody directed against human apo B (catalogue no. AB742, Millipore, Billerica, MA, USA), and 2.5 µL of 10-times diluted anti-CD45 (Beckman Coulter), and incubated for 30 minutes in the dark on ice. In case of a leukocyte count above 10 x $10^9$ cells/L, only 10 µL of leukocyte-suspension was added. The cells were washed once in 1 mL PBS-BSA and the supernatant was drained away. The cells were then incubated with 2.5 µL of a 10-times diluted rabbit anti-goat antibody conjugated with fluorescein isothiocynate (RAG-FITC, Nordic Immunological Laboratories, Tilburg, The Netherlands) for an additional 30 minutes in the dark on ice. In order to determine background signal, each sample was incubated in parallel without the apo B antibody, but with RAG-FITC. To be able to differentiate between different types of leukocytes, a CD45 antibody labeled with PE-Texas Red (Beckman Coulter, Miami, USA) was added simultaneously with the apo B antibodies. Samples were kept in the dark on ice until measurement. A total of 15000 leukocytes per sample were analyzed with an Epics XL-flow cytometer (Beckman Coulter). The different types of leukocytes (lymphocytes, monocytes and granulocytes) were identified based on their side scatter and the level of CD45 on their cell surface. The fluorescent intensity of each cell was expressed as the mean fluorescent intensity, given in arbitrary units (au). The mean fluorescent intensity of each leukocyte population was determined by subtracting the background signal obtained with the RAG-FITC control from the signal measured with the apo B antibody. Erythrocyte-bound apo B was determined as has been described previously [15,16].



### Statistics

For normally distributed continuous variables, data are given as mean ± standard deviation in the text, tables and figures and differences between groups were tested with the Independent Samples T Test. Continuous variables with skewed distributions (triglycerides, C-reactive protein, neutrophil-, monocyte- and lymphocyte-bound apo B) are given as median (interquartile range (IQR)), and differences between groups were tested with the Mann Whitney-U test. Differences in neutrophil- and monocyte-bound

apo B before and after statin withdrawal were tested with the Wilcoxon Signed Ranks test. The Chi-square test was used to test for differences between groups for discrete variables. Correlations were determined with Spearman correlation statistics. Because of anticipated differences in baseline characteristics between patients with and without CVD, the influence of age, gender and waist circumference on neutrophil- and monocyte-bound apo B was assessed in a multiple linear regression analysis. Age was found to be of significant influence, while gender and waist circumference were not. In addition, variables such as statin use, ACE-inhibitor use and chronic kidney disease did not influence leukocyte-bound apo B in multiple regression analysis. Results were therefore also stratified for age below or above 58 years. Statistical analysis was carried out with PASW statistics version 22.0 (IBM SPSS Statistics, New York, United States). P-values below 0.05 (2-tailed) were considered statistically significant.

## RESULTS

### Baseline characteristics

Table 9.1 shows the baseline characteristics of the 396 included subjects, of whom 183 (46%) had a history of CVD (CVD+) and 213 had no CVD (CVD-). The CVD+ group consisted of more males and more patients with diabetes mellitus. CVD+ patients were on average older, had higher body mass index, waist circumference, systolic blood pressure, fasting glucose, triglycerides, neutrophil count, monocyte count and cIMT than CVD- subjects. CVD+ subjects had lower low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, apo B and apo A-I than the CVD- subjects. The use of statins, antihypertensive drugs and acetylsalicylic acid was significantly higher in the CVD+ group. Subclinical atherosclerosis, defined as a cIMT above 0.700 mm, was present in 131 patients (mean age 65 years, range 44-81). In 252 patients (mean age 54 years, range 22-79), no subclinical atherosclerosis was observed. In the remaining 13 patients, no cIMT value was available, due to technical problems during measurement.

**Table 9.1** General characteristics of study participants (n=396)

| | CVD+ (n=183) | CVD- (n=213) | P-value |
|---|---|---|---|
| Male gender | 127 (69) | 81 (38) | <0.001 |
| Diabetes mellitus | 51 (28) | 19 (9) | <0.001 |
| Smoking | 36 (20) | 33 (16) | 0.291 |
| Age (years) | 63 ± 9 | 54 ± 12 | <0.001 |
| Body mass index (kg/m$^2$) | 28.1 ± 4.7 | 26.0 ± 4.4 | <0.001 |
| Waist circumference (cm) | 102 ± 13 | 93 ± 14 | <0.001 |
| Systolic blood pressure (mmHg) | 136 ± 18 | 129 ± 17 | <0.001 |
| Diastolic blood pressure (mmHg) | 78 ± 10 | 79 ± 10 | 0.362 |
| Glucose (mmol/L) | 6.8 ± 2.6 | 5.8 ± 1.9 | <0.001 |
| Triglycerides (mmol/L) | 1.38 (0.99-2.18) | 1.20 (0.86-1.77) | 0.020 |
| Total cholesterol (mmol/L) | 4.4 ± 0.9 | 5.3 ± 1.2 | <0.001 |
| LDL cholesterol (mmol/L) | 2.4 ± 0.8 | 3.2 ± 1.0 | <0.001 |
| HDL cholesterol (mmol/L) | 1.3 ± 0.4 | 1.5 ± 0.4 | <0.001 |
| Apolipoprotein B (g/L) | 0.85 ± 0.22 | 1.00 ± 0.28 | <0.001 |
| Apolipoprotein A-I (g/L) | 1.48 ± 0.28 | 1.61 ± 0.32 | <0.001 |
| C-reactive protein (mg/L) | 2.0 (1.0-4.0) | 2.0 (1.0-4.0) | 0.672 |
| Leukocyte counts (10$^9$ cells/L) | 6.9 ± 1.9 | 6.4 ± 1.9 | 0.030 |
| Neutrophil counts (10$^9$ cells/L) | 4.2 ± 1.6 | 3.8 ± 1.6 | 0.028 |
| Monocyte counts (10$^9$ cells/L) | 0.60 ± .18 | 0.52 ± 0.17 | <0.001 |
| Lymphocyte counts (10$^9$ cells/L) | 1.89 ± 0.55 | 1.92 ± 0.59 | 0.608 |
| Carotid intima media thickness (mm) | 0.702 ± 0.134 | 0.592 ± 0.124 | <0.001 |
| Use of statins | 165 (90) | 35 (16) | <0.001 |
| Use of acetylsalicylic acid | 150 (82) | 22 (10) | <0.001 |
| Use of beta blockers | 89 (49) | 21 (10) | <0.001 |
| Use of diuretics | 49 (27) | 19 (9) | <0.001 |
| Use of ACE-inhibitors | 84 (46) | 10 (5) | <0.001 |
| Use of angiotensin II receptor antagonists | 45 (25) | 22 (10) | <0.001 |
| Use of calcium channel antagonists | 55 (30) | 7 (3) | <0.001 |

Data are given as mean ± standard deviation for normally distributed continuous variables, as median (interquartile range) for continuous variables with skewed distributions (triglycerides, C-reactive protein, erythrocyte-bound apo B), or as number (percentage). P-value for difference between groups.

### Leukocyte-bound apolipoprotein B and clinical atherosclerosis

Compared to CVD- subjects, CVD+ patients had lower apo B bound to neutrophils (12.7 au (9.8-16.2) and 14.2 au (10.1-17.5), respectively, p=0.038) and to monocytes (2.5 au (1.7-3.1) and 2.7 (1.9-3.6) au, respectively, p=0.025) (Figure 9.1). Lymphocyte-bound apo B did not differ between those with and without CVD (0.2 au (0.1-0.3) and 0.2 au (0.1-0.3), respectively, p=0.51). When stratified for age, for patients older than 58 years, monocyte-bound apo B remained significantly lower in patients with CVD (2.5 au (1.7-3.0) and 3.1 au (2.2-3.7), p<0.001), with a trend for lower neutrophil-bound apo B (12.4 au (9.4-16.0) and 13.5 au (10.4-17.3), p=0.092). Neutrophil- and monocyte-bound apo B were no longer significantly different between CVD+ and CVD- patients younger than 58 years.

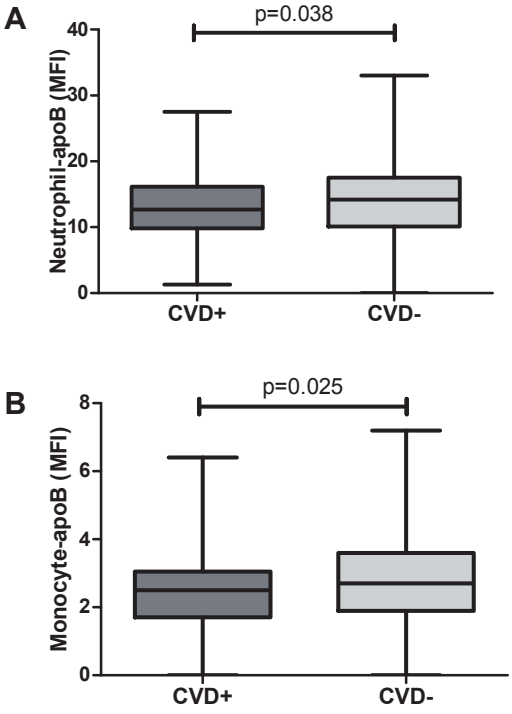


**Figure 9.1.** Neutrophil- and monocyte-bound apo B is lower in subjects with cardiovascular disease (CVD)
Boxplot showing the difference in neutrophil-bound apo B (A) and monocyte-bound apo B (B) between patients with a history of cardiovascular disease (CVD+, n=183, dark grey bars) and those without previous cardiovascular disease (CVD-, n=213, light grey bars). Data are shown as median with the 25$^{th}$ and 75$^{th}$ percentile, with the whiskers depicting minimal and maximal values. P-value for difference between both groups (Mann Whitney-U test). Both neutrophil- and monocyte-bound apo B were lower in CVD+ patients than in CVD- patients.

## Leukocyte-bound apolipoprotein B and subclinical atherosclerosis

Neutrophil-bound apo B and cIMT as well as monocyte-bound apo B and cIMT were weakly inversely correlated (Figure 9.2). No significant correlation was observed between lymphocyte-bound apo B and cIMT. When stratified for age, correlations remained significant for patients younger than 58 years (Spearman's rho -0.173, p=0.020 for cIMT and monocyte-bound apo B; and rho -0.156, p=0.037 for cIMT and neutrophil-bound apo B). For patients older than 58 years, no significant correlations were found.

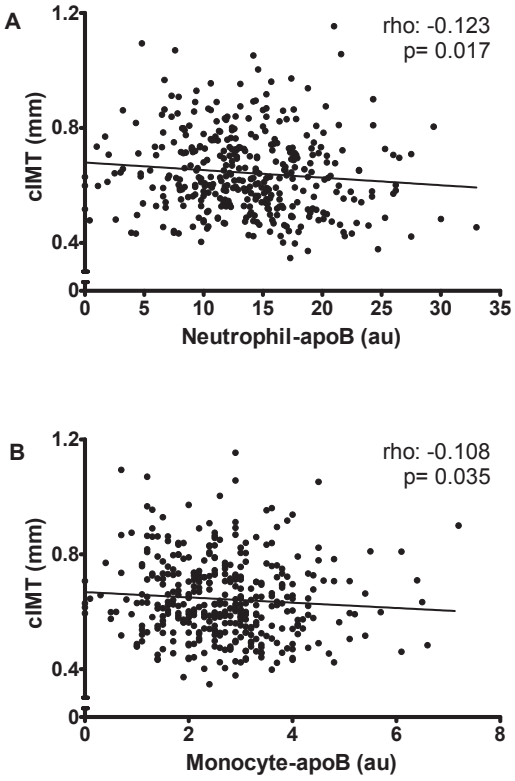


**Figure 9.2.** Neutrophil- and monocyte-bound apo B is inversely correlated with carotid intima media thickness (cIMT)

Correlation plot of neutrophil-bound apo B (A) and monocyte-bound apo B (B) with carotid intima media thickness (cIMT), a marker of subclinical atherosclerosis (Spearman's rho with corresponding p-value). Neutrophil- and monocyte-bound apo B both correlated inversely with cIMT.

### Determinants of leukocyte-bound apo B

Table 9.2 shows the correlation statistics for monocyte- and neutrophil-bound apo B with apo B bound to erythrocytes and lymphocytes, as well as the correlation with several anthropometric and laboratory measurements. Monocyte-bound apo B was positively associated with apo B bound to neutrophils, lymphocytes and erythrocytes. Neutrophil-bound apo B correlated positively with monocyte-, lymphocyte- and erythrocyte-bound apo B. Monocyte and neutrophil-bound apo B were inversely associated with age, body mass index, waist circumference, triglycerides, C-reactive protein, complement C3 and total leukocyte count. We found no correlations between plasma apo B levels and leukocyte-bound apo B (data not shown).

**Table 9.2** Determinants of leukocyte-bound apo B

| | Monocyte-bound apo B | Neutrophil-bound apo B |
|---|---|---|
| Monocyte-bound apo B | - | 0.582 (<0.001) |
| Neutrophil-bound apo B | 0.582 (<0.001) | - |
| Lymphocyte-bound apo B | 0.419 (<0.001) | 0.376 (<0.001) |
| Erythrocyte-bound apo B | 0.313 (<0.001) | 0.190 (<0.001) |
| Age | -0.037 (0.462) | -0.136 (0.007) |
| BMI | -0.133 (0.009) | -0.081 (0.112) |
| Waist | -0.147 (0.004) | -0.098 (0.056) |
| Triglycerides | -0.132 (0.009) | -0.144 (0.004) |
| C-reactive protein | -0.104 (0.045) | -0.132 (0.011) |
| Complement C3 | -0.100 (0.048) | -0.094 (0.063) |
| Leukocyte count | -0.027 (0.591) | -0.139 (0.006) |

Table with correlation statistics (Spearman's rho and corresponding p-value), with monocyte- and neutrophil bound apo B in the vertical columns, and apo B bound to several cell types and anthropometric and laboratory measurements in the horizontal rows.

### Leukocyte-bound apo B after discontinuation of statins

In the separate statin withdrawal cohort, 54 subjects were included. Their baseline characteristics have been published elsewhere [16]. No correlations were found between fasting plasma apo B and neutrophil- or monocyte-bound apo B (Spearman's rho: 0.005, p=0.92 and 0.050, p=0.32, respectively). After statin withdrawal, plasma apo B increased by 59.3 ± 30.8% (p<0.001), while median neutrophil- and monocyte-bound apo B did not change significantly (-1.1 ± 0.6%, p=0.92, and +4.2 ± 4.1%, p=0.49, respectively).

## DISCUSSION

This is the first study describing an association between atherosclerosis and the decreased presence of apo B-containing lipoproteins on circulating neutrophils and monocytes. These results are in contrast with our hypothesis and with current concepts. Since the uptake of lipoproteins by leukocytes results in activation of these leukocytes [2,3,13,14], which has been associated with the development of atherosclerosis [20,21], we hypothesized that the binding of apo B-containing lipoproteins would result in a pro-atherogenic condition. Our data suggest the opposite.

It should be underlined that the observed differences in leukocyte-bound apo B are relatively small. However, the results are consistent across different cell lines (neutrophil granulocytes, monocytes and erythrocytes) and the results are in line with our previous finding of an inverse relationship between apo B-containing lipoproteins bound to circulating erythrocytes with the presence of atherosclerosis [15,16]. These *in vivo* data do not fit into the current concepts regarding the interaction between atherogenic lipoproteins and leukocytes and its effect on the arterial wall.



