Interplay between Lipoproteins, the Complement System and Blood Cells in Atherosclerosis

Boudewijn Klop

ISBN: 978-94-6169-534-5

Cover: The bronze sculpture Ery-apoB shown throughout this thesis was specially crafted by the author for the described research. The sculpture resembles an abstracted lipoprotein adhered to the cellular surface of an erythrocyte. Only a part of the erythrocyte has been sculpted resulting in distinguished borders since research is characterized by a clear description of methodologies, results and context. The ever changing atmosphere surrounding the sculpture was strongly influenced by using photograpy from different perspectives and changing lighting conditions.

Layout and print: Optima Grafische Communicatie B.V.

Interplay between Lipoproteins, the Complement System and Blood Cells in Atherosclerosis

Proefschrift

Ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof. mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op donderdag 11 september 2014
klokke 16:15 uur

door

Boudewijn Klop
geboren te Woerdense Verlaat, Nederland
in 1984

Promotor:

Prof. dr. J. W. Jukema (Leids Universitair Medisch Centrum)

Co-promotores:

Dr. M. Castro Cabezas (Sint Franciscus Gasthuis, Rotterdam)
Dr. J. W. F. Elte (Sint Franciscus Gasthuis, Rotterdam)

Overige leden:

Prof. dr. A.J. Rabelink (Leids Universitair Medisch Centrum)
Prof. dr. C. van Kooten (Leids Universitair Medisch Centrum)
Prof. dr. P.C.N. Rensen (Leids Universitair Medisch Centrum)
Prof. dr. J. de Graaf (Radboud Universitair Medisch Centrum, Nijmegen)
Dr. R. van Bruggen (Sanquin, Amsterdam)

De drukkosten van het proefschrift werden vriendelijk gesponsord door:

Stichting Sint Christophorileen tot Oldehove
Sint Franciscus Gasthuis
Merck Sharp and Dohme (MSD)
Bayer HealthCare
Novo Nordisk
Servier
Het verschijnen van dit proefschrift werd mede mogelijk gemaakt door de steun van de Nederlandse Hartstichting

Voor papa en mama

Table of contents

Chapter 1

General introduction and outline of the thesis

Published in part in:
Int J Vasc Med 2012;2012:271030
Curr Cardiovasc Risk Rep 2012;6:27-34

Introduction

Atherosclerosis is the primary cause of death in the world [1]. Classical risk factors such as smoking, hypertension, hyperlipidemia, insulin resistance, increased body fat mass and unfavourable body fat distribution are strongly interrelated and can often be found in one and the same subject. Subjects with fasting hypertriglyceridemia usually have elevated postprandial triglycerides (TG) due to the close correlation of fasting and postprandial TG [2]. Postprandial lipemia is interesting because of recent reports showing that non-fasting TG independently predict the risk for atherosclerosis [3,4] and are possibly, even stronger predictors of cardiovascular disease (CVD) than fasting TG [3,5]. The postprandial state is characterized by accumulation of TG-rich lipoproteins inducing leukocyte activation [6,7], which is of special interest since atherosclerosis is considered a low-grade chronic inflammatory disease [8]. One of the early signs of atherosclerosis is the development of endothelial dysfunction and postprandial leukocyte activation has been related to temporary impaired endothelial function [9,10]. Besides leukocytes, there is also evidence of an interaction between lipoproteins and erythrocytes, which make up the majority of circulating blood cells [11]. A mouse model has shown that erythrocytes may contribute to reverse cholesterol transport, but factors influencing the binding of lipoproteins to erythrocytes need yet to be determined [12]. Therefore, the focus of this thesis is on postprandial lipemia and the interplay between lipids and circulating blood cells in relation to atherosclerosis.

Triglycerides and atherosclerosis

One of the first reports linking hypertriglyceridemia to cardiovascular disease is the description by Nicolaes Tulp (1593-1674), who was immortalized in the "Anatomical lesson" by Rembrandt, describing a patient with severe hypertriglyceridemia who probably died from a seizure [13]. Since the mid-twentieth century several publications appeared showing that patients with coronary heart disease have increased postprandial lipemia [14-16]. Convincing evidence establishing the role of postprandial hyperlipidemia and TG-rich lipoproteins in atherosclerosis was provided by research coming from different groups in the last three decades [17-20].

