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Author: Klop, Boudewijn
Title: Interplay between lipoproteins, the complement system and blood cells in atherosclerosis
Issue Date: 2014-09-11
Chapter 9

General discussion and summary
Triglycerides: from measurement to treatment

The metabolism of cholesterol and triglycerides is a complex process and relates strongly to cardiovascular disease. All apolipoprotein (apo) B containing lipoproteins, which include chylomicrons, chylomicron remnants, VLDL, IDL and LDL are atherogenic [1-4]. Not surprisingly, non-HDL-C, LDL-C and apo B have all been associated with cardiovascular disease [5,6]. Apo B48, which is the specific apolipoprotein for chylomicrons, was also found to correlate with the carotid intima media thickness [7]. In addition, triglycerides have also been associated with cardiovascular disease since elevated triglycerides reflect a surplus in circulating chylomicrons and VLDL [8-10]. Recently, multiple genetic studies confirmed this relation via strong associations between genes involved in primarily elevated triglycerides and cardiovascular disease [1,11]. Triglycerides are not only associated with cardiovascular disease, but high levels of triglycerides (> 10 mmol/l) may also cause pancreatitis [12]. Therefore, treatment of elevated triglycerides is important, but the treatment strategy depends on the underlying disorder leading to hypertriglyceridemia.

A physician oriented guideline for the treatment of hypertriglyceridemia based on the literature is provided in Chapter 2. Weight loss and improvement of dietary habits are the main treatment of hypertriglyceridemia and should be sufficient if hypertriglyceridemia is caused by obesity without existing co-morbidity. However, pharmacological treatment is necessary in hypertriglyceridemic patients due to a primary lipid disorder like familial hypertriglyceridemia or familial combined hyperlipidemia [13]. The lipolysis of triglycerides is greatly disturbed in familial hypertriglyceridemia, which is best treated with fibrates since they stimulate lipolysis and fatty acid oxidation, and subsequently, the metabolism of chylomicrons into chylomicron remnants and VLDL into LDL. However, hypertriglyceridemia in patients with familial combined hyperlipidemia, cardiovascular disease or type 2 diabetes mellitus is most often a reflection of an elevated number of circulating triglyceride-rich lipoproteins with a surplus in remnant cholesterol. Recently, it was demonstrated that genetically elevated remnant cholesterol levels in hypertriglyceridemic subjects has a causal association with coronary artery disease [1]. Therefore, this condition is best treated with statin therapy to reduce specifically the number of circulating chylomicrons and VLDL, which may be combined with fibrates to further improve the clearance of these lipoproteins.

Current guidelines advise to use apo B or non-HDL-C as a secondary treatment target next to LDL-C in patients with a combined hyperlipidemia [14]. Apo B represents the total number of atherogenic particles (chylomicrons, chylomicron remnants, VLDL, IDL and LDL), whereas non-HDL-C represents the amount of cholesterol in both the triglyceride-rich lipoproteins and LDL. Recently, a meta-analysis has shown that implementation of non-HDL-C or apo B as treatment target over LDL-C would prevent an
additional 300,000 - 500,000 cardiovascular events in the US population over a 10-year period [15]. Apo B and non-HDL-C are also not affected by food intake in contrast to LDL-C, which can only be measured reliably in the fasting state. Current guidelines still recommend to measure lipid profiles in the fasting state [14,16], but emerging evidence consisting of large epidemiological studies show that measuring non-fasting lipids only affects the measured lipid parameters to a small extent [17,18].

In Chapter 3 we compared the intra-individual variability of triglycerides during the day. A problem with measuring triglycerides is the high intra-individual variability (approximately 25%) despite standardized fasting measurements. It is believed that the intra-individual variability of triglycerides is even higher in the non-fasting state, but this has never been investigated. We were able to demonstrate that the intra-individual variability of triglycerides does not differ between fasting and non-fasting states [19]. Only in men the intra-individual variability of triglycerides increased after dinner and at bedtime, but remained unchanged during daylight hours. With these data it would be possible to propose reference values for non-fasting triglycerides, which can contribute to the use of non-fasting triglycerides and other lipid parameters in clinical practise. The measurement of non-fasting over fasting lipid profiles is much more convenient for both patients and physicians. It helps patient adherence to follow-up programs and it may decrease the necessity to perform blood tests during the early morning, which reduces waiting times and further improves patient convenience [17,20].