Atherosclerosis is considered to be an inflammatory disease, and it has been suggested that the uptake of apo B-containing lipoproteins by leukocytes in the subendothelial space is a pro-atherogenic process [6]. Increasing *in vitro* and *in vivo* evidence from different groups suggests that leukocytes also interact with atherogenic lipoproteins in the circulation [2,13]. A possible explanation for the paradoxical inverse relationship between leukocyte-bound apo B and atherosclerosis may be that the binding of atherogenic apo B-containing lipoproteins to leukocytes in the circulation might prevent these lipoproteins from interacting with the endothelium. We assume that the binding of atherogenic apo B-containing lipoproteins to circulating leukocytes may lead to internalization and possibly catabolism in the fasting situation. It has been shown that interaction between endothelial cells and apo B-containing lipoproteins results in increased expression of cell-adhesion molecules on endothelial cells, thereby facilitating the binding of leukocytes to the endothelium, inducing a pro-inflammatory and pro-atherogenic state [22,23]. In this respect, leukocyte-lipoprotein interaction in the circulation may reflect a first line defense mechanism, protecting the vessel wall. This hypothesis is supported by the weak inverse correlation between leukocyte-bound apo B and markers of inflammation and cIMT.

At present, it is unknown how apo B-containing lipoproteins bind to leukocytes and erythrocytes. Multiple receptors for the binding of apo B-containing lipoproteins to circulating leukocytes are known, including the LDL-receptor, LRP-1, the apo B48-receptor and LOX-1 [13,24–26]. We have recently demonstrated that the complement receptor 1 (CR1) may also be involved in the binding of lipoproteins to blood cells [19]. The strong positive association between apo B bound to erythrocytes and to each of the leukocyte

classes suggests a common binding mechanism. Since CR1, in contrast to the aforementioned lipoprotein receptors, is present both on erythrocytes and leukocytes, this may be a candidate receptor. In this respect we proposed a role for erythrocytes as an alternative transport system for atherogenic lipoproteins, whereby these lipoproteins may be cleared from the circulation when erythrocytes circulate through the liver. This is an established clearance mechanism for immune complexes and micro-organisms bound to CR1 on erythrocytes known as "immune adherence" [27]. Lipoproteins bound to leukocytes may follow the same pathway, which would explain the protective effect described here.

One of the main limitations of the present study is the heterogeneity of patients with and without CVD. Several factors associated with the metabolic syndrome, such as triglycerides, body mass index and C3, were inversely correlated to monocyte and neutrophil-bound apo B. With the design of the current study, we cannot determine whether leukocyte-bound apo B is affected by these factors. It would be interesting to investigate in an interventional study whether for instance weight loss or reduction of triglyceride levels positively influences leukocyte-bound apo B. In addition, it would be valuable to investigate the metabolism of leukocyte-bound apo B in a population with high neutrophil or monocyte count.

Another difference between patients with and without CVD in our study was the use of statins. We have shown previously that statins have no effect on erythrocyte-bound apo B [16]. We now demonstrate that also monocyte- and neutrophil-bound apo B did not change 6 weeks after discontinuation of statins in 54 subjects. We cannot exclude the possibility that statin discontinuation beyond six weeks could affect leukocyte-bound apo B, although this seems not very likely due to the dynamic metabolism of these cells.

We need to underline that the investigated population consisted mainly of a selected group of subjects visiting our hospital for cardiovascular risk assessment. Although the population also comprised some healthy volunteers, the observed results may not be representative for the general population.

In order to further establish the role of the binding of apo B-containing lipoproteins to circulating leukocytes in the development of atherosclerosis, large prospective studies are needed, and more detailed investigation of the mechanisms involved will be necessary. The number of apo B-containing lipoproteins bound to monocytes and neutrophils at saturation needs to be established, so that the relative contribution of these cells to the lipoprotein metabolism may be estimated.

In conclusion, similarly to erythrocyte-bound apo B, the presence of clinical and subclinical atherosclerosis was associated with a lower level of apo B bound to circulating monocytes and neutrophils. The binding of apo B-containing lipoproteins to the surface of circulating leukocytes may represent a protective mechanism against atherosclerosis.

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# Chapter 10

## Complement receptor 1 gene polymorphisms are associated with cardiovascular risk

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Atherosclerosis. 2016;257:16-21

## ABSTRACT

**Background:** Inflammation plays a key role in atherosclerosis. The complement system is involved in atherogenesis, and the complement receptor 1 (CR1) plays a role in facilitating the clearance of immune complexes from the circulation. Limited evidence suggests that CR1 may be involved in cardiovascular disease. We investigated the relationship between CR1 gene polymorphisms and cardiovascular risk.

**Methods:** Single nucleotide polymorphisms (SNPs) within the CR1 region (n=73) on chromosome 1 were assessed in 5244 participants in PROSPER (PROspective Study of Pravastatin in the Elderly at Risk) (mean age 75.3 years), who had been randomized to pravastatin 40 mg/day or placebo and followed for a mean of 3.2 years. Logistic regression adjusted for gender, age, country and use of pravastatin was used to assess the association between the SNPs and cardiovascular disease.

**Results:** All 73 SNPs within the genomic region of the CR1 gene on Chromosome 1 were extracted. In this region, strong LD was present leading to the occurrence of two haploblocks. Twelve of the 73 investigated CR1 SNPs were significantly associated with the risk of fatal or nonfatal myocardial infarction (all $p<0.05$). Moreover, most of the associated SNPs were also associated with levels of serum C-reactive protein (CRP). The global p-value for the tail strength method to control for multiple testing was 0.0489, implying that the null hypothesis of no associated SNPs can be rejected.

**Conclusion:** These data indicate that genetic variation within the CR1 gene is associated with inflammation and the risk of incident coronary artery disease.

## INTRODUCTION

Inflammation plays a key role in the development of atherosclerosis. Lipoproteins can migrate into the subendothelial space, where they can induce inflammation, foam cell formation and atherosclerotic plaque development [1,2]. Several inflammatory markers, such as C-reactive protein (CRP), interleukin-6 and leukocyte count have been associated with the risk of cardiovascular disease [3,4], and after myocardial infarction, levels of interleukin-6, CRP and leukocyte count increase [5]. Interleukin-6 is the main stimulant of the hepatic synthesis of acute phase proteins, such as CRP [6].

The complement system is involved in this inflammatory condition leading to atherogenesis. The terminal complement complex, C5b-9, colocalizes with CRP in human atherosclerotic lesions [7]. Furthermore, elevated levels of serum complement component 3 (C3) have been associated with the presence of myocardial infarction, and predict the risk of future coronary events [8,9].



The complement receptor 1 (CR1) is found on the membranes of many types of cells, including erythrocytes, granulocytes, monocytes and macrophages [10]. CR1 is a receptor for the complement proteins C3b and C4b [10]. Via this receptor, erythrocytes carry immune complexes from the circulation to the spleen and liver, where the immune complexes are transferred to phagocytic cells [11]. Recently, it has been postulated that CR1 on erythrocytes may also be involved in the clearance of atherogenic lipoproteins [12–14]. Besides a role in the clearance of immune complexes and lipoproteins, CR1 can also inhibit complement activation, by acting as a co-factor for the factor I-mediated breakdown of C3b into iC3b [10,15].

A large intra-individual variation in the number of CR1 molecules per erythrocyte has been described, with values ranging between less than 100 to 1200 molecules per cell [16,17]. Erythrocytes loose CR1 molecules during their aging process in the circulation [18]. Accelerated and sometimes reversible loss of erythrocyte-CR1 has been described in patients with several types of inflammatory and non-inflammatory diseases, such as severe acute respiratory syndrome [19], tuberculosis [20], insulin-dependent diabetes mellitus [21] and systemic lupus erythematosus [22]. In addition to these conditions, several polymorphisms in the CR1 gene have been related to erythrocyte CR1 expression, including the Pro1827Arg (C5507G, rs3811381) SNP in exon 33, the His1208Arg (A3650G, rs2274567) SNP in exon 22 and the HindIII restriction fragment length polymorphism (RFLP, T520C, rs11118133) in intron 27 [17,23,24]. These three polymorphisms are in strong linkage disequilibrium (LD) [25].

The role of the CR1 gene in cardiovascular disease and atherosclerosis remains unclear. The goal of the present study was to investigate the relationship between CR1 polymorphisms and cardiovascular disease.

## MATERIALS AND METHODS

### Study population

All data come from the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER). A detailed description of the study has been published elsewhere [26,27]. In short, elderly subjects (aged 70-82 years) with a history of vascular disease, or increased vascular risk, were enrolled in Scotland, Ireland and the Netherlands. The primary study endpoint was death from coronary heart disease, non-fatal myocardial infarction (MI), and fatal and non-fatal stroke. Secondary endpoints were the separate coronary and cerebrovascular components of the primary endpoint. The study protocol was approved by the medical ethics committees of each participating institution. All study subjects gave written informed consent. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

### Genotyping

In the PHASE project, whole genome wide screening has been performed, as has been described in detail previously [28]. From this GWAS study, we selected all single nucleotide polymorphisms within the CR1 region on chromosome 1 (n=73) with PLINK software. Taking a relatively stringent R2 threshold (>0.8), using LDlink (https://analysistools.nci.nih.gov/LDlink/) [29], we observed three sets of SNPs. The R2-matrix for these 12 SNPs is displayed in Table 10.1.

### Laboratory measurements

All measurements were performed on samples stored at -80°C. CRP was measured by automated particle-enhanced immunoturbidimetric assay (Roche, UK). This method has an inter- and intra-assay coefficient of variation of 3%. IL-6 was determined using a high-sensitivity enzyme-linked immunosorbent assay (R&D Systems, Abingdon, UK) with inter- and intra-assay coefficients of variation of <6% and sensitivity of 0.16 pg/mL. White blood cell count (WBC) was measured by a fully automated system Sysmex XE-2100 (TOA Medical Electronics, Kobe, Japan).

### Statistical analysis

Allele frequencies were estimated and pairwise LD between the investigated SNPs was estimated and plotted with the program Haploview. Associations between the CR1 SNPs and laboratory measurements were assessed with linear regression adjusted for sex, age, and country. Logistic regression was used to associate the CR1 SNPs with cardiovascular outcomes adjusted for sex, age, country, and pravastatin treatment. All statistical analyses were performed with PLINK statistical software (http://pngu.mgh.harvard.edu/~purcell/plink/download.shtml#download). To control for multiple testing, we

**Table 10.1** $R^2$-matrix for the 12 SNPs associated with myocardial Infarction during follow-up

| RS number | rs10127904 | rs17259038 | rs11118157 | rs6691117 | rs3886100 | rs2274566 | rs7519119 | rs7542544 | rs11803956 | rs12041437 | rs12034383 | rs11803366 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| rs10127904 | 1 | 0.995 | 0.128 | 0.127 | 0.293 | 0.293 | 0.292 | 0.292 | 0.283 | 0.283 | 0.283 | 0.284 |
| rs17259038 | 0.995 | 1 | 0.128 | 0.127 | 0.29 | 0.29 | 0.289 | 0.289 | 0.279 | 0.279 | 0.279 | 0.28 |
| rs11118157 | 0.128 | 0.128 | 1 | 0.994 | 0.404 | 0.422 | 0.424 | 0.424 | 0.424 | 0.424 | 0.424 | 0.422 |
| rs6691117 | 0.127 | 0.127 | 0.994 | 1 | 0.401 | 0.42 | 0.422 | 0.422 | 0.422 | 0.422 | 0.422 | 0.42 |
| rs3886100 | 0.293 | 0.29 | 0.404 | 0.401 | 1 | 0.975 | 0.963 | 0.963 | 0.947 | 0.947 | 0.947 | 0.935 |
| rs2274566 | 0.293 | 0.29 | 0.422 | 0.42 | 0.975 | 1 | 0.988 | 0.988 | 0.971 | 0.971 | 0.971 | 0.959 |
| rs7519119 | 0.292 | 0.289 | 0.424 | 0.422 | 0.963 | 0.988 | 1 | 1 | 0.984 | 0.984 | 0.984 | 0.971 |
| rs7542544 | 0.292 | 0.289 | 0.424 | 0.422 | 0.963 | 0.988 | 1 | 1 | 0.984 | 0.984 | 0.984 | 0.971 |
| rs11803956 | 0.283 | 0.279 | 0.424 | 0.422 | 0.947 | 0.971 | 0.984 | 0.984 | 1 | 1 | 1 | 0.988 |
| rs12041437 | 0.283 | 0.279 | 0.424 | 0.422 | 0.947 | 0.971 | 0.984 | 0.984 | 1 | 1 | 1 | 0.988 |
| rs12034383 | 0.283 | 0.279 | 0.424 | 0.422 | 0.947 | 0.971 | 0.984 | 0.984 | 1 | 1 | 1 | 0.988 |
| rs11803366 | 0.284 | 0.28 | 0.422 | 0.42 | 0.935 | 0.959 | 0.971 | 0.971 | 0.988 | 0.988 | 0.988 | 1 |

Generated using LDlink (https://analysistools.nci.nih.gov/LDlink/) [29].

calculated global p-values using the tail strength method [30]. In short, the tail strength measures how much p-values in a set differ from the expected uniform distribution under the null hypothesis and sums up these differences into a single test statistics. The tail strength is powerful when many small effects exist in the data [30]. Since SNPs are not independent, empirical p-values were computed using permutations. SNPs were permuted as a block, keeping intact the relationship between covariates and outcome. Individual tests were based on a cox-model (*coxph*) and 2×104 permutations were used. Computations were parallelized using package *parallelize.dynamic* [31]. Global p-values were computed using R version 3.2.

## RESULTS

Table 10.2 shows the baseline characteristics of the 5,244 participants of the PROSPER Study. Table 10.3 shows the characteristics of subjects who developed fatal or nonfatal myocardial infarction versus those without myocardial infarction. The mean age of the

**Table 10.2** Baseline characteristics of the 5,244 subjects of the PROSPER study

| | **Participants** |
|---|---|
| **Demographics** | |
| Female, n (%) | 2,720 (51.9) |
| Age, years | 75.3 ± 3.4 |
| Current smoker, n (%) | 1,392 (26.5) |
| Body Mass Index, kg/m$^2$ | 26.8 ± 4.2 |
| History of Diabetes, n (%) | 544 (10.4) |
| History of Hypertension, n (%) | 3,257 (62.1) |
| History of Myocardial infarction, n (%) | 708 (13.5) |
| History of Stroke or TIA, n (%) | 586 (11.2) |
| History of Vascular disease*, n (%) | 2,336 (44.5) |
| Hyperlipidemia**, n (%) | 1,424 (27.2) |
| **Laboratory measurements** | |
| White blood cell count , x10$^9$/L | 6.43 ± 1.62 |
| C-reactive protein, LN transformed, mg/L | 1.13 ± 1.13 |
| Interleukin-6, LN transformed, ng/L | 0.97 ± 0.66 |
| LDL-cholesterol, mmol/L | 3.79 ± 0.80 |
| HDL-cholesterol, mmol/L | 1.28 ± 0.35 |

Data are presented as number (percentage) or mean ± SD. *Any of stable angina, intermittent claudication, stroke, transient ischemic attack, myocardial infarction, peripheral artery disease surgery, or amputation for vascular disease more than 6 months before study entry. **Hyperlipidemia was defined according to NCEP criteria as a total cholesterol > 6.21 mmol/L (240 mg/dL) or triglycerides > 5.5 mmol/L (400 mg/dL).