Recently large, prospective epidemiological studies have shown that a single measurement of non-fasting TG is associated with an increased risk of cardiovascular disease independent from classical risk factors [3,5]. For every 1 mmol/l increase in non-fasting TG a significant increase of death was found with multifactorially adjusted hazard ratios of 1.2-1.8 for men and 1.3-3.3 for women. Similar significant trends were found for myocardial infarction and ischemic heart disease in both men and women [5].

Therefore, it was suggested that non-fasting TG are stronger predictors of cardiovascular disease than fasting TG [3,5]. Abnormalities in TG reflect metabolic derangements and may be used as biomarkers for cardiovascular risk because of their strong relation with atherogenic remnant lipoproteins (chylomicron remnants and hepatic derived VLDL-remnants) [21]. **Chapter 2** provides an overview from the literature regarding the interpretation of elevated TG and subsequent treatment of hypertriglyceridemia with clear instructions for physicians.

Triglycerides and postprandial lipemia

Atherosclerosis is a chronic disease which takes years to develop, but endothelial function is already transiently impaired after a single high-fat meal and is associated with an abnormal postprandial TG response [9,10,22]. Most of the postprandial studies are performed using experimental situations where participants undergo standardized oral fat loading tests. However, these tests are not suitable to screen large populations or to use as a diagnostic tool for clinical practice. They are laborious, require a metabolic ward and are inconvenient for subjects, who need to fast for a long period and to be hospitalized during the test. Work from our laboratory described the technique of self-measured capillary TG to determine postprandial lipemia in individual subjects without the need for laborious and time-consuming oral fat loading tests. Daytime triglyceridemia measured in this way is also increased in patients with premature cardiovascular disease compared to healthy controls [23-27]. The intra-individual variability in fasting TG is approximately 20-25%, but variation in TG and postprandial lipemia between individuals is even higher [28-33]. However, little is known about the variability of non-fasting TG, which may affect the clinical usability of non-fasting TG in clinical practice. **Chapter 3** describes the results from a study, which investigated the intra-individual variability of diurnal TG using the technique of self-measured capillary TG.

Postprandial lipemia and leukocyte activation

As mentioned before, atherosclerosis is in part an inflammatory disease. Many inflammatory markers such as C-reactive protein (CRP), leukocyte count and complement component 3 (C3) have been associated with cardiovascular disease [34-39]. Several studies with animal models showed a reduction in atherosclerotic plaque formation [40,41] and prevention of endothelial dysfunction [42], when adherence of leukocytes to the endothelium was prevented. These findings support the theory that atherogenesis, in part, starts with leukocyte-endothelium interaction and adherence. Obligatory for