A growing body of evidence concerning the use of non-fasting triglycerides for cardiovascular risk prediction in a clinical setting are emerging. Both fasting and non-fasting triglycerides were predictive for cardiovascular disease in patients with systemic lupus erythematoses [21]. The results from the Women’s Health Study even suggest that non-fasting triglycerides are a stronger predictor for cardiovascular disease than fasting triglycerides [9]. In addition, A large observational study showed little association between lipid subclass levels and fasting time illustrating the potential small effect on clinical practice by using non-fasting samples [17]. However, these large studies compared lipid samples from different fasting intervals, but all samples were from different subjects. Little data is available related to the comparison of the clinical relevance of fasting versus non-fasting lipids within subjects. Therefore, a randomized-controlled trial is necessary where subjects are randomized between a group who measures a fasting plus non-fasting sample versus a group who measures two times a fasting sample. This will provide the best data to compare the effects of fasting times on lipid changes and whether this would affect clinical decision making such as initiating or adjusting lipid lowering therapy.
The measurement and modification of postprandial leukocyte activation

The development of atherosclerosis is not only initiated by mere cholesterol deposition in the arterial wall, but inflammation is an important contributor to atherosclerosis as well. It was recently demonstrated in a large population study with 60,608 participants that remnant cholesterol is causally associated with inflammation and cardiovascular disease [22]. Chylomicrons and VLDL can activate leukocytes directly ex vivo and in vivo, which is associated with endothelial dysfunction [23,24]. It is generally accepted that postprandial lipemia with accumulating chylomicrons and VLDL reflected by an increase in triglycerides induces an inflammatory response [25].

Normally, circulating leukocytes are present for host defence, but leukocytes recognise VLDL and chylomicrons as pathogens just like bacteria and immune complexes. Activated leukocytes are able to enter the arterial wall due to upregulation of cellular adhesion molecules like CD11b, which contributes to the development of atherosclerosis. Upon activation, leukocytes increase in volume and degranulate and these parameters are normally used by hematology analysers for 5-part leukocyte differentiation. Our research described in Chapter 4 showed that standard a hematology analyser was able to detect activation of monocytes and neutrophils after a fat load with comparable changes in activation during infectious diseases, but to less extent [26]. Light scatter was decreased in monocytes and neutrophils together with an increase in size of monocytes postprandially. However, an oral fat load did not disturb the automatic differentiation of leukocytes to such extent that it would interfere with the potential use of leukocyte cell population data for infection detection in clinical practise.

In general, the measurement of leukocyte activation using flow cytometry requires extensive labour, suitable equipment, highly trained technicians, time consuming blood handling and costly antibodies. This can be one of the reasons why little is known about postprandial leukocyte activation in specific conditions such as familial hypertriglyceridemia and familial combined hyperlipidemia. It is tempting to speculate that in conditions with postprandial hyperlipidemia, leukocyte activation is exaggerated postprandially. Therefore, leukocyte cell population data determined by standard hematology analyzers can provide an easy, cheap and fast method to measure postprandial leukocyte activation in a wide array of conditions and study designs.

Little research has been done to search for interventions to reduce the atherogenic postprandial leukocyte activation. So far, only exercise shortly before a meal has been found to reduce postprandial leukocyte activation [27]. We investigated in Chapter 5 whether vitamin D3 supplementation reduced postprandial leukocyte activation. Vitamin D3 is not only important for the metabolism of bone mineralisation. Vitamin D3 has also shown beneficial effects on arterial elasticity [28,29] and affects leukocyte
activation. In vitro studies had shown that 1,25-dihydroxyvitamin D3, which is the active metabolite of vitamin D3, reduced the activation state of leukocytes and formation of atherogenic foam cells [30,31].