**Table 10.3** Baseline characteristics, stratified on occurrence of myocardial infarction during follow-up

| | No event (n=4654) | Event (n=590) |
|---|---|---|
| **Demographics** | | |
| Female, n (%) | 2,486 (53.4) | 234 (39.7) |
| Age, years | 75.3 ± 3.3 | 75.7 ± 3.4 |
| Current smoker, n (%) | 1,240 (26.6) | 152 (25.8) |
| Body Mass Index, kg/m$^2$ | 26.8 ± 4.2 | 27.0 ± 4.1 |
| History of Diabetes, n (%) | 461 (9.9) | 83 (14.1) |
| History of Hypertension, n (%) | 2896 (62.2) | 361 (61.2) |
| History of Myocardial infarction, n (%) | 555 (11.9) | 153 (25.9) |
| History of Stroke or TIA, n (%) | 514 (11.0) | 72 (12.2) |
| History of Vascular disease$_a$, n (%) | 1,992 (42.8) | 344 (58.3) |
| Hyperlipidemia | 1283 (27.6) | 141 (23.9) |
| **Laboratory measurements** | | |
| White blood cell count, x10$^9$/L | 6.40 ± 1.61 | 6.64 ± 1.69 |
| C-reactive protein, LN transformed, mg/L | 1.11 ± 1.12 | 1.30 ± 1.12 |
| Interleukin-6, LN transformed, ng/L | 0.96 ± 0.66 | 1.06 ± 0.64 |
| C-reactive protein, mg/L | 5.90 ± 11.54 | 6.87 ± 10.23 |
| Interleukin-6, ng/L | 3.37 ± 3.09 | 3.63 ± 3.11 |
| LDL-cholesterol, mmol/L | 3.80 ± 0.81 | 3.74 ± 0.72 |
| HDL-cholesterol, mmol/L | 1.29 ± 0.35 | 1.24 ± 0.34 |



participants was 75.3 years and approximately 50% were female. Due to the inclusion criteria of PROSPER, almost 50% of the participants had a history of vascular disease. Mean follow-up of study subjects was 3.2 years (range 2.8-4.0).

From the GWAS database including 2.5 million SNPs, we extracted all SNPs within the genomic region of the CR1 gene on Chromosome 1 (n=73). Figure 1 shows the CR1 gene structure with the location of the investigated SNPS. Strong LD is present within the genomic region of CR1 leading to the occurrence of two haploblocks as shown in Figure 1. All SNPs were in Hardy Weinberg equilibrium (p>0.05).

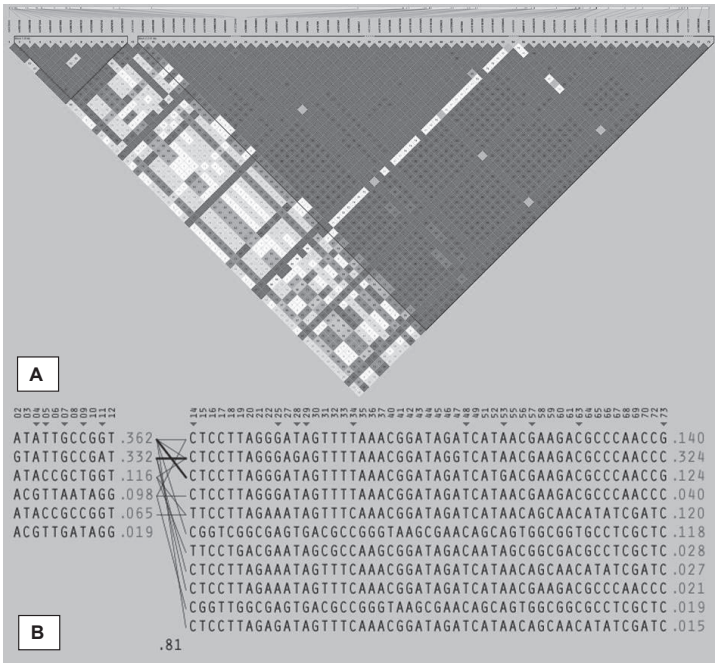


**Figure 10.1.** Details of the genomic region of the CR1 gene
Linkage disequilibrium (LD) between the single nucleotide polymorphisms examined. Two haploblocks are shown with strong LD (dark blocks present LD>95%), with the existing haplotypes with corresponding frequencies.

During follow-up, 12.7% of the patients developed a fatal or nonfatal MI, 4.8% experienced fatal or nonfatal stroke, 5.3% died from cardiovascular disease and the overall mortality was 11.7%.

Twelve of the 73 investigated CR1 SNPs were significantly associated with the risk of fatal or nonfatal MI. These SNPs, and their corresponding odds ratio for MI with 95% confidence intervals, are depicted in panel A of Figure 10.2. The minor allele frequency of the CR1 SNPs rs3886100, rs2274566, rs11118157, rs6691117, rs7519119, rs7542544, rs11803956, rs12041437, rs12034383 and rs11803366 was associated with a decreased risk of coronary artery disease, while the minor allele frequency of SNPs rs10127904 and rs17259038 were associated with increased risk. Seven out of 10 SNPs associated with decreased risk of MI, were also associated with lower levels of CRP. Hence, the two SNPs associated with an increased risk of MI showed an association with higher CRP levels, although not statistically significant. The 12 SNPs were not associated with leukocyte count or levels of Interleukin-6 (data not shown). The global p-value for the tail strength method to control for multiple testing was 0.0489, implying that the null hypothesis of no associated SNPs can be rejected.

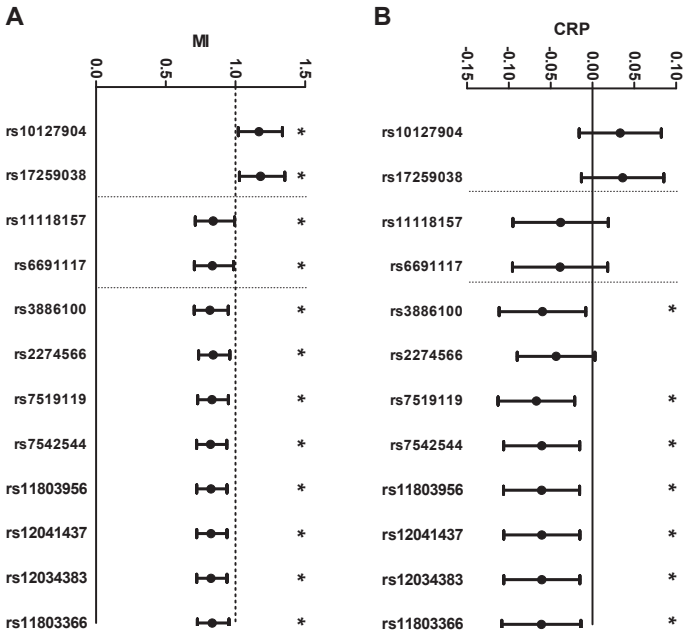


**Figure 10.2.** CR1 SNPs in relation to risk of MI and CRP
The relationship all investigated SNPs significantly associated with risk of future fatal or nonfatal myocardial infarction (odds ratio with 95% confidence interval) (A). The relationship of these SNPs with serum C-reactive protein levels (beta-coefficient with 95% confidence interval) (B). There were three sets of SNPs, which are separated by the dotted lines. *p<0.05

Figure 10.3 shows the association of the investigated SNPs with vascular and all-cause mortality and with fatal and nonfatal stroke. In line with the results for risk of MI, SNPs rs3886100 and rs2274566 were associated with decreased risk of vascular and all-cause mortality, while SNPs rs10127904 and rs17259038 were associated with increased vascular and all-cause mortality. SNPs rs7542544, rs11803956, rs12041437 and rs12034383 were associated with decreased vascular mortality, but not with all-cause mortality. None of the investigated SNPs were associated with the risk of stroke.

Further adjustments for smoking, diabetes, hypertension, average LDL cholesterol during follow-up, history of MI, history of stroke, and log-transformed CRP did not materially change the results (data not shown).

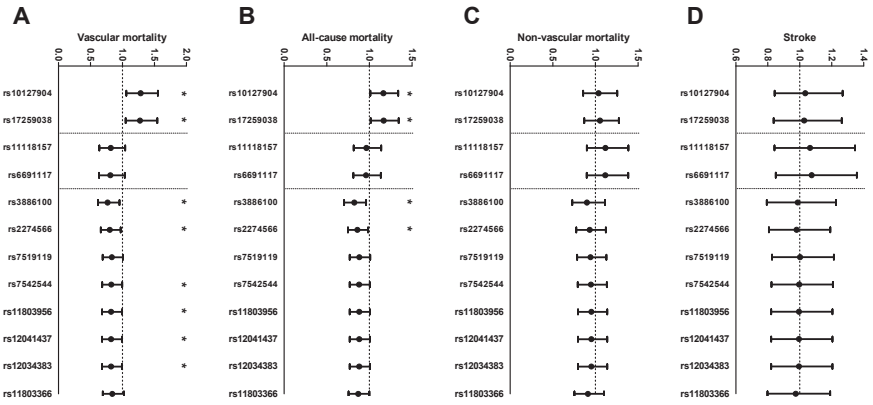


**Figure 10.3.** CR1 SNPs in relation to risk of stroke and mortality

The investigated SNPs in relation to risk of vascular mortality (A), all-cause mortality (B), non-vascular mortality (C) and fatal or nonfatal stroke (D) (OR with 95% CI). There were three sets of SNPs, which are separated by the dotted lines. *p<0.05

## DISCUSSION

We investigated the relationship between CR1 polymorphisms and cardiovascular risk in PROSPER, a prospective randomized trial in which elderly subjects received pravastatin or placebo. After a mean follow-up of 3.2 years, 12 SNPs in the CR1 gene were associated with risk of fatal and nonfatal MI. In addition, several SNPs associated with decreased risk of MI were also associated with lower levels of CRP. Furthermore, many of the investigated SNPs were associated with the risk of vascular and all-cause mortality.

To date, two previous studies have investigated the relationship between CR1 polymorphisms and cardiovascular risk. Buraczynska et al found that the GG phenotype of the Pro1827Arg polymorphism, corresponding with decreased erythrocyte-CR1 expression [23,32], was more prevalent in end-stage renal disease patients with a history of cardiovascular disease than in those without cardiovascular disease [33]. In contrast, Boiocchi et al described a lower prevalence of the GG variant in hypercholesterolemic patients with a history of coronary artery disease than in healthy controls [34]. However, in that study, the total number of patients with the GG variant was only 20. Unfortunately, this polymorphism was not present in the database of our GWAS.

No previous studies have assessed the relationship between the 12 presently described CR1 SNPs with cardiovascular disease. However, the minor alleles of the rs12034383 and rs6691117 SNPs have been associated with lower erythrocyte sedimentation rate [35]. The lower risk of MI which we observed in carriers of the minor allele rs12034383 and rs6691117, and lower levels of CRP in rs12034383, are in line with the previously

reported lower inflammation in these subjects [35]. Several markers of inflammation, including CRP [3], IL-6 [3] and white blood cell count [4], have been associated with increased cardiovascular risk. Several CR1 SNPs that were significantly associated with cardiovascular risk in the present study, were also associated with levels of CRP, but not with IL-6 or white blood cell count. CRP is synthesized in the liver, primarily in response to IL-6 [36]. When CRP binds to phosphocholine groups on the surface of for instance bacteria, this activates the complement system and induces an inflammatory cascade to destroy the ligand [37]. Complexes of CRP with soluble ligands may bind to CR1 on the erythrocyte surface and thus be cleared from the circulation [38]. This direct interaction between CRP and CR1 may explain the observed association with the CR1 SNPs. In the present study we found no evidence for a direct effect of CR1 gene polymorphisms on levels of IL-6 or leukocyte count.



We hypothesize that the mechanism behind the observed relationship between CR1 polymorphisms and the risk of coronary artery disease involves the level of CR1 on circulating erythrocytes. This may affect cardiovascular risk in several ways. Firstly, polymorphisms leading to lower expression of CR1 on these cells may result in reduced clearance of immune complexes from the circulation, resulting in a pro-inflammatory and therefore, a pro-atherogenic situation. Secondly, lower erythrocyte-CR1 expression may be pro-atherogenic due to less binding of atherogenic lipoproteins to erythrocytes. We have previously demonstrated *in vivo* that circulating human erythrocytes are able to bind atherogenic apolipoprotein B-containing lipoproteins [12,39]. The binding of these lipoproteins by circulating blood cells was associated with a reduced prevalence of atherosclerosis [12,39]. Possibly, this binding of atherogenic particles by blood cells prevents their interaction with the endothelium, and we have speculated that erythrocytes contribute to removal of the lipoproteins from the circulation [40]. *In vitro* and *ex vivo* work from our group indicates that CR1 is a likely candidate receptor for the binding of lipoproteins to circulating blood cells [13].

A limitation of the present study is that we did not quantify erythrocyte-bound CR1 in our patients. We do not know whether these SNPs lead to functional changes in the CR1 protein, although the observed relationship with CRP levels and the previously reported relationship with erythrocyte sedimentation rate suggest functionality. Another limitation is that we did not correct for multiple testing with the more common Bonferroni correction. Due to the large number of SNPs analyzed, the Bonferroni adjusted p-value would have been $6.8 \times 10^{-6}$ (0.05 / 73). However, we noticed by checking the haplotype structure within the gene, that there were only two major haploblocks with very strong LD. This indicates that the 73 investigated SNPs were not all independent of each other and that using the Bonferroni correction would have been too conservative. Instead we used the tail strength method to control for multiple testing, since this test is powerful for studies with strong LD (dependency) and with small effect sizes. Since we found

a significant p-value with this test as well, we consider our results to be valid and not false-positive. Another limitation is that these data were obtained in a single cohort, and these findings need to be confirmed in an independent cohort. In addition, we investigated a group of subjects aged 70 years and above. While the impact of CR1 gene polymorphisms, and their influence on inflammation and atherosclerosis, may be most pronounced in a group of elderly individuals, if CR1 gene polymorphisms lead to premature death before the age of 70, we may underestimate the true effect of these polymorphisms. Furthermore, future studies investigating the functionality of these CR1 SNPs are necessary.

In conclusion, genetic variation within the CR1 gene is associated with inflammation and risk of incident coronary artery disease. These data further strengthen the evidence for the role of the complement system in atherosclerosis.

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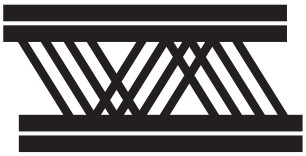
# Chapter 11



## Summary and general discussion

## INTRODUCTION

Atherosclerosis, the underlying pathology of cardiovascular disease, is an inflammatory disease [1,2]. It is thought that especially inflammation induced by different stimuli, such as postprandial lipemia, oxidized lipoproteins leading to the production of radical oxygen species and many others, may be atherogenic. Since humans in Western countries are postprandial during most of the day, this postprandial inflammation may have a large impact on the risk of cardiovascular disease. In **Chapter 1**, we reviewed the contribution of different nutrients on postprandial inflammation and endothelial dysfunction, and the effects of lifestyle and pharmaceutical interventions. After a high-fat meal, both triglyceride-rich lipoproteins and free fatty acids released upon hydrolysis of these particles may contribute to inflammation. This inflammation consists of increased expression of adhesion molecules on endothelial cells [3], neutrophils and monocytes [4–6], increased leukocyte count [7], the production of pro-inflammatory molecules [8], and leads to transient endothelial dysfunction [7]. Not only a high-fat meal, but also a meal containing glucose may induce inflammation and impair endothelial function [7,9,10]. Recent evidence suggests that this postprandial inflammation, considered to be pro-atherogenic and detrimental to endothelial function, can be reduced by a diet rich in anti-oxidants, which can be found in tomatoes [11], strawberries [12], black raspberries [13], orange juice [14] and red wine [15], and monounsaturated fatty acids, such as in extra virgin olive oil [16,17]. In addition, weight loss [18,19] and the use of lipid and glucose lowering drugs [20,21] may be effective in reducing postprandial inflammation.