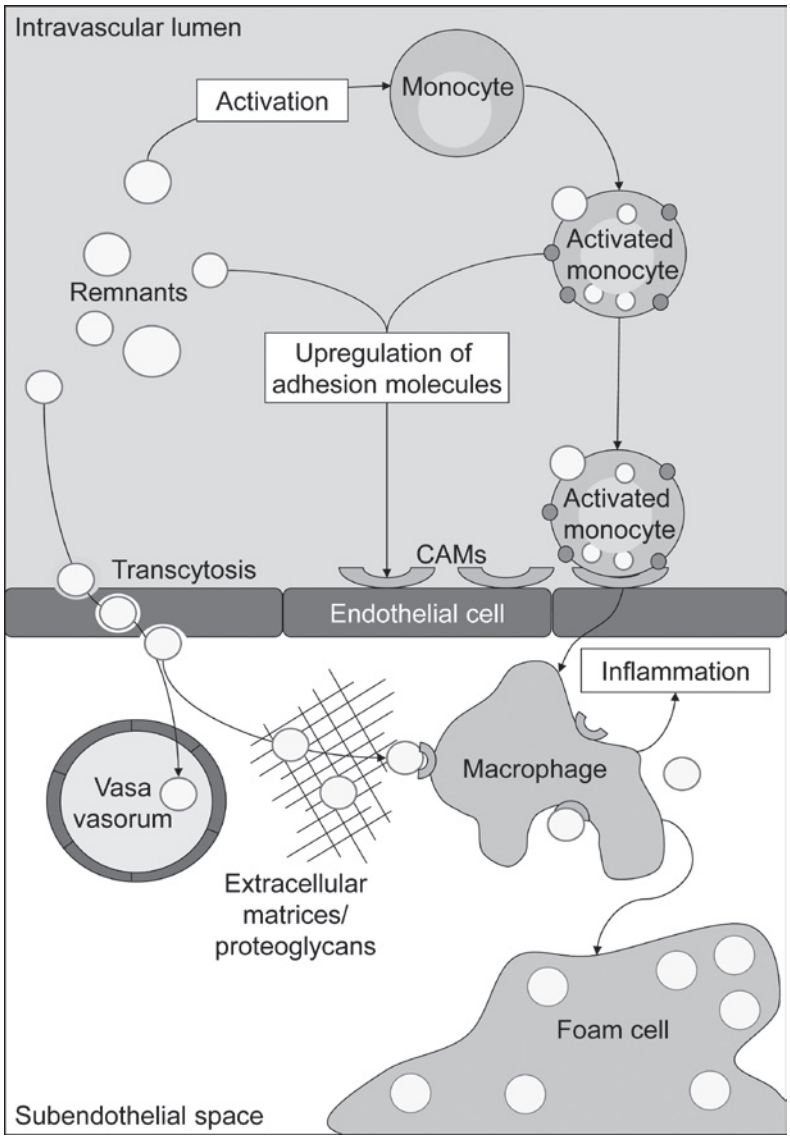

Figure 1: Concept of the initiation of atherosclerosis by remnant lipoproteins: Remnants enter the subendothelial space via non-specific transcytotic processes. This is often a non-pathologic process, because the remnants leave the subendothelial space again via the vasa vasorum. However, retention of remnants may occur in the presence of proteoglycans and excess extracellular matrices. Remnants can be easily taken up by macrophages, in contrast to LDL, which need to become modified first. Circulating remnants themselves also contribute to the presence of subendothelial macrophages. Monocytes can bind and take up remnants, which stimulate the monocytes to become activated. Subsequently, activated monocytes express adhesion molecules on the outer membrane and stimulate the expression of endothelial cellular adhesion molecules (CAMs), which allows monocytes to home on the endothelium and migrate into the subendothelial space. Finally, the macrophages change into highly atherogenic foam cells when lipid uptake exceeds lipid efflux.

this adherence is a cytokine-controlled sequential upregulation of selectins and adhesion molecules on activated leukocytes and endothelial cells (Figure 1) [43].

Van Oostrom et al have shown that postprandially, when TG rise, neutrophil counts increase with concomitant production of pro-inflammatory cytokines and oxidative stress; and that these changes may contribute to endothelial dysfunction [9,44]. Furthermore, TG are able to induce leukocyte activation, as has been shown *in vitro* [45,46] and *ex vivo* in hypertriglyceridemic patients [47]. In healthy volunteers and in patients with premature cardiovascular disease, postprandial lipemia upregulates leukocyte activation markers [48,49]. Leukocytes of fasting patients with cardiovascular disease have an increased lipid content, compared to controls and this may be due to uptake of chylomicrons [50]. Furthermore, uptake of remnant lipoproteins by primary human monocytes has been demonstrated in experiments *in vitro* [46]. Leukocytes are also able to take up retinyl esters, as markers of intestinally derived TG-rich lipoproteins [51]. Recently, we have shown that apolipoprotein (apo) B, the structural molecule of the atherogenic lipoproteins, binds to neutrophils and monocytes and that postprandial leukocytes transport dietary fatty acids [6]. This indicates that activation of leukocytes may occur in the circulation by direct interaction with TG-rich lipoproteins.

Since TG-rich lipoproteins induce leukocyte activation, postprandial lipemia may interfere with routine diagnostic procedures used to differentiate leukocytes into lymphocytes, monocytes, neutrophils, eosinophils and basophils. The automatic 5-part differentiation of leukocytes is performed by automatic cell counters in the laboratory using leukocyte cell population data (VCS), which consists of measurements of cell volume, conductivity and light scatter. The influence of postprandial lipemia on these parameters is described in **Chapter 4**.