We hypothesized that the reported beneficial effects of vitamin D3 on arterial elasticity may be related to a reduction in postprandial leukocyte activation. Our results showed indeed an improvement in arterial elasticity postprandially in men and women, but this was accompanied by a reduction in postprandial leukocyte activation markers only in women. Therefore, a direct relationship between the vitamin D3 associated improvement in arterial elasticity and reduction in postprandial leukocyte activation is questionable. A possible explanation for our observed gender difference may be related to sex steroids. It has been demonstrated that estradiol reduces the CD11b expression on monocytes in vitro [32], whereas testosterone potentiates neutrophil activation [33]. Others have also shown that women have fewer CYP24A1 transcripts encoding the 1,25-dihydroxyvitamin D3-inactivating enzyme when compared to men. Therefore, binding and cellular accumulation of 1,25-dihydroxyvitamin D3 is increased in women with subsequent increased anti-inflammatory effects [34]. Recently, it was also demonstrated in a large observational study that 25-hydroxyvitamin D3 serum concentrations is not consistently associated with the carotid intima media thickness, which demonstrates the complex relationship between vitamin D3 and cardiovascular disease [35]. Current studies in our laboratory are carried out to further investigate the relationship between vitamin D3, postprandial leukocyte activation and its interaction with sex steroids. Moreover, a large prospective randomized trial is currently carried out to investigate whether vitamin D3 supplementation will actually reduce incident cardiovascular disease.

Transport of atherogenic lipoproteins by erythrocytes

Our research group and others have demonstrated the uptake of lipoproteins by leukocytes [36,37] including the presence of apo B on the membrane of leukocytes using flow cytometry [24]. We were interested whether apo B was also detectable on erythrocytes and it was indeed possible to detect apo B on erythrocytes using similar techniques. A pilot study in 166 subjects described in Chapter 6 demonstrated that patients with cardiovascular disease express less erythrocyte-bound apo B (ery-apoB) compared to control subjects. There was also a negative correlation between the carotid intima media thickness, which is a marker of subclinical atherosclerosis, and ery-apoB [38]. Chapter 7 described the results of the extended study with a total of 409 subjects. Very low values to absence of ery-apoB in particular was associated with cardiovascular disease. Ery-apoB remained higher in subjects with a normal carotid intima media thick-
ness when compared to subjects with an increased carotid intima media thickness [39]. Therefore, the capability of erythrocytes to bind apo B containing lipoproteins seems to be atheroprotective. So far, the exact molecular basis for this phenomenon remains unknown. Erythrocyte mediated reverse cholesterol transport by binding of atherogenic apo B-containing lipoproteins may explain the atheroprotective effect of ery-apoB since it was recently demonstrated in a mouse model that erythrocytes can contribute to reverse cholesterol transport in a similar way as high density lipoproteins [40]. In addition, we hypothesized that apo B containing lipoproteins bound to erythrocytes less likely interact with the endothelium when compared to unbound lipoproteins [41].

We continued our research investigating potential determinants of ery-apoB since the expression of apo B on erythrocytes was highly variable among study subjects. Since elevated serum concentrations of apo B are associated with cardiovascular disease it would be logical to hypothesize that more ery-apoB just reflects low serum concentrations of apo B. However, we did not observe any association between serum apo B and ery-apoB, nor was ery-apoB affected by temporary discontinuation of statin therapy despite an increase in serum apo B as described in Chapter 7. Therefore, ery-apoB is independent from the standard lipid parameters and may provide additional information as a clinical biomarker for cardiovascular risk prediction. However, its predictive and clinical value still needs validation in future studies.

Ery-apoB was measured using a polyclonal antibody against apo B, which is present on all different atherogenic lipoproteins including modified forms of LDL. This antibody should be able to bind different epitopes of apo B. Nevertheless, we presume that the apo B associated with erythrocytes represents most probably LDL or modified LDL. We

![Figure 1](image-url)

**Figure 1:** A postprandial change in erythrocyte-bound apolipoprotein B (ery-apoB) could not be detected. Ery-apoB was measured up to eight hours after the ingestion of a standardized oral fat loading test (n = 12). Data represent mean ± SEM. MFI = mean fluorescent intensity.
performed oral fat loading tests in 12 healthy subjects, which did not affect ery-apoB up to eight hours postprandially, which suggests that the triglyceride-rich lipoproteins are not involved (Figure 1). In addition, we were unable to detect a positive signal on erythrocytes with flow cytometry by using an antibody specifically targeted against apo B48.

Interestingly, we found that the ABO blood group was strongly associated with ery-apoB, which was measured in a relatively small subgroup (n = 104) [39]. In Chapter 7 it was shown that the expression of ery-apoB was almost twice as high in subjects with blood group O when compared to subjects with blood group A, B or AB. The prevalence of blood group O was lowest in the lowest ery-apoB tertile and highest in the highest ery-apoB tertile, whereas the prevalence of blood group A was exactly the opposite. It should be noted that the number of study subjects with blood group B and AB was very low. Therefore, our group is currently further exploring the relationship between blood groups and ery-apoB. Nevertheless, these results may indicate a direction for future research to investigate possible mechanisms for apo B to bind erythrocytes.