Despite this increasing evidence for the role of postprandial lipids in inflammation and atherosclerosis, many guidelines for cardiovascular risk management still advise to measure the lipid profile in the fasting state. However, since we are nonfasting for the major part of the day, a fasting measurement may not be representative. In addition, the need to be fasting for blood sampling imposes practical limitations for both patients and physicians. As we describe in **Chapter 2**, a nonfasting lipid profile, i.e. measurement of apolipoprotein (apo) B and non-high-density lipoprotein cholesterol (non-HDL-C), is a sensitive indicator of cardiovascular risk and may even be superior in cardiovascular risk prediction to fasting low-density lipoprotein cholesterol measurement [22,23]. In addition, a nonfasting lipid profile is usually adequate in distinguishing between familial hypercholesterolemia (FH), familial combined hyperlipidemia (FCH) and familial hypertriglyceridemia (FHTG) [24–26]. We therefore, advocate the use of nonfasting lipid profile with apo B or non-HDL-C for cardiovascular risk assessment, evaluation of lipid lowering therapy and differentiation between primary lipid disorders. Very recently, this proposal has been implemented in the European Atherosclerosis Society guidelines.

In part 1 of this thesis, we further focused on inflammation in the postprandial situation, and specifically on the role of lipids and glucose in this inflammation. In part 2,

we investigated potentially beneficial effects of cell-bound atherogenic lipoproteins on atherosclerosis and mortality, and the role of inflammation in this process.

## POSTPRANDIAL GLUCOSE AND LIPIDS INDUCE LEUKOCYTE ACTIVATION

### Glucose-induced leukocyte activation in different degrees of insulin-sensitivity

Although it has been shown that glucose may induce inflammation and leukocyte activation in both type 2 diabetes mellitus (T2DM) patients and in healthy controls [27,28], no previous studies have directly compared glucose-induced leukocyte activation between T2DM patients and healthy controls. In **Chapter 3**, we investigated the effect of glucose ingestion on leukocyte activation in subjects with different degrees of insulin sensitivity. We included insulin-sensitive healthy subjects and insulin-resistant T2DM patients. In addition, we included a group of subjects with non-diabetic reduced insulin sensitivity, i.e. patients with FCH. We demonstrated that both fasting and postprandial leukocyte activation was highest in the most insulin-resistant subjects, the T2DM patients, intermediate in the reduced insulin-sensitive FCH patients and lowest in healthy, insulin-sensitive controls. Furthermore, both acute and chronic glycemia, assessed by measurement of glucose and HbA1c levels, were associated with fasting as well as postprandial leukocyte activation. Leukocyte activation has been linked to the development of diabetic complications, such as nephropathy [29] and retinopathy [30]. Therefore, our results may have implications for the prevention of diabetic microvascular complications, and underline the need for studies investigating the effect of different glucose lowering drugs or lifestyle interventions on fasting and postprandial leukocyte activation.

### Chylomicron remnants induce leukocyte activation

In **Chapter 4**, we focused on a specific atherogenic lipoprotein, the chylomicron remnant, in relation to inflammation. Chylomicrons are synthesized in the intestine. They transport diet-ingested lipids to the circulation, where triglycerides are removed from their content, and chylomicron remnants remain [31]. These remnants are known to be atherogenic, because they can penetrate the vessel wall, where they are trapped, and can induce foam cell formation, inflammation and atherosclerosis [32,33]. No previous studies have investigated whether circulating chylomicron remnants also induce leukocyte activation. We assessed the relation between these particles and leukocyte activation in the circulation. The measurement of apo B48 is a good indicator of the total number of chylomicron remnants, since each particle contains a single apo B48 protein [31]. We demonstrated that in patients with and without atherosclerosis, in the fasting situation, a high level of apo B48 is associated with increased leukocyte activation. Furthermore,

in a group of healthy volunteers, after a high-fat meal, both fasting and postprandial apo B48 were associated with postprandial leukocyte activation. Interestingly, this correlation with postprandial inflammation was not found for triglycerides. We therefore propose that chylomicron remnants not only directly contribute to atherosclerosis in the arterial wall, but also indirectly, by stimulating and activating leukocytes in the circulation, resulting in leukocyte adhesion to the endothelium and transmigration, further stimulating atherosclerosis (Figure 11.1).

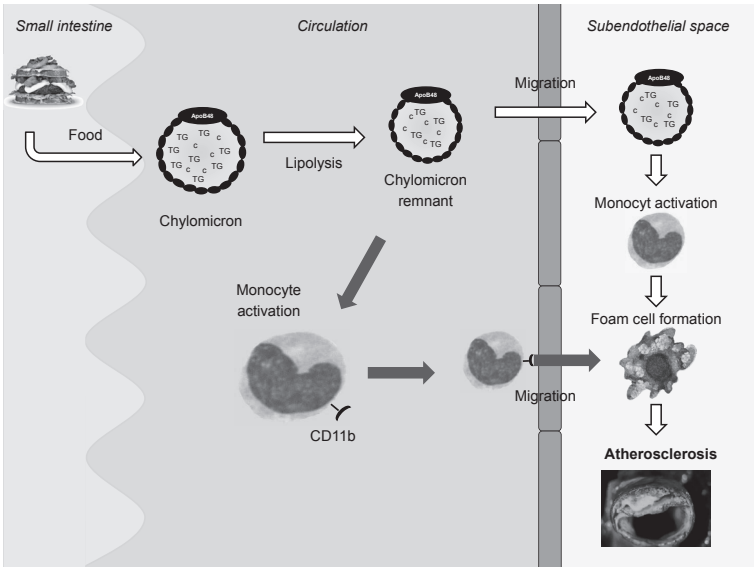



**Figure 11.1.** Proposed pro-atherogenic mechanisms of chylomicron remnants
According to classical concepts, chylomicron remnants directly induce atherosclerosis by migrating to the subendothelial space, where they induce monocyte activation and foam cell formation (white arrows). We propose that in addition, chylomicron remnants may also indirectly induce atherosclerosis, by stimulating and activating leukocytes in the circulation, resulting in leukocyte adhesion to the endothelium and transmigration, further stimulating atherosclerosis (dark arrows).

## Vitamin D and postprandial leukocyte activation

In the first chapters of this thesis, we have shown which factors elicit postprandial inflammation, and which interventions may help to reduce this process. In addition to the factors mentioned in Chapter 1, in theory also vitamin D may have the potential to reduce postprandial inflammation. An unblinded and uncontrolled pilot study by our group in healthy, lean subjects demonstrated that a single dose of 100 000 IU of cholecalciferol (vitamin D3) improved postprandial arterial elasticity in both men and women, while postprandial leukocyte activation was only reduced in women [6]. In **Chapter 5**, we describe the results of a randomized controlled double-blind trial, aiming to confirm these data. In this trial, healthy pre-menopausal overweight or obese vitamin D deficient

women underwent an oral fat loading test, before and after a single low (75 000 IU) or high dose (300 000 IU) vitamin D3. In this select group of female subjects, in contrast to results of the pilot study [6], vitamin D3 did not reduce postprandial arterial stiffness or leukocyte activation. This discrepancy may have several causes. The young and obese volunteers of the randomized trial had higher fasting and postprandial inflammation, reflecting increased inflammation in obesity. This increased inflammation may be more difficult to improve with a single dose of vitamin D. In contrast, arterial stiffness in this study population was lower, in line with previous studies reporting decreased arterial stiffness in young and in obese subjects [34–36]. Although cholecalciferol supplementation increased vitamin D levels significantly in both groups, after vitamin D3 supplementation, 42% of the study subjects still had a vitamin D level below 50 nmol/l. These data suggest that possibly higher vitamin D levels must be reached to achieve beneficial effects. A comparison of several trials investigating inflammation and vascular function after vitamin D supplementation supports this assumption, since in studies reporting positive effects [6,37–41], higher vitamin D levels were reached than in studies reporting no effect of vitamin D supplementation [42–45].

While no short-term beneficial effect of a single dose of 75 000 or 300 000 vitamin D3 on arterial function or leukocyte activation was observed, we found that vitamin D3 may possibly affect complement C3 levels. Fasting complement C3 levels were strikingly high in the study population, and decreased after vitamin D3. This may be of clinical relevance, since C3 has been positively associated with CVD [46,47] and the metabolic syndrome [48]. We speculate that vitamin D reduces C3 secretion by adipocytes, and that this reduced C3 production may lead to increased sensitivity for the effect of the hormone acylation-stimulating hormone, which increases fat storage and glucose transport [49,50].

### Leukocyte activation in the prediction of cardiovascular disease

In the first chapters of this thesis, we have shown that both lipids and glucose elicit leukocyte activation. Several inflammatory markers, including leukocyte count, CRP and C3, have been demonstrated to predict cardiovascular risk [47,51,52]. While increased leukocyte activation has been associated with the presence of CVD [53–56], and we have shown an inflammatory gradient of intracellular MPO towards the coronary circulation in patients with stable CAD [57], little is known about the value of leukocyte activation markers in the prediction of cardiovascular risk in humans. In **Chapter 6**, we describe the results of a prospective study investigating whether the measurement of leukocyte activation in the coronary circulation may help to identify which subjects are at increased risk of future coronary artery disease. We included 99 subjects, and after approximately six years of follow-up, coronary monocyte CD11b, neutrophil CD11b and CD66b and intracellular MPO did not predict progression of coronary artery disease. We

speculate that this negative outcome may be due to the fact that activated leukocytes, with high expression of CD11b or CD66b, more readily adhere to the endothelium and are therefore missed by blood sampling. Furthermore, activated neutrophils transfer MPO to endothelial cells. In this respect, the measurement of CD11b and CD66b on the leukocyte surface, and intracellular MPO, may in fact be an underestimation of the true level of leukocyte activation and inflammation. In addition, studies with larger number of patients and more events may be needed to firmly establish whether the measurement of coronary leukocyte activation may help to predict cardiovascular risk.

### Blood cell-bound apoloprotein B and atherosclerosis

In Part 1 of this thesis, we focused on the negative effects of inflammation. However, inflammation is a physiologic response and it is vital for survival. Inflammation is crucial in recovery from infections and trauma, such as severe burn injury [58]. Children with inherited severe combined immunodeficiencies (SCID), which are the result of a block in T lymphocyte differentiation, often die before the age of one [59]. In addition, inflammation has been demonstrated to be not only detrimental, but also beneficial in cardiovascular disease. For instance, monocytes and macrophages have been proposed to be key regulators of the restoration of damaged myocardial tissue in myocardial infarction [60].



Another mechanism by which inflammation may have a beneficial effect, in this case on the development of atherosclerosis, is by facilitating the binding of atherogenic apo B-containing lipoproteins to erythrocytes (ery-apoB) in the circulation. The association between increased serum apo B and the development of atherosclerosis has been firmly established [23,61,62]. Our research group had developed a method to measure apo B bound to circulating erythrocytes and leukocytes by flow cytometry. We have shown that ery-apoB is inversely associated with the presence of clinical and subclinical atherosclerosis. We hypothesize that this inverse relationship may be due to decreased interaction between atherogenic particles and the endothelium, and increased removal of harmful particles from the circulation by erythrocytes. This process is very likely dependent on inflammation, since the complement receptor 1 (CR1) is the main receptor involved in the binding of harmful particles, such as immune complexes, to circulating erythrocytes, a process known as 'immune adherence' [63]. Once bound to erythrocytes, these particles are transported to the liver and spleen, where they are detached from the erythrocyte surface. The harmful particles are then eliminated from the body, and the intact erythrocyte returns to the circulation.

### Erythrocyte-bound apo B in relation to inflammation, cardiometabolic factors, ABO blood group and future cardiovascular disease

In Part 2 of this thesis, we have further investigated the phenomenon of blood cell-bound apo B. In **Chapter 7**, we describe the results of a clinical cross-sectional study, in

which we explored which factors possibly influence ery-apoB. We measured ery-apoB and several clinical factors in a group of 427 patients and healthy volunteers. We found that ery-apoB was inversely associated with levels of CRP, complement C3 and the presence of the metabolic syndrome.

Interestingly, a major determinant of ery-apoB was the ABO blood group, and subjects with the O blood group, which are known to have lower cardiovascular risk [64,65], had on average almost threefold higher ery-apoB than those with a non-O blood group. While this observation is in line with our results of decreased prevalence of atherosclerosis in those with high ery-apoB [66,67], at this point we have no explanation for this association. We speculate that the presence of A or B antigens on the erythrocyte surface may prevent lipoproteins from interacting with the erythrocytes. All erythrocytes carry a chain of glycoproteins and glycolipids, of which the basis is the H antigen, consisting of a terminal galactose monosaccharide [68]. Blood group O, A, B and AB erythrocytes differ in the number and type of monosaccharides attached to the H antigen [68]. In blood group O, no additional antigens exist. Blood group A erythrocytes have an N-acetylgalactosamine sugar linked to the H antigen, in blood group B this is sugar is galactose. In blood group AB, a mix of both A and B chains are present [68]. Several enzymes capable of enzymatic conversion of blood group A and B erythrocytes to O erythrocytes have been developed [69]. These enzymes digest the N-acetylgalactosamine (A-zyme) of galactose (B-zyme), resulting in A and B erythrocytes with the phenotype of blood group O [69]. We conducted several experiments with erythrocytes treated with such enzymes, kindly provided by professor Martin Olsson, to test whether removal of A or B antigens affects the presence of apo B-containing lipoproteins on the erythrocyte surface. We tested the untreated and treated erythrocytes of several subjects with A, B and O blood group. In enzyme-treated erythrocytes of all blood groups, ery-apoB was higher than in untreated erythrocytes from the same donor (Figure 11.2A), suggesting that the enzyme itself rather than the removal of the A or B antigen may influence the binding of apo B to these cells. Simultaneously, we measured the expression of CR1. On enzyme-treated erythrocytes, CR1 expression decreased, which may implicate that the enzyme treatment affects more than just the N-acetylgalactosamine and galactose monosaccharides (Figure 11.2B). In conclusion, with these experiments we were unable to prove our hypothesis of impaired binding of apo B-containing lipoproteins to erythrocytes due to the presence of A or B antigens on the erythrocyte surface.

In addition to the factors described above, CR1 polymorphism genotyping demonstrated that ery-apoB was higher in subjects with a genetic predisposition for a high expression of CR1 on erythrocytes. These data indicate that while increased inflammation in itself

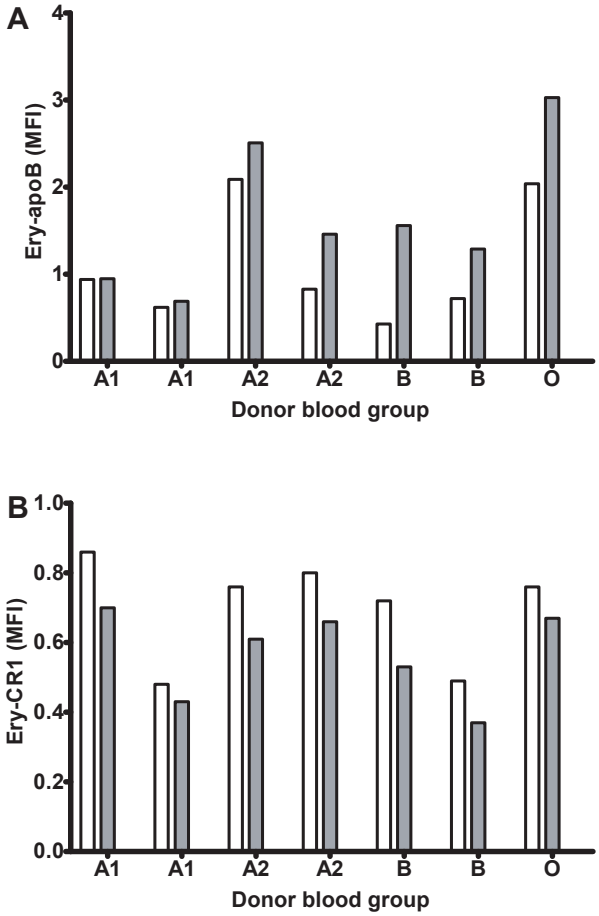



**Figure 11.2.** Effect of enzymatic conversion of erythrocytes on erythrocyte-bound apolipoprotein B and complement receptor 1

Erythrocyte-bound apo B (ery-apoB, mean of measurement in triplicate) (A) and erythrocyte CR1 expression (ery-CR1, mean of measurement in duplicate) (B) on native (white bars) and enzyme-treated (grey bars) erythrocytes of several donors with blood group A1, A2, B and O.