Vitamin D deficiency has been associated with cardiovascular disease and a potential beneficial effect of vitamin D on cardiovascular disease has been suggested by the immunomodulatory effects of vitamin D. In vitro, it has been shown that 1,25-dihydroxyvitamin D3, the active metabolite from vitamin D3, reduces the activated state of leukocytes as expressed by a reduction in CD11b expression [52]. When 1,25-dihydroxyvitamin D3 is present in the culture medium macrophages take up less cholesterol with a concomitant reduction in atherogenic foam cell formation [53]. Therefore, we investigated the possible immunomodulatory effects of vitamin D on postprandial leukocyte activation in relation to arterial stiffness. The results of this study are described in **Chapter 5**.

Erythrocyte-bound apolipoprotein B in relation to atherosclerosis

Every atherogenic lipoprotein contains exactly one apo B molecule and the measurement of apo B provides an indication of the amount of atherogenic lipoproteins. Therefore, it is not surprising that studies have shown that the concentration of serum apo B is a strong predictor of cardiovascular disease [4,54,55]. Lipoproteins are found in fluid phase where they are metabolized and transported to specific organs. However, there is also evidence of a marginated pool of apo B containing lipoproteins notably attached to the endothelium and to circulating blood cells [11,56]. Erythrocytes represent the largest blood cell population and make up more than 99% of the total cellular space in blood [57]. The exchange of cholesterolesters between LDL and erythrocyte membranes may be substantial, which can only be explained by binding of LDL to erythrocytes and not by accidental collision [58,59]. In addition, erythrocytes may contribute to reverse cholesterol transport and a trend of impaired reverse cholesterol transport was observed in anemic mice [12].

Binding of atherogenic apo B containing lipoproteins to erythrocytes as a mechanism for reverse cholesterol transport may provide an atheroprotective effect of erythrocyte-bound apo B. In addition, we hypothesized a contributing protective mechanism that apo B containing lipoproteins bound to erythrocytes are less likely to interact with the endothelium [11]. **Chapter 6** presents a pilot study showing that patients with known cardiovascular disease have lower values of erythrocyte-bound apo B when compared to control subjects without cardiovascular disease. Potential determinants like serum apo B concentrations, the use of statins and the ABO blood group in relation to erythrocyte-bound apo B are explored in **Chapter 7**.

The complement receptor 1 as a potential mechanism for lipoprotein binding to erythrocytes

Another inflammatory pathway related to cardiovascular disease and lipid metabolism is the complement system. Serum complement component (C) 3 is a strong predictor of myocardial infarction [37] and it has been positively associated with obesity, cardiovascular disease, insulin resistance, the metabolic syndrome, fasting and postprandial TG and hypertension [2,37,60]. Chylomicrons are the strongest *in vitro* and *in vivo* stimulators of adipocyte C3 production via activation of the alternative complement cascade [39,61]. A postprandial increase in serum C3 after a fat meal has been shown in healthy subjects, in patients with cardiovascular disease and in patients with familial combined hyperlipidemia [2,36,39]. Moreover, this postprandial increment has been related to TG and FFA metabolism [62].

The complement receptor 1 (CR1) is known for its capacity to bind C3b-opsonized immune complexes and pathogens. CR1 is present on monocytes and neutrophils as well as on erythrocytes. In leukocytes CR1 internalizes C3b-opsonized immune complexes and pathogens for phagocytosis, whereas CR1 can not be internalized by erythrocytes. Therefore, erythrocytes use CR1 as a transport mechanism of C3b-opsonized immune complexes called immune adherence [63-65]. We hypothesized that CR1 may be involved in the binding and transportation of apo B containing lipoproteins by erythrocytes. **Chapter 8** presents *in vitro* experiments, investigating the binding capacity of LDL and modified LDL to CR1.

Summary

The topics in the upcoming chapters will slowly shift from a more clinical perspective to a more fundamental level of research. The up to date literature regarding the treatment of hypertriglyceridemia is reviewed in **Chapter 2**. **Chapter 3** focuses on determinants regarding the intra-individual variability in fasting and non-fasting plasma triglycerides. The effects of postprandial lipemia and subsequent leukocyte activation on the automated 5-part leukocyte differentiation by a hematology analyzer are described in **Chapter 4**. The effects of vitamin D3 supplementation on postprandial leukocyte activation and arterial stiffness are investigated in **Chapter 5**. The association between erythrocyte-bound apo B and cardiovascular disease, serum apo B, the use of statins and ABO blood group are described in **Chapters 6 and 7**. Finally, a potential contribution of the complement receptor 1 to bind lipoproteins as a model for cellular binding of LDL and modified LDL is described in **Chapter 8** followed by a general discussion in **Chapter 9**.