The complement receptor 1 may contribute to cellular transport of lipoproteins

Since apo B can be detected on both erythrocytes and different types of leukocytes we explored possible binding mechanisms. Multiple receptors such as the scavenger receptor CD36, LDL receptor, LOX-1 and apo B48 receptor have already been identified, which are involved in binding and uptake of lipoproteins by leukocytes [37,42,43]. Besides these classical lipoprotein receptors, complement receptors may also be involved, especially because the complement system has been linked to lipoprotein metabolism and the complement receptor 1 (CR1) is present on both leukocytes and erythrocytes [41,44]. The in vitro experiments described in Chapter 8 demonstrate that CR1 is indeed capable of binding LDL and modified LDL, but only once the lipoproteins were first opsonized with C3b by complement activation. The classical pathway contributed to C3b opsonisation of both LDL and modified LDL, whereas the alternative pathway only contributed to C3b opsonisation of modified LDL. Whether the lectin pathway was important in C3b opsonisation of LDL or modified LDL remained unclear, but it was clearly demonstrated that mannose binding lectin (MBL) did not bind LDL nor modified LDL.

The capability of CR1 to bind C3b opsonized forms of LDL may explain the presence of apo B on erythrocytes and in part on leukocytes. In contrast to leukocytes, erythrocytes do not express any established receptors capable of binding LDL or modified forms of LDL. CR1 on erythrocytes functions as an immune complex clearing mechanism.
and a similar mechanism may contribute to apo B-containing lipoproteins bound to erythrocytes, explaining the atheroprotective mechanism of ery-apoB [45,46].

It must be noted that all described results were obtained from in vitro experiments and that C3b was not originally present on the isolated lipoproteins. The absence of C3b on freshly isolated LDL may have been induced by the ultracentrifugation process or because C3b opsonized LDL are not present in serum anymore due to avid binding to CR1 expressing cells. Therefore, we have started experiments to investigate the relationship between CR1 and ery-apoB in vivo. Others have shown a positive relationship between the number of CR1 per erythrocyte and the capacity of erythrocytes to bind immune complexes [47]. We measured the expression of CR1 by erythrocytes in subjects with absence or very low levels of ery-apoB and in subjects with very high levels of ery-apoB. Ery-apoB was significantly different between the groups, but we did not observe a significant difference in CR1 expression by erythrocytes. In addition, no correlation was found between ery-apoB and the CR1 expression by erythrocytes (Figure 2). Other factors than just the amount of CR1 per erythrocyte must be responsible to initiate binding of apo B to erythrocytes (Figure 3). Future studies need to address whether specific CR1 polymorphisms like the A3650G and C5507G polymorphisms [48], CR1 gene methylation, modified LDL serum concentrations and different states of complement activation or blood groups [39] are the major determinants of ery-apoB (Figure 3).

Figure 2: Erythrocyte-bound apolipoprotein B (ery-apoB) was unrelated to the expression of complement receptor 1 (CR1 or CD35) by erythrocytes. Subjects with low (n = 25) and high (n = 28) ery-apoB (≤ 0.20 a.u. and ≥ 2.00 a.u., respectively) were asked to have their ery-apoB measured again on a separate occasion, which was combined with measuring their erythrocyte CR1 expression simultaneously. Ery-apoB remained significantly higher in subjects with high ery-apoB on their first occasion when compared to subjects with low ery-apoB on their initial visit (A). CD35 or CR1 expression was similar between subjects with low or high ery-apoB (B). No correlation between CD35 expression by erythrocytes and ery-apoB was found as shown in the scatter dotplot (n = 53) (C). Data in A and B represent the mean ± SEM. MFI = mean fluorescent intensity. *P < 0.001
Final remarks

The studies performed in this thesis illustrate the complex pathophysiology of atherosclerosis: from postprandial lipemia with continuous diurnal accumulation of triglycerides to complex cross-talk between circulating lipoproteins and blood cells. From a clinical point of view, the relatively simple implementation of non-fasting lipid profiles in clinical practice can already provide an improvement in patient convenience. Future studies are necessary to prove the effectiveness of vitamin D3 in reducing cardiovascular disease and whether binding of apo B containing lipoproteins to erythrocytes can be modified in a beneficial way to reduce the atherosclerotic burden and subsequent cardiovascular disease.
References


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