(high CRP or C3 levels) may be detrimental to health, the capacity of erythrocytes to utilize the complement system may protect against atherosclerosis.

Previous studies on ery-apoB have been based on cross-sectional data [66,67], or have focused on the mechanisms behind this phenomenon [70]. In **Chapter 8**, we describe the results of the first prospective study investigating the role of ery-apoB in future cardiovascular disease and mortality. We assessed the occurrence of fatal and nonfatal cardiovascular events and all-cause mortality after a median follow-up of 4.8 years in 384 subjects. Study participants were divided into three groups: those with low (<0.2 au), intermediate (0.2-2.0 au) and high (>2.0 au) ery-apoB. The occurrence of cardiovascular events differed significantly between subjects with low, intermediate and high ery-apoB. There was a trend for a higher cardiovascular event rate in the low ery-apoB group, although the hazard ratio did not reach significance, possibly due to lack of power. Subjects with low ery-apoB had a significantly higher risk of all-cause mortality or the occurrence of either death or a fatal or nonfatal cardiovascular event than those with high ery-apoB. The difference in all-cause mortality and cardiovascular disease between the three groups was also present in a subgroup analysis with only subjects with a history of prior CVD, but not in those without previous CVD. These data indicate that ery-apoB could potentially be used as a novel cardiovascular risk marker, especially for secondary prevention purposes.

### Leukocyte-bound apo B in relation to cardiovascular disease

We have previously shown that not only erythrocytes, but also monocytes and neutrophil granulocytes carry apo B on their surface [71]. In Chapters 1 and 4 of this thesis, we described the pro-inflammatory effect of atherogenic apo B-containing lipoproteins on leukocytes, where these lipoproteins induce increased leukocyte activation [72] and the production of pro-inflammatory cytokines [7,8]. In addition, leukocyte-lipoprotein interaction may result in the uptake of free fatty acids by leukocytes [71]. We therefore hypothesized that increased binding of apo B to circulating leukocytes (leuko-apoB) would be a pro-atherogenic process. We investigated the relation between leuko-apoB and the presence of clinical and subclinical atherosclerosis in a group of 396 subjects. The results of this study are described in **Chapter 9**. Contrary to our hypothesis, and to the current concepts of the effect of interaction between leukocytes and atherogenic lipoproteins, apo B bound to monocytes or neutrophils was *lower* in subjects with a history of cardiovascular disease, and was *inversely* associated with carotid intima media thickness, a measurement of subclinical atherosclerosis.

Both monocyte- and neutrophil-bound apo B were positively correlated with ery-apoB. In addition, similar to the associations found for ery-apoB, as described in Chapter 7, inverse associations were found between monocyte- and neutrophil-bound apo B with cardiometabolic and inflammatory factors, such as body mass index, waist circum-

ference, triglycerides, CRP and complement C3. Combined, these data suggest that a similar binding mechanism may be involved in erythrocytes and leukocytes. A viable candidate receptor for this binding is CR1, since this is one of the few receptors present on both erythrocytes, monocytes and neutrophil granulocytes [4,70,73–75].

A possible explanation for the inverse relation between leuko-apoB and atherosclerosis may be that the binding of atherogenic lipoproteins to circulating leukocytes may prevent interaction between these lipoproteins and the endothelium. Possibly, leukocytes contribute to immune adherence, similar to erythrocytes [76], and in this way remove harmful lipoproteins from the circulation. In addition, we speculate that binding of atherogenic lipoproteins to the leukocyte surface may lead to internalization and degradation of these lipoproteins.

### Complement Receptor 1 gene polymorphisms are associated with cardiovascular risk



As we have mentioned previously, CR1 is very likely involved in the binding of apo B-containing lipoproteins to circulating erythrocytes and leukocytes. Several single nucleotide polymorphisms (SNPs) in the CR1 gene have been associated with erythrocyte CR1 expression [77–79]. We therefore hypothesized that CR1 polymorphisms would be associated with cardiovascular disease, since both high erythrocyte- and leukocyte-bound apo B are associated with less cardiovascular disease. In **Chapter 10**, we investigated the association between SNPs in the CR1 gene with the risk of future cardiovascular disease, in the PROSPER study, a prospective multicenter randomized trial investigating the effect of pravastatin treatment on major cardiovascular events. Data on CR1 SNPs were available in 5244 subjects. After a mean follow-up of 3.2 years, 12 of the 73 investigated CR1 SNPs were significantly associated with the risk of fatal and nonfatal myocardial infarction, and several of these SNPs were also associated with vascular mortality and all-cause mortality. We speculate that this relationship may be (partly) due to differences in erythrocyte- or leukocyte-bound apo B between carriers of the major and minor allele frequencies. Unfortunately, the presence of apo B-containing lipoproteins nor CR1 expression on erythrocytes or leukocytes was quantified in this study. Furthermore, little is known about the functionality of the investigated SNPs, and if functionality is present, which allele frequency would be associated with increased CR1 expression. However, several of the SNPs associated with cardiovascular risk in this study were also associated with levels of CRP, and the minor alleles of two of these SNPs have previously been associated with reduced erythrocyte sedimentation rate [80], which suggests that these SNPs have consequences for levels of inflammation and possibly expression of CR1.

## CLINICAL IMPLEMENTATIONS AND FUTURE DIRECTIONS

In this thesis we have shown that inflammation may have both detrimental and beneficial effects on cardiovascular disease. While glucose and lipids induce inflammation in the postprandial phase, and this postprandial inflammation is linked to the development of atherosclerosis, to some extent inflammation may protect against atherosclerosis as well. In this thesis, we added data to the existing body of literature demonstrating that the binding of apo B-containing lipoproteins to circulating erythrocytes may convey protection against atherosclerosis. In addition, we have shown for the first time that the binding of apo B to circulating leukocytes may be atheroprotective as well. This process is very likely dependent on some level of inflammation, since the binding of native LDL to CR1 is mediated via the classical pathway of complement activation, which is dependent on IgM or IgG antibodies and CRP [70]. For the complement-mediated binding of immune complexes or lipids to CR1, opsonization is necessary [81]. Upon complement activation, C3 is cleaved into C3a and C3b [82], and C3b can then bind to target surfaces and to CR1, facilitating the binding of harmful particles to CR1 [81]. Thus, for the CR1-mediated beneficial effect of the binding of apolipoprotein-B containing lipoproteins to circulating erythrocytes and leukocytes, a low level of inflammation and complement activation is crucial.

Much more research is needed into the binding of apo B-containing lipoproteins to circulating blood cells. Mechanistic studies into the exact processes involved in this binding are necessary, as well as studies determining whether this binding can be influenced. The effect of for instance weight loss or glucose-lowering drugs on ery-apoB is currently under investigation. In addition, it needs to be established whether indeed ery-apoB contributes to the so-called 'residual risk': the occurrence of cardiovascular events despite aggressive lipid-lowering therapy [83]. While our data so far indicate that ery-apoB measurement may help to select patients who may benefit from intensive secondary prevention strategies, this needs to be established in an independent cohort and validated as a cardiovascular risk marker. If ery-apoB is found to be modifiable, studies investigating the causality of the association between ery-apoB and CVD or mortality are needed.

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# Chapter 12

**Appendices**

**APPENDIX A**

# Nederlandse samenvatting

## NIEUWE PRO- EN ANTI-ATHEROGENE EFFECTEN VAN APOLIPOPROTEÏNE B-BEVATTENDE LIPOPROTEÏNEN

### INLEIDING

Hart- en vaatziekten zijn wereldwijd een groot gezondheidsprobleem. Naar schatting overleden in 2012 17,5 miljoen mensen aan de gevolgen van hart- en vaatziekten, dit was ongeveer 31% van alle sterfgevallen. De meeste mensen overleden aan de gevolgen van een hartinfarct of een beroerte. Hart- en vaatziekten zijn niet alleen een belangrijke doodsoorzaak, maar veroorzaken ook veel gezondheidsproblemen. Veel mensen leven met beperkingen die veroorzaakt worden door bijvoorbeeld coronaire hartziekten, verlamming na een beroerte of verminderde mobiliteit door perifeer vaatlijden. De onderliggende oorzaak van hart- en vaatziekten is aderverkalking, ofwel atherosclerose. Atherosclerose kan ontstaan onder invloed van meerdere risicofactoren. De belangrijkste hiervan zijn roken, diabetes mellitus, hoog cholesterol, hoge bloeddruk, overgewicht, lichamelijke inactiviteit, een ongezond dieet (weinig fruit en groenten), alcoholgebruik en psychosociale factoren.



Bij het ontstaan van atherosclerose speelt ontsteking, of inflammatie, een belangrijke rol. Vooral de inflammatie die in de bloedbaan ontstaat na het eten, postprandiale inflammatie, blijkt een ongunstig effect te hebben op atherosclerose. Dit is van groot belang, omdat we de hele dag door eten en dus niet nuchter zijn. In **Hoofdstuk 1** hebben we literatuuronderzoek gedaan naar de bijdrage van allerlei verschillende voedingsstoffen op postprandiale inflammatie, en naar het effect van verschillende leefstijlveranderingen en medicijnen. Na een vetrijke maaltijd ontstaat inflammatie door het vrijkomen van vetten en vrije vetzuren, waardoor er meer en actievere witte bloedcellen (leukocyten) ontstaan. Ook krijgen endotheelcellen, die de binnenkant van de bloedvaten bedekken, meer moleculen op hun oppervlak waaraan deze leukocyten kunnen hechten, en worden er meer moleculen geproduceerd die inflammatie in de hand werken. Al deze veranderingen leiden tot tijdelijke achteruitgang van de vaatfunctie. Niet alleen een vetrijke maaltijd, maar ook een maaltijd met veel glucose kan deze ontsteking en verslechterde vaatfunctie veroorzaken. Postprandiale inflammatie kan geremd worden door een dieet met veel antioxidanten, die onder andere in tomaten,

aardbeien, zwarte frambozen, sinaasappelsap en rode wijn zitten, en met meervoudig onverzadigde vetzuren, zoals in extra vergine olijfolie, maar ook door gewichtsverlies en het gebruik van cholesterol of glucose verlagende medicatie.

Hoewel er dus veel bewijs is dat juist ontsteking in niet-nuchtere toestand bijdraagt aan het ontstaan van hart- en vaatziekten, adviseren veel internationale richtlijnen nog altijd om het lipiden profiel nuchter te bepalen. Deze nuchtere meting heeft verschillende nadelen. Ten eerste is deze meting geen goede afspiegeling van de rest van de dag, omdat we het grootste deel van de dag niet nuchter zijn. Ten tweede is het belastend voor patiënten om niet te mogen eten of drinken voor de bloedafname, en kan een arts niet op een willekeurig moment van de dag het lipiden profiel laten bepalen. In **Hoofdstuk 2** beschrijven we dat een niet-nuchtere meting van bepaalde onderdelen van het lipidenprofiel, namelijk het apolipoproteïne (apo) B en het non-HDL-cholesterol (non-HDL-c) een even goede, en misschien zelfs betere inschatting van het risico op hart- en vaatziekten mogelijk maakt dan een nuchtere meting van het LDL-cholesterol. Bovendien kan met een niet-nuchter lipiden profiel in de meeste gevallen goed onderscheid gemaakt worden tussen verschillende familiare aandoeningen die tot verhoogd cholesterol of triglyceriden leiden.

In het eerste deel van dit proefschrift hebben we ons gericht op ontsteking na de maaltijd, vooral op het effect van glucose en verschillende typen lipiden hierop. In het tweede deel van het proefschrift beschrijven we de gunstige effecten van de binding van bepaalde lipoproteïnen (verbindingen van vetten en eiwitten) aan rode en witte bloedcellen op atherosclerose, en de rol van ontsteking hierbij.

## GLUCOSE EN LIPOPROTEINEN ACTIVEREN LEUKOCYTEN

### Activatie van leukocyten door glucose

De activatie van leukocyten is geassocieerd met allerlei complicaties die kunnen optreden bij suikerziekte, zoals aandoeningen van de ogen en van de nieren. Eerder onderzoek heeft aangetoond dat inname van glucose ontsteking en activatie van leukocyten veroorzaakt bij zowel patiënten met type 2 diabetes mellitus als bij gezonde proefpersonen, maar er was nog nooit onderzoek gedaan waarbij het effect van glucose op deze ontsteking werd vergeleken tussen deze groepen. In **Hoofdstuk 3** hebben we het effect van glucose inname op activatie van leukocyten onderzocht in verschillende groepen: gezonde proefpersonen, een patiëntengroep die een verminderde gevoeligheid voor het hormoon insuline heeft (patiënten met familiaire gecombineerde hyperlipidemie) en patiënten met type 2 diabetes mellitus, die insuline ongevoelig zijn.

Met dit onderzoek hebben we laten zien dat de activatie van leukocyten na inname van glucose het grootst was bij de patiënten met suikerziekte, gemiddeld was bij de patiënten met familiaire gecombineerde hyperlipidemie en het laagst bij de gezonde proefpersonen. Het effect van glucose op ontsteking nam dus toe bij een toenemende mate van insuline-ongevoeligheid. Daarnaast was zowel een kortdurend als een langdurig hoog glucose geassocieerd met leukocyten activatie, zowel in nuchtere toestand als na de maaltijd. Dit onderzoek heeft mogelijk gevolgen voor het voorkomen van de complicaties van suikerziekte, en toont aan dat er meer onderzoek nodig is naar het effect van medicijnen die de bloedsuiker verlagen en leefstijl interventies op leukocyten activatie na de maaltijd.

### Activatie van leukocyten door chylomicron remnants

In **Hoofdstuk 4** hebben we onderzoek gedaan naar de rol die een bepaald type atherogeen lipoproteïne, het chylomicron remnant, speelt bij inflammatie. Chylomicronen worgen gesynthetiseerd in de darm en transporteren lipiden vanuit ons voedsel naar de circulatie, waar triglyceriden worden afgesplitst, waardoor er chylomicron remnants overblijven. Deze remnants kunnen de vaatwand binnendringen en daar schuimcelvorming, inflammatie en atherosclerose stimuleren. Dit was het eerste onderzoek naar het directe effect van chylomicron remnants op leukocyten activatie in de circulatie. Omdat ieder chylomicron remnant één apo B48 eiwit op het oppervlak heeft, is een meting van apo B48 een goede afspiegeling van het totale aantal chylomicron remnants. Ons onderzoek toont aan dat in de nuchtere situatie een hoog apo B48 geassocieerd is met meer leukocyten activatie, zowel bij patiënten met als zonder atherosclerose. Na een vetrijke maaltijd was zowel nuchter als postprandiaal apo B48 geassocieerd met postprandiale leukocyten activatie. Dit wijst erop dat chylomicron remnants mogelijk niet alleen direct in de vaatwand bijdragen aan atherosclerose, zoals nu wordt aangenomen, maar ook indirect, door het stimuleren en activeren van leukocyten in de circulatie.



## VITAMINE D EN POSTPRANDIALE LEUKOCYTEN ACTIVATIE

Er is steeds meer bewijs dat vitamine D mogelijk ook postprandiale inflammatie remt. Een pilot studie van onze onderzoeksgroep liet zien dat een enkele dosis van 100 000 IE vitamine D3 postprandiale vaatelasticiteit bij zowel mannen als vrouwen verbeterde, terwijl postprandiale leukocyten activatie alleen werd geremd bij vrouwen. In **Hoofdstuk 5** hebben we in een dubbelblinde gerandomiseerde studie geprobeerd deze resultaten bij vrouwen te bevestigen. Bij deze studie ondergingen gezonde, premenopauzale vrouwen met overgewicht een vetbelastingstest, voor en week na een enkele lage (75 000 IE) of hoge dosis (300 000 IE) vitamine D3. Bij deze studie vonden we geen

gunstig effect van vitamine D3 op postprandiale vaatelasticiteit of leukocyten activatie, in tegenstelling tot onze eerdere studie. Dit verschil kan met meerdere factoren te maken hebben. Ten eerste hadden de proefpersonen in de gerandomiseerde studie meer inflammatie, mogelijk door hun overgewicht, waardoor mogelijk meer vitamine D nodig is voor een gunstig effect. Ten tweede was de vaatelasticiteit bij deze relatief jongere vrouwen lager. Ten derde had 42% van de proefpersonen in de gerandomiseerde studie ook na suppletie nog een vitamine D tekort, en mogelijk moeten hogere vitamine D spiegels bereikt worden voor gunstige effecten. Hoewel vitamine D in dit onderzoek geen effect had op leukocyten activatie, was de nuchtere complement C3 spiegel na beide doseringen vitamine D significant lager. Dit kan van belang zijn, omdat C3 geassocieerd is met het atherosclerose en het metabool syndroom. Mogelijk remt vitamine D de productie van C3 door adipocyten, en zorgt dit voor betere gevoeligheid voor het hormoon 'acetylation-stimulating hormone', wat vetopslag en glucose transport bevordert.

## LEUKOCYTEN ACTIVATIE IN HET VOORSPELLEN VAN HART- EN VAATZIEKTEN

Verschillende inflammatoire markers, waaronder leukocyten aantal, CRP en C3, zijn geassocieerd met cardiovasculair risico. In **Hoofdstuk 6** hebben we onderzocht of het meten van leukocyten activatie in de coronaire circulatie kan voorspellen welke patiënten een verhoogd cardiovasculair risico hebben. Na gemiddeld zes jaar follow-up bleek coronaire leukocyten activatie (de marker CD11b op monocyten en neutrofiele granulocyten, CD66b op neutrofiele granulocyten en intracellulair MPO) niet geassocieerd te zijn met de progressie van coronaire hartziekten. Een mogelijke verklaring voor deze negatieve uitkomst is dat leukocyten met de integrines CD11b en CD66b makkelijker aan het endotheel hechten en deze geactiveerde leukocyten dus gemist worden bij bloedafname. Bovendien geven geactiveerde leukocyten MPO af aan endotheelcellen en is een meting hiervan een onderschatting van het daadwerkelijke niveau van leukocyten activatie.

## BLOEDCEL GEBONDEN APOLIPOPROTEINE B EN ATHEROSCLEROSE

In het eerste deel van dit proefschrift hebben we ons gericht op de negatieve kanten van inflammatie. Inflammatie is echter ook een fysiologische reactie die essentieel is voor overleving. Zo is inflammatie belangrijk bij het bestrijden van infecties en herstel van letsel. Er zijn ook aanwijzingen dat inflammatie niet alleen atherogeen is, maar dat

bijvoorbeeld monocyten en macrofagen ook een belangrijke rol spelen bij het herstel van beschadigd myocardweefsel na een myocardinfarct.

Een andere manier waarop inflammatie wellicht een positief effect heeft op atherosclerose, is door het faciliteren van de binding van atherogene apo-B bevattende lipoproteïnen aan erytrocyten in de circulatie. Serum apo B is sterk positief geassocieerd met het ontstaan van atherosclerose. Onze onderzoeksgroep heeft een methode ontwikkeld om apo B gebonden aan het oppervlak van erytrocyten en leukocyten te meten met een flow cytometer. We hebben aangetoond dat de binding van apo B-bevattende lipoproteïnen aan erytrocyten (ery-apoB) omgekeerd geassocieerd is met de aanwezigheid van atherosclerose, mogelijk door een verminderde interactie tussen deze atherogene deeltjes en het endotheel, en door het verwijderen van deze deeltjes uit de circulatie. Dit proces is zeer waarschijnlijk afhankelijk van inflammatie, en een belangrijke receptor hierbij is de complement receptor 1 (CR1). Deze receptor bindt schadelijke deeltjes aan erytrocyten, zodat deze via de lever en milt kunnen worden afgevoerd.



**Erytrocyt-gebonden apolipoproteïne B in relatie tot inflammatie, cardiometabole factoren, ABO bloedgroep en toekomstige hart- en vaatziekten**

In het tweede deel van dit proefschrift hebben we het fenomeen van bloedcel gebonden apo B verder onderzocht. In **Hoofdstuk 7** hebben we onderzocht welke factoren het ery-apoB mogelijk beïnvloeden. Ery-apoB was omgekeerd geassocieerd met CRP, complement C3 en de aanwezigheid van het metabool syndroom. Ook vonden we een sterke associatie met de ABO bloedgroep: het ery-apoB was gemiddeld bijna 3 keer hoger bij proefpersonen met bloedgroep O, die een lager cardiovasculair risico hebben, dan bij proefpersonen met bloedgroep A, B of AB. Een andere factor die gerelateerd was aan het ery-apoB was het CR1 polymorfisme: ery-apoB was hoger bij diegenen met een genetische aanleg voor een hoge expressie van CR1 op erytrocyten. Deze resultaten laten zien dat inflammatie mogelijk ook een gunstige kant heeft, doordat het gebruik van het complement systeem door erytrocyten om atherogene lipoproteïnen te vervoeren mogelijk beschermt tegen atherosclerose.

In **Hoofdstuk 8** beschrijven we de resultaten van het eerste prospectieve onderzoek naar de rol van ery-apoB in cardiovasculaire aandoeningen en sterfte. De incidentie van cardiovasculaire aandoeningen verschilde significant tussen proefpersonen met laag, intermediair of hoog ery-apoB, met een trend voor een hogere incidentie in personen met een laag ery-apoB. Degenen met een laag ery-apoB hadden een hoger risico op sterfte en op het krijgen van een fatale of niet-fatale cardiovasculaire aandoening. In een subgroep analyse was dit verhoogde risico aanwezig in de groep proefpersonen met een cardiovasculaire voorgeschiedenis, maar niet bij degenen die niet eerder hart- en

vaatziekten hadden gehad. Hieruit concluderen we dat ery-apoB mogelijk een nieuwe marker voor cardiovasculair risico is voor secundaire preventie.

### Leukocyt-gebonden apo B en cardiovasculaire ziekte

Niet alleen erytrocyten, maar ook monocyten en neutrofiele granulocyten hebben apo B op hun oppervlak. In **Hoofdstuk 9** hebben we onderzoek gedaan naar de relatie tussen leukocyt-gebonden apo B en hart- en vaatziekten. Zoals we in Hoofdstuk 1 en 4 van dit proefschrift beschrijven, kunnen atherogene lipoproteïnen met apo B leukocyten activeren en de productie van pro-inflammatoire cytokinen stimuleren. Onze hypothese was daarom dat een hoog leuko-apoB geassocieerd zou zijn met meer hart- en vaatziekten. Apo B gebonden aan monocyten (mono-apoB) en neutrofiele granulocyten (neutro-apoB) was lager in proefpersonen met een cardiovasculaire voorgeschiedenis, en was omgekeerd geassocieerd met de intima media dikte van de arteria carotis, een maat voor subklinische atherosclerose. Het mono-apoB en neutro-apoB was, net als ery-apoB, omgekeerd geassocieerd met cardiometabole factoren en inflammatie, zoals body mass index, tailleomtrek, triglyceriden, CRP en C3. Deze resultaten waren tegengesteld aan onze hypothese, en aan de huidige opvattingen over het effect van de interactie tussen leukocyten en atherogene lipoproteïnen. Mogelijk beschermt een hoog mono- en neutro-apoB tegen atherosclerose door het binden en wegvangen van atherogene lipoproteïnen uit de circulatie, waardoor zij minder in aanraking komen met het endotheel. Mono- en neutro-apoB was positief geassocieerd met ery-apoB. Deze associatie, en het feit dat de associatie met cardiometabole factoren, inflammatie en hart- en vaatziekten hetzelfde was als voor het ery-apoB, suggereert dat er mogelijk een gezamenlijk bindingsmechanisme is. Een waarschijnlijk mechanisme is via CR1, aangezien dit één van de weinige receptoren is die zich op zowel erytrocyten als monocyten en neutrofiele granulocyten bevindt.

### Complement Receptor 1 gen polymorfismen zijn geassocieerd met cardiovasculair risico

Verschillende 'single nucleotide polymorfismen' (SNPs), ofwel kleine variaties in het DNA, in het CR1 gen zijn gerelateerd aan de expressie van CR1 op erytrocyten. Omdat de binding van apo B aan erytrocyten en leukocyten waarschijnlijk via de CR1 receptor gaat, en ery-apoB en leuko-apoB is gerelateerd aan cardiovasculaire ziekten, hebben we onderzocht of deze CR1 polymorfismen geassocieerd zijn met cardiovasculaire ziekten. In **Hoofdstuk 10** beschrijven we dat verschillende CR1 polymorfismen geassocieerd waren met het risico op een fataal of niet-fataal myocardinfarct, en een aantal van deze polymorfismen was ook geassocieerd met het risico op vasculaire mortaliteit en met totale mortaliteit. Mogelijk komt dit door een verschil in de binding van apo B aan erytrocyten, monocyten en/of neutrofiele granulocyten. Verder onderzoek naar dit

verband is nodig, omdat we in deze studie niet de binding van apo B aan deze bloedcellen hebben gemeten. Ook is er nog weinig bekend over de mate waarin de onderzochte polymorfismen het CR1 op erytrocyten beïnvloeden.

## CONCLUSIES

In dit proefschrift hebben we laten zien dat inflammatie zowel nadelige als gunstige effecten heeft op hart- en vaatziekten. Glucose en lipiden veroorzaken inflammatie na de maaltijd, en deze postprandiale inflammatie is geassocieerd met het ontstaan van atherosclerose. Aan de andere kant lijkt enige mate van inflammatie ook te beschermen tegen hart- en vaatziekten. Met dit proefschrift hebben we eerdere bevindingen over het beschermende effect van de binding van apolipoproteïne B aan erytrocyten op atherosclerose uitgebreid. Ook hebben we voor het eerst aangetoond dat de binding van apolipoproteïne B aan leukocyten mogelijk ook beschermt tegen atherosclerose. Voor dit proces is zeer waarschijnlijk een lage mate van inflammatie nodig, omdat de binding van immuuncomplexen en lipiden aan de complement receptor 1 afhankelijk is van het complement systeem.

**APPENDIX B**

# Curriculum vitae

Marijke Akua de Vries was born on Wednesday 25 March 1987 in Rotterdam. She spent the first three years of her life in the small town of Hwidiem in Ghana, in West-Africa, where her parents were working as doctors for the non-governmental organization Memisa. Her middle name, Akua, means 'Wednesday-born girl' in the local language of the Akan, the original population of the south of Ghana. The Adinkra symbols used throughout this thesis are part of the Akan culture as well. She attended secondary school at the Helinium in Hellevoetsluis. She graduated in 2005, and started medical school at the Erasmus University in Rotterdam in the same year. During her medical training, she did an internship in Obstetrics in Sawla, Ghana in 2008, and an internship in Ophthalmology in Gitarama, Rwanda in 2011. Her interest in scientific research developed during her doctoral research on the subject of the selection of human antibody fragments directed against tumor T-cell epitopes for adoptive T-cell therapy, at the Department of Medical Oncology in the Erasmus Medical Center - Daniel den Hoed Cancer Center. From June 2012 to March 2013, she worked as a resident in Internal Medicine at the Maasstad Hospital in Rotterdam, under the supervision of dr. M.A. van den Dorpel. She started the work for this thesis in the Franciscus Gasthuis in March 2013, under the supervision of prof. dr. W.W. de Herder and dr. M. Castro Cabezas. The majority of the work presented in this thesis was carried out in close collaboration with the Department of Clinical Chemistry (dr. G.J. van de Geijn, dr. L. Prinzen, dr. E. van der Zwan). In January 2016, she started her training in Internal Medicine, under the supervision of dr. A.P. Rietveld, to be continued at the Erasmus Medical Center, under the supervision of dr. S.C.E. Klein Nagelvoort-Schuit.

## APPENDIX C

# List of abbreviations

| | |
|---|---|
| 25OHD | 25-hydroxyvitamin D |
| ACE-i | angiotensin-converting enzyme inhibitor |
| AGEs | advanced glycation end products |
| AIx | augmentation index |
| ANOVA | analysis of variance |
| Apo | apolipoprotein |
| ASA | acetylsalicylic acid |
| ASP | acylation-stimulating protein |
| au | arbitrary units |
| AUC | area under the curve |
| BMI | body mass index |
| BP | blood pressure |
| C3 | complement component 3 |
| CAD | coronary artery disease |
| CAG | coronary angiography |
| CCA | common carotid artery |
| CI | confidence interval |
| cIMT | carotid intima media thickness |
| CR1 | complement receptor 1 |
| CRP | C-reactive protein |
| CV | coefficient of variation |
| CVD | cardiovascular disease |
| CVE | cardiovascular event |
| DAG | diacylglycerol |
| dAUC | delta area under the curve |
| DBP | diastolic blood pressure |
| DM | diabetes mellitus |
| EAS | European Atherosclerosis Society |
| ECD | PE-Texas Red |
| ER | endoplasmatic reticulum |
| ery-apoB | erythrocyte-bound apolipoprotein B |
| ESC | European Society of Cardiology |
| FCH | familial combined hyperlipidemia |
| FFA | free fatty acid |

| | |
|---|---|
| FH | familial hypercholesterolemia |
| FHTG | familial hypertriglyceridemia |
| FITC | fluorescein isothiocynate |
| HDL | high-density lipoprotein |
| HDL-C | high-density lipoprotein cholesterol |
| HR | hazard ratio |
| hs-CRP | high-sensitivity C-reactive protein |
| ICAM-1 | intercellular adhesion molecule-1 |
| IDL | intermediate-density lipoprotein |
| IFN-γ | interferon-gamma |
| IL-1β | interleukin-1 beta |
| IL-6 | interleukin-6 |
| IMT | intima media thickness |
| IQR | interquartile range |
| LCA | left coronary artery |
| LD | linkage disequilibrium |
| LDL | low-density lipoprotein |
| LDL-C | low-density lipoprotein cholesterol |
| LDL-r | low-density lipoprotein receptor |
| leuko | leukocyte |
| leuko-apoB | leukocyte-bound apolipoprotein B |
| LF-L | lactoferrin-like polypeptide |
| LOX-1 | lectin-like oxidized LDL receptor 1 |
| LPL | lipoprotein lipase |
| LPS | lipopolysaccharide |
| LRP-1 | LDL receptor-related protein 1 |
| LSD | least significant difference |
| MAG | monoacylglycerols |
| MAPK | mitogen-activated protein kinase |
| MCP-1 | monocyte chemotactic protein-1 |
| MDA | malondialdehyde |
| MetS | metabolic syndrome |
| MFI | mean fluorescent intensity |
| MI | myocardial infarction |
| MIP-1α | macrophage inflammatory protein-1 alpha |
| MMP-9 | matrix metalloproteinase 9 |
| mono | monocyte |
| MPO | myeloperoxidase |
| MTP | microsomal transfer protein |

| | |
|---|---|
| MUFA | monounsaturated fatty acid |
| NCEP | National Cholesterol Education Program |
| neutro | neutrophil |
| NF-κB | nuclear factor kappaB |
| non-HDL-C | non-high-density lipoprotein-cholesterol |
| OFLT | oral fat loading test |
| OGTT | oral glucose tolerance test |
| oxLDL | oxidized low-density lipoprotein |
| PE | phycoerythrin |
| PKC | protein kinase C |
| PON-1 | paraoxonase 1 |
| PTH | parathyroid hormone |
| PUFA | polyunsaturated fatty acid |
| PWV | pulse wave velocity |
| RAG | rabbit anti-goat |
| RAGE | receptor for AGE |
| RCA | right coronary artery |
| RER | rough endoplasmatic reticulum |
| RFLP | restriction fragment length polymorphism |
| ROS | reactive oxygen species |
| RRR | relative risk ratio |
| SBP | systolic blood pressure |
| SCID | severe combined immunodeficiencie |
| SD | standard deviation |
| SEM | standard error of the mean |
| SER | smooth endoplasmatic reticulum |
| SFA | saturated fatty acid |
| s-ICAM-1 | soluble intercellular adhesion molecule 1 |
| SNP | single nucleotide polymorphism |
| T2DM | type 2 diabetes mellitus |
| TC | total cholesterol |
| TG | triglyceride |
| TNF-α | tumor necrosis factor alpha |
| TRL | triglyceride-rich lipoprotein |
| VCAM-1 | vascular cell adhesion molecule 1 |
| VLDL | very low-density lipoprotein |
| WBC | white blood cell count |
| WT | wild type |

**APPENDIX D**

# List of publications

**M.A. de Vries**, B. Klop, H.W. Janssen, T.L. Njo, E.M. Westerman, M. Castro Cabezas. Postprandial inflammation: targeting glucose and lipids. Adv Exp Med Biol 2014;824:161-70

**M.A. de Vries**, B. Klop, M. Castro Cabezas. The use of the non-fasting lipid profile for lipid-lowering therapy in clinical practice - point of view. Atherosclerosis 2014;234(2):473-5

**M.A. de Vries**, B. Klop, S.A. Eskes, T.L. van der Loos, F.J. Klessens-Godfroy, J. Wiebolt, H.W. Janssen, E.M. Westerman, M. Castro Cabezas. The postprandial situation as a pro-inflammatory condition. Clin Investig Arterioscler 2014;26(4):184-92

**M.A. de Vries**, J.P. Samijn, R. de Man, J.M. Boots. Hepatitis E-associated encephalopathy in a renal transplant recipient. BMJ Case Rep 2014;2014



B. Klop, J.P. van der Pol, R. van Bruggen, Y. Wang, **M.A. de Vries**, S. van Santen, J. O'Flynn, G.J. van de Geijn, T.L. Njo, H.W. Janssen, P. de Man, J.W. Jukema, T.J. Rabelink, P.C. Rensen, C. van Kooten, M. Castro Cabezas. Differential complement activation pathways promote C3b deposition on native and acetylated LDL thereby inducing lipoprotein binding to the complement receptor 1. J Biol Chem 2014;289(51):35421-30

**M.A. de Vries**, A. Alipour, B. Klop, G.J. van de Geijn, H.W. Janssen, T.L. Njo, N. van der Meulen, A.P. Rietveld, A.H. Liem, E.M. Westerman, W.W. de Herder, M. Castro Cabezas. Glucose-dependent leukocyte activation in patients with type 2 diabetes mellitus, familial combined hyperlipidemia and healthy controls. Metabolism 2015;64(2):213-7

**M.A. de Vries**, B. Klop, A. Alipour, G.J. van de Geijn, L. Prinzen, A.H. Liem, P. Valdivielso, J. Rioja Villodres, J. Ramírez-Bollero, M. Castro Cabezas. In vivo evidence for chylomicrons as mediators of postprandial inflammation. Atherosclerosis 2015;243(2):540-5

D.F. van Breukelen-van der Stoep, D. van Zeben, B. Klop, G.J. van de Geijn, H.J. Janssen, M.J. Hazes, E. Birnie, N. van der Meulen, **M.A. de Vries**, M. Castro Cabezas. Association of Cardiovascular Risk Factors with Carotid Intima Media Thickness in Patients with Rheumatoid Arthritis with Low Disease Activity Compared to Controls: A Cross-Sectional Study. PLoS One. 2015;10(10):e0140844

**M.A. de Vries**, A. Alipour, E. Birnie, A. Westzaan, S. van Santen, E. van der Zwan, A.H. Liem, N. van der Meulen, M. Castro Cabezas. Coronary leukocyte activation in relation to progression of coronary artery disease. Front Med 2016;10(1):85-90

**M.A. de Vries**, B. Klop, N. van der Meulen, G.J. van de Geijn, L. Prinzen, E. van der Zwan, E. Birnie, J.W. Cohen Tervaert, A.H. Liem, W.W. de Herder, M. Castro Cabezas. Leucocyte-bound apolipoprotein B in the circulation is inversely associated with the presence of clinical and subclinical atherosclerosis. Eur J Clin Invest 2016;46(8):690-7

D.F. van Breukelen-van der Stoep, D. van Zeben, B. Klop, G.J. van de Geijn, H.J. Janssen, N. van der Meulen, **M.A. de Vries**, M. Hazes, E. Birnie, M. Castro Cabezas. Marked underdiagnosis and undertreatment of hypertension and hypercholesterolaemia in rheumatoid arthritis. Rheumatology 2016;55(7):1210-6

**M.A. de Vries**, S. Trompet, S.P. Mooijaart, R.A. Smit, S. Böhringer, M. Castro Cabezas M, J.W. Jukema. Complement receptor 1 gene polymorphisms are associated with cardiovascular risk. Atherosclerosis 2016;257:16-21

**M.A. de Vries**, N. van der Meulen, G.J.M. van de Geijn, B. Klop, E.M. van der Zwan, L. Prinzen, E. Birnie, E.M. Westerman, W. W. de Herder, M. Castro Cabezas. Effect of a single dose vitamin D3 on postprandial arterial stiffness and inflammation in vitamin D deficient women. J Clin Endocrinol Metab 2016:jc20163394

**APPENDIX E**

# PhD portfolio

PhD portfolio

| | |
|---|---|
| Name PhD student: | Marijke Akua de Vries |
| Erasmus MC department: | Internal Medicine – Section of Endocrinology |
| Research school: | Netherlands Institute of Health Sciences |
| PhD period: | March 2013 – December 2015 |
| Promotor: | Prof. Dr. W.W. de Herder |
| Supervisor: | Dr. M. Castro Cabezas (Franciscus Gasthuis) |

| **General academic courses** | **Year** | **ECTS** |
|---|---|---|
| Good clinical practice | 2013 | 1 |
| Wetenschappelijk schrijven in het Engels | 2013 | 1 |
| **Research skills** | **Year** | **ECTS** |
| Erasmus Summer School – Introduction to data management | | 1 |
| Leergang 'Wetenschappelijk onderzoek' – Franciscus Gasthuis | 2015 | 0.5 |
| **(Internal) conferences – oral presentations** | **Year** | **ECTS** |
| North European Young Diabetologists – Denmark<br>*Acute and chronic effects of glycaemia on leukocyte activation in patients with type 2 diabetes mellitus, insulin resistance and healthy controls* | 2013 | 1 |
| Annual Dutch Diabetes Research Meeting – Oosterbeek<br>*Gender differences in postprandial glucose-dependent leukocyte activation in patients with T2DM, FCH and healthy controls* | 2013 | 1 |
| Wetenschapsdag – SFG Rotterdam<br>*Coronary leukocyte activation as predictor of cardiovascular events in young subjects: five year follow-up data* | 2014 | 1 |
| Obesitas Forum – SFG Rotterdam<br>*Vitamin D and atherosclerosis: DOSFEM study* | 2014 | 1 |

| | | |
|---|---|---|
| Symposium Triglyceriden, vitamine D en bloedcellen en atherosclerose SFG<br>*Vitamine D en atherosclerose* | 2014 | 1 |
| Jong talent presenteert wetenschappelijk onderzoek – SFG Rotterdam<br>*Vitamine D en atherosclerose: toekomstig onderzoek* | 2014 | 1 |
| Nederlandse Lipoproteïnen Club – Leiden<br>*Complement receptor 1 is involved in the binding of apo B-containing lipoproteins to circulating blood cells* | 2014 | 1 |
| Regionale refereeravond Vasculaire Geneeskunde – SFG Rotterdam<br>*Vitamine D en atherosclerose* | 2014 | 1 |
| Wetenschapsdag – SFG Rotterdam<br>*Leukocyt-gebonden apolipoproteïne B en atherosclerose* | 2015 | 1 |
| Internistendagen - Maastricht<br>*Chylomicronen en postprandiale inflammatie* | 2015 | 1 |
| American Heart Association – Orlando (Florida, VS)<br>*Clinical Characteristics of Subjects with Different Levels of Systemic Erythrocyte-bound Apolipoprotein B: Association with Metabolic Syndrome and ABO blood group* | 2015 | 1 |
| Wetenschapsdag – Franciscus Gasthuis Rotterdam<br>*Erythrocyte-bound Apolipoprotein B Predicts Mortality and Cardiovascular Events in Patients with High Cardiovascular Risk: Results from a 5-year Follow-up Study* | 2016 | 1 |
| **(Internal) conferences – poster presentations** | **Year** | **ECTS** |
| Wetenschapsdag SFG<br>*Postprandial glucose-dependent leukocyte activation in patients with different ranges of insulin sensitivity* | 2014 | 1 |
| European Atherosclerosis Society – Madrid (Spain)<br>*Coronary leukocyte activation as predictor of coronary events in young subjects: five year follow-up data* | 2014 | 1 |

| | | |
|---|---|---|
| International Society of Atherosclerosis – Amsterdam<br>*Leukocyte-bound Apolipoprotein B in the Circulation is Inversely Associated with the Presence of Clinical and Subclinical Atherosclerosis* | 2015 | 1 |
| International Society of Atherosclerosis – Amsterdam<br>*In Vivo Evidence for Chylomicrons as Mediators of Postprandial Inflammation* | 2015 | 1 |
| Wetenschapsdag SFG<br>*In Vivo Evidence for Chylomicrons as Mediators of Postprandial Inflammation* | 2015 | 1 |
| Wetenschapsdag – Franciscus Gasthuis Rotterdam<br>*Clinical Determinants of Systemic Erythrocyte-bound Apolipoprotein B: Association with Metabolic Syndrome, Complement Receptor 1 and ABO Blood Group* | 2016 | 1 |
| Wetenschapsdag – Franciscus Gasthuis Rotterdam<br>*The effect of single dose of vitamin D on postprandial arterial stiffness and leukocyte activation in premenopausal vitamin D deficient overweight or obese women* | 2016 | 1 |



| **Clinical meetings/projects/participation** | **Year** | **ECTS** |
|---|---|---|
| Outpatient clinic Cardiovascular risk management in RA patients | 2013-2015 | 6 |
| Two-weekly Scientific meeting Cardiovascular Research – Franciscus Gasthuis Rotterdam | 2013-2015 | 3 |
| Monthly Franciscus Education And Research meeting | 2013-2016 | 1.5 |

| **Symposia, seminars & workshops** | **Year** | **ECTS** |
|---|---|---|
| Nascholing Cardiovasculair risico management | 2013 | 0.2 |
| Wetenschapsdag – SFG Rotterdam | 2013 | 0.2 |
| Internistendagen - Maastricht | 2013 | 1 |
| European Atherosclerosis Society – Lyon (France) | 2013 | 1 |
| Rotterdamse Internistendag | 2013 | 0.4 |
| Hyperlipidemia Academy – Berlin (Germany) | 2013 | 1 |

| | | |
|---|---|---|
| Annual Dutch Diabetes Research Meeting – Oosterbeek | 2014 | 0.5 |
| Symposium Vasculair Spreekuur – Rotterdam | 2014 | 0.2 |
| Internistendagen - Maastricht | 2014 | 1 |
| Symposium Brown Adipose Tissue – LUMC Leiden | 2014 | 0.5 |
| Cardiovascular Conference – Amersfoort | 2014 | 0.5 |
| Internistendagen - Maastricht | 2016 | 1 |
| **Teaching activities** | **Year** | **ECTS** |
| KOW 'XXL management' 2013/2014 – SFG Rotterdam<br>*Vaardigheidsonderwijs - onderzoek aan lipiden* | 2014 | 1 |
| KOW 'XXL management' 2014/2015 – SFG Rotterdam<br>*Vaardigheidsonderwijs - onderzoek aan lipiden* | 2015 | 1 |
| Onderwijs analisten KCHL – SFG Rotterdam<br>*Update vasculaire research* | 2014 | 1 |
| **Congress organization** | **Year** | **ECTS** |
| Wetenschapsdag – Franciscus Gasthuis | 2015 | 3 |
| Wetenschapsdag – Franciscus Gasthuis | 2016 | 3 |

**APPENDIX F**

# Legend Adrinkra symbols

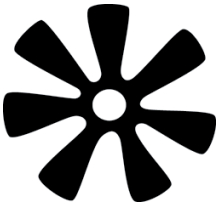
The symbols used throughout this thesis are Adinkra symbols. These symbols are believed to have been created ages ago by Ashanti craftsmen. The Ashanti are an ethnical group in the South of Ghana. They speak Twi, a dialect of the Akan language. The symbols are used to decorate items such as clothes and furniture. Apart from their decorative use, the symbols have philosophical, educational and historical value. I have chosen these particular symbols, because in my opinion, they represent life lessons or meanings that are applicable to the challenges experienced with (medical) science. On the following pages, the meaning of the different symbols will be explained.

*De symbolen die in dit proefschrift zijn gebruikt zijn Adinkra symbolen. Men denkt dat de symbolen lang geleden gecreëerd zijn door Ashanti ambachtslieden. De Ashanti zijn een ethische groep uit het zuiden van Ghana. Ze spreken Twi, een dialect van de Akan taal. De symbolen worden gebruikt om voorwerpen zoals kleding en meubels te versieren. Behalve hun decoratieve waarde, hebben de symbolen ook filosofische, educatieve en historische waarde. Ik heb deze specifieke symbolen gekozen, omdat ze naar mijn mening levenslessen of betekenissen weergeven die van toepassing zijn op het doen van (medisch) wetenschappelijk onderzoek, en het doorlopen van een promotietraject. Op de volgende pagina's zal de betekenis van de verschillende symbolen worden uitgelegd.*



Source: Agbo AH. Values of Adinkra Symbols. 1st ed. Kumasi: Ebony designs and publications; 1999

**Ananse Ntontan**

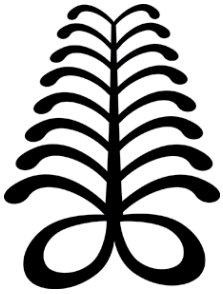


***Wisdom***
**'Ananse Ntontan' – Spider's web**

The spider Ananse is a well-known character in West African folktales. The spider is known for its wisdom and creativity.

*De spin Ananse is een bekend figuur in West-Afrikaanse volksverhalen. Hij staat bekend om zijn wijsheid en creativiteit.*

**Aya**

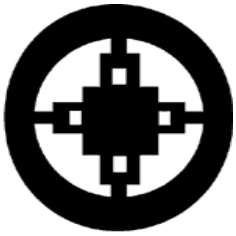


***Endurance***
**'Aya' – Fern**

The fern is a hardy plant, able to endure any weather conditions and soil types. This symbol teaches that the survival of mankind requires the will to face all challenges, to be self-reliant and resourceful.

*De varen is een robuuste plant, die bestand is tegen elke weersomstandigheid en ieder type grond. Dit symbool toont dat om te kunnen overleven, men alle veranderingen het hoofd moet bieden en onafhankelijk en vindingrijk moet zijn.*

**Dame Dame**



***Intelligence***

This symbol represents a board game. It signifies the power of the mind to reason and solve problems.

*Dit symbool stelt een bordspel voor. Het staat voor de kracht van de geest om te redeneren en problemen op te lossen.*

**Denkyem**

***Adaptability***
**'Denkyem' – Crocodile**

The crocodile is noted for its ability to live in the water, while it needs air to breathe. The symbol signifies the importance of adaptation to changing circumstances, especially those that seem difficult.

*De krokodil is in staat om in het water te leven, terwijl het lucht nodig heeft om te ademen. Dit symbool staat voor het belang van het aanpassen aan veranderende omstandigheden, vooral aanpassingen die moeilijk lijken.*



**Dwennimmen**

***Humility and strength***
**'Odwennini' – Ram, 'Mmen' – Horns**

This symbol refers to the ram, noted for its strength in a fight, but also its submission upon slaughter. It signifies the importance of modesty and toughness.

*Dit symbool verwijst naar de horens van de ram, een dier dat sterk is in een gevecht, maar zich ook onderwerpt voor de slacht. Het staat voor het belang van bescheidenheid en onverzettelijkheid.*

**Hwe Mu Dua**

***Strive for quality***
**'Hwe mu' – Look in, 'Dua' – Stick**

This symbol represents a measuring stick, and encourages examination of all aspects of human life, and to strive for high quality.

*Dit symbool stelt een meetlat voor. Het zet aan tot grondige inspectie van alle aspecten van het leven, en tot het streven naar hoge kwaliteit.*

**Mate Masie**

***Wisdom and consideration***
**'Mate' – I have heard, 'Sie' – To keep**

This symbol represents the need to take in wisdom and knowledge, but also to keep secrets and to overthink issues before taking decisions.

*Dit symbool staat voor het belang van het vergaren van wijsheid en kennis, maar ook van het bewaren van geheimen en het overdenken van zaken voordat een beslissing wordt genomen.*

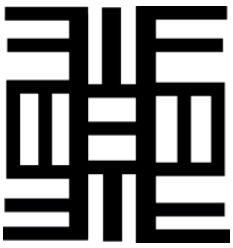
**Mmere dane**

***Change and dynamics***
**'Mmere dane' – Time changes**

This symbol represents change and the dynamics of life.

*Dit symbool staat voor de verandering en de dynamiek van het leven.*

**Nea onnim do sue a ohu**



***Knowledge and education***
**'Nea onnim do sue a ohu' – He who does not know can know from learning**

This symbol represents the value of knowledge and education.

*Dit symbool staat voor het belang van kennis en van onderwijs.*

**Owo Foro Adobe**

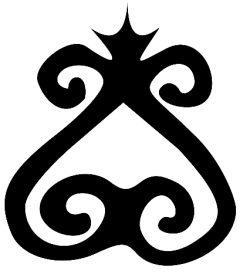
***Persistence and diligence***
**'Owo' – Snake, 'Foro' – Climb, 'Dobe' – Raffia palm**

The raffia palm is a thorny tree, which is difficult to climb. A snake climbing this tree has accomplished an impossible task. This symbol encourages people to be persistent and diligent in their efforts in order to achieve success.

*De raffia palm is een boom met doornen die moeilijk te beklimmen is. Een slang die deze boom beklimt heeft een onmogelijke taak volbracht. Dit symbool moedigt aan om volhardend en ijverig te zijn om succesvol te zijn.*



**Sankofa**

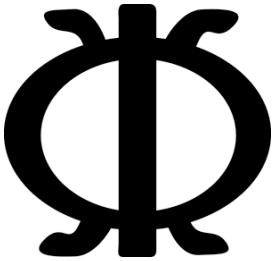


***Learn from the past***
**'Sanko' – Go back, 'Fa' – Take**

This symbol signifies that progress is based on the right use of the positive contributions of the past, and that one must learn from the past. The symbol exists in two forms: that of a heart turned upside down, and a bird taking an egg of its back.

*Dit symbool geeft weer dat vooruitgang gebaseerd is op de positieve bijdrage van het verleden, en dat men moet leren van het verleden. Het symbool bestaat in twee vormen: die van een omgekeerd hart, en een vogel die een ei van zijn rug pakt.*

**Wawa aba**



***Toughness and perseverance***

**'Wawa' – A type of tropical rainforest tree, 'Aba' – Seed**

The seed of a wawa tree has a very hard outer covering, but it is broken by the slowly developing seedling from within, which in life defies all types of weather and grows into a big tree. This signifies the meaning to persist even in the face of difficulty.

*De zaden van de wawa boom, een type boom in het tropisch regenwoud, heeft een harde buitenkant, maar wordt van binnenuit gebroken door het langzaam ontwikkelende kiemplantje, dat gedurende het leven alle weersoorten doorstaat en uitgroeit tot een grote boom. Dit symbool staat voor het belang van volhouden, ook in moeilijke omstandigheden.*

**APPENDIX G**

# Dankwoord

Een gezegde onder reizigers is "It's not the destination that matters, but the journey". Het gaat dus niet om de uiteindelijke bestemming, maar om de reis daarnaar toe. Dat geldt voor reizen, maar zeker ook voor het doen van promotieonderzoek. Natuurlijk heb ik de afgelopen jaren toegewerkt naar Het Boekje dat je nu in handen hebt, maar tijdens mijn onderzoekstijd heb ik vooral ook veel geleerd. Het doen van promotieonderzoek leert je onder andere als een zelfstandige wetenschapper te denken, onderzoeksvragen gericht om te zetten in een experiment of onderzoeksprotocol, om te gaan met tegenslagen (als de resultaten van je experiment bijvoorbeeld tegenvallen) en het presenteren van je data, zowel op papier als voor een publiek. Mijn 'reis' naar dit proefschrift heb ik niet alleen gemaakt: ik heb onderweg hulp en steun gehad van veel personen.



**Prof. dr. De Herder**, beste **Wouter**, met mij als perifere promovenda hadden we een wat ongebruikelijke promotor-promovendus verhouding. We ontmoetten elkaar voor het eerst toen het eerste jaar van mijn promotieonderzoek achter de rug was. Desalniettemin heb ik veel gehad aan onze gesprekken over de voortgang van mijn proefschrift en je input bij mijn artikelen.

**Dr. Castro Cabezas**, beste **Manuel**, ik heb het enorm getroffen met jou als betrokken co-promotor. Bijna wekelijks nam je uitgebreid de tijd om alle lopende projecten samen door te nemen, en een manuscript dat ik je toestuurde voor correcties had ik altijd binnen twee dagen weer terug. Het gebeurde zelfs een keer dat ik een manuscript 's avonds naar je mailde, blij om er even van af te zijn, om het de volgende ochtend nagekeken op mijn bureau terug te vinden. Samen op congres was altijd een feest: niet alleen naar praatjes luisteren, maar ook genieten van bezienswaardigheden, goed eten en uiteraard goede wijn of cognac. Na mijn allereerste slagroomproef heette je me welkom in het "selecte gezelschap van postprandiale weirdo's". Ik ben trots om net als jij een postprandiale weirdo te mogen zijn!

**Gert-Jan**, zonder jouw hulp was het helemaal niets geworden op het lab. Je stond altijd voor me klaar voor mijn kleine en grote vragen. Daarnaast was je altijd kritisch op mijn experimenten en de resultaten daarvan. Ik heb niet alleen inhoudelijk veel aan je gehad, maar het was met jou om de hoek ook altijd gezellig in het lab.

**Noëlle**, lieve Noëlle, jij hebt je eindeloos ingezet voor mij en mijn onderzoek, daarvoor ben ik je enorm dankbaar. We maakten samen lange dagen voor de slagroomproeven,

maar liefst achtenveertig ochtenden ben jij voor dag en dauw naar het Franciscus Gasthuis gekomen om al rond 7 uur te kunnen opstarten voor weer zo'n proef. Je bent een kanjer.

**Boudewijn**, ontzettend bedankt voor je hulp gedurende mijn hele promotietijd. De eerste weken heb je me wegwijs gemaakt in het ziekenhuis en op het lab. Het was ontzettend handig om met jouw tips en trics aan de slag te kunnen. Ook nadat je voor je opleiding was vertrokken naar Dordrecht was je altijd bereikbaar en bereid om me te helpen met de artikelen die ik schreef, je aanvullingen en suggesties hielpen me altijd verder. Dankzij jou, en natuurlijk ook dankzij **Arash** en **Sarah**, stapte ik in een lopende onderzoekstrein die mede dankzij jullie inzet flink op stoom was.

**Erwin**, bij jou kon ik altijd terecht voor al mijn statistische en methodologische vragen. Ontzettend bedankt voor al je hulp en geduld.

Ik wil ook de klinisch chemici en artsen van het lab bedanken voor al hun hulp. In het begin van mijn onderzoekstijd waren dat **Hans** en **Tjin**, later hebben **Ellen**, **Lenneke** en **Fokke** het stokje overgenomen. Bedankt voor jullie tijd en input tijdens de vele vasculaire research besprekingen, bij mijn artikelen en voor al mijn vragen op het lab. **John**, bedankt voor je hulp bij het meedenken over de planning van de slagroomproeven, je hebt altijd je best gedaan om mijn onderzoek qua bezetting op de flow cytometer zo goed mogelijk te faciliteren. **Hamid**, **René B**, **Jochem** en **Rita**, bedankt voor jullie hulp met pipetteren bij de slagroomproeven. Ook de andere flowspecialisten, **Martijn**, **Yvonne** en **Monique**, bedankt voor jullie geduld en jullie gezelligheid tijdens de lange dagen op het lab. Ook alle andere analisten en medewerkers van het KCHL wil ik bedanken voor jullie gastvrijheid en bereidheid tot hulp als ik bijvoorbeeld weer op zoek was naar een serumbuis van mijn slagroomproeven. Mijn dank gaat ook uit naar **Henk**, die eindeloze hoeveelheden LPS voor mijn incubatie experimenten heeft vervaardigd.

Als arts-onderzoeker in een perifeer ziekenhuis is het soms een beetje zoeken naar je plek. Gelukkig heb ik veel steun gehad van mijn collega arts-onderzoekers **Stefanie** en **Yasmine**, die net als ik in hetzelfde schuitje zaten. Daarnaast heb ik veel gezellige borrels en skiweekenden mogen meemaken met de groep **arts-assistenten interne**.

Tijdens mijn onderzoek heb ik veel hulp gehad van stagiaires uit binnen- en buitenland. **Selvetta**, **Lana** en **Ashnaa**, bedankt voor jullie hulp en gezelligheid! **Fernando** y **José**, muchas gracias por su ayuda y sociabilidad.

**Benvinda** en **Simone**, bedankt voor het steeds weer geblindeerd klaarzetten van de vitamine D drank voor mijn slagroomproeven. Zonder jullie hulp had ik mijn proeven niet kunnen afronden.

Uiteraard bedank ik ook alle **proefpersonen** die hebben meegedaan aan mijn verschillende studies en zich hebben onderworpen aan alle bloedafnames en sommigen zelfs het drinken van een grote beker ongeklopte slagroom op lege maag! Zonder jullie was dit proefschrift niet tot stand gekomen.

Dames van de bibliotheek, **Carmen** en **Katja**, bedankt voor de eindeloze hoeveelheid artikelen die jullie altijd binnen no-time voor me hebben opgevraagd. Vaak kreeg ik binnen een paar uur van jullie de artikelen die ik nodig had om mijn onderzoek te kunnen voortzetten.

Alle dames (en heer) van het Diabetes en Vasculair Centrum: **Ayse**, **Denise**, **Don**, **Evelien**, **Ineke**, **Jacqueline**, **Jeanette**, **Jolanda**, **Mavis**, **Monique**, **Noëlle**, **Paulien**, en **Petra**, vanaf dag één voelde ik me ontzettend welkom bij jullie op de poli. De gezellige koffiepauzes waren altijd een welkome afleiding van de uren achter de computer, en de uitjes met het team waren altijd geslaagd. De kers op de taart was het verrassingsetentje dat jullie voor mijn afscheid hadden georganiseerd. Ik zal jullie nooit vergeten! Ook de **doktersassistenten** bij balie 18 en **Els** wil ik bedanken voor alle ondersteuning bij mijn onderzoek.

Ook de **maatschap interne specialismen**, en in het bijzonder **Arie Rietveld**, ben ik grote dank verschuldigd voor de steun tijdens mijn promotietijd. Ontzettend fijn ook dat ik aansluitend aan mijn onderzoek mijn opleiding tot internist bij jullie mocht beginnen.

De **Raad van Bestuur** van het Franciscus Gasthuis, en in het bijzonder **Karen Kruijthof**, wil ik bedanken dat ze mij de gelegenheid hebben gegeven om promotieonderzoek te doen in hun mooie ziekenhuis.

Welkome afleiding van het onderzoek en het werk vond en vind ik bij de yuppengroep, **Lisanne**, **Sanne**, **Martijn**, **Karsten**, **Mirelle** en **Tim**, altijd in voor een drankje in de Locus, uitgebreid eten en Sinterkersternieuw. **Toke**, genieten van je heerlijke kookkunsten onder het genot van een goed glas wijn doet me altijd goed. **Kirsten**, met jou de stad onveilig maken, of het nou Rotterdam of Eindhoven is, is altijd gezellig. Ook de hele **club uit Dordrecht**, en **Peter** en **René**, jullie zijn altijd present bij feestjes bij ons thuis of elders.

En dan mijn lieve familie. **Papa** en **mama**, jullie hebben mij van jongs af aan altijd de kans gegeven om mij te ontwikkelen zoals ik dat zelf wilde, en me tegelijkertijd gemotiveerd om mijn dromen na te streven. Ik had me geen lievere en fijnere ouders kunnen wensen, jullie zijn altijd mijn voorbeelden geweest. Mijn broer en zussen, **Erik**, **Janneke** en **Dorien,** en natuurlijk **Renzo,** die ik ook beschouw als mijn broer, wat is het altijd heerlijk thuiskomen als jullie er zijn. Bedankt voor alle steun en gezelligheid de afgelopen jaren. Lieve opa, **Walther Jan**, je bent altijd geïnteresseerd en bij jou kan ik altijd terecht met mijn verhalen over mijn onderzoek en opleiding. Ik ben gezegend met zo'n lieve opa. Mijn schoonfamilie, **John**, **Esther**, **Marsha**, **Jeroen**, **oma Agnes** en **ome Antoon**, bij jullie voel ik me helemaal thuis en ik wil ook jullie bedanken voor jullie gezelligheid en steun de afgelopen jaren.

Lieve **Nikolaj**, het allermooiste dat de reis naar mijn promotie me heeft opgeleverd ben jij. Ik leerde jou halverwege mijn promotie kennen tijdens de afterparty van het personeelsfeest. Sindsdien delen we lief en leed, en ben jij mijn klankbord geweest voor alles wat goed, maar soms ook minder goed ging tijdens het onderzoek. Je motiveerde me om toch nog even die paar uurtjes in het weekend aan mijn artikelen te werken, en deelde mijn vreugde als een artikel na zo vaak proberen eindelijk geaccepteerd werd voor publicatie. Ik denk nog altijd met veel plezier terug aan onze reis door Florida, waar we samen, na een congres in Orlando, met de auto doorheen zijn gereisd. Lief, ik hou meer van je dan ik op papier kan uitdrukken! Wat ben ik dankbaar dat ik jou tegen het lijf ben gedanst.