References

1. Braunwald E. Shattuck lecture--cardiovascular medicine at the turn of the millennium: Triumphs, concerns, and opportunities. N Engl J Med 1997;337:1360-1369
2. Halkes CJ, van Dijk H, de Jaegere PPT, Plokker HWM, van der Helm Y, Erkelens DW, et al. Postprandial increase of complement component 3 in normolipidemic patients with coronary artery disease. Arterioscler Thromb Vasc Biol 2001;21:1526-1530
3. Bansal S, Buring JE, Rifai N, Mora S, Sacks FM, Ridker PM. Fasting compared with nonfasting triglycerides and risk of cardiovascular events in women. JAMA 2007;298:309-316
4. Mora S, Rifai N, Buring JE, Ridker PM. Fasting compared with nonfasting lipids and apolipoproteins for predicting incident cardiovascular events. Circulation 2008;118:993-1001
5. Nordestgaard BG, Benn M, Schnohr P, Tybjaerg-Hansen A. Nonfasting triglycerides and risk of myocardial infarction, ischemic heart disease, and death in men and women. JAMA 2007;298:299-308
6. Alipour A, van Oostrom AJ, Izraeljan A, Verseyden C, Collins JM, Frayn KN, et al. Leukocyte activation by triglyceride-rich lipoproteins. Arterioscler Thromb Vasc Biol 2008;28:792-797
7. Gower RM, Wu H, Foster GA, Devaraj S, Jialal I, Ballantyne CM, et al. CD11c/CD18 expression is upregulated on blood monocytes during hypertriglyceridemia and enhances adhesion to vascular cell adhesion molecule-1. Arterioscler Thromb Vasc Biol 2011;31:160-166
8. Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med 1999;340:115-126
9. van Oostrom AJ, Sijmonsma TP, Verseyden C, Jansen EH, de Koning EJ, Rabelink TJ, et al. Postprandial recruitment of neutrophils may contribute to endothelial dysfunction. J Lipid Res 2003;44:576-583
10. Vogel RA, Corretti MC, Plotnick GD. Effect of a single high-fat meal on endothelial function in healthy subjects. Am J Cardiol 1997;79:350-354
11. Bovenberg SA, Alipour A, Elte JW, Rietveld AP, Janssen JW, van de Geijn GJ, et al. Cell-mediated lipoprotein transport: A novel anti-atherogenic concept. Atheroscler Suppl 2010;11:25-29
12. Hung KT, Berisha SZ, Ritchey BM, Santore J, Smith JD. Red blood cells play a role in reverse cholesterol transport. Arterioscler Thromb Vasc Biol 2012;32:1460-1465
13. Erkelens DW, de Bruin TW, Castro Cabezas M. Tulp syndrome. Lancet 1993;342:1536-1537
14. Barritt DW. Alimentary lipaemia in men with coronary artery disease and in controls. Br Med J 1956;2:640-644
15. Brown DF, Kinch SH, Doyle JT. Serum triglycerides in health and in ischemic heart disease. N Engl J Med 1965;273:947-952
16. Zilversmit DB. Atherogenesis: A postprandial phenomenon. Circulation 1979;60:473-485
17. de Groot LC, van Staveren WA, Burema J. Survival beyond age 70 in relation to diet. Nutr Rev 1996;54:211-212
18. Karpe F, Steiner G, Uffelman K, Olivecrona T, Hamsten A. Postprandial lipoproteins and progression of coronary atherosclerosis. Atherosclerosis 1994;106:83-97
19. Patsch JR, Miesenbock G, Hopferwieser T, Muhlberger V, Knapp E, Dunn JK, et al. Relation of triglyceride metabolism and coronary artery disease. Studies in the postprandial state. Arterioscler Thromb 1992;12:1336-1345
20. Weintraub MS, Grosskopf I, Rassin T, Miller H, Charach G, Rotmensch HH, et al. Clearance of chylomicron remnants in normolipidaemic patients with coronary artery disease: Case control study over three years. BMJ 1996;312:935-939

21. Miller M, Stone NJ, Ballantyne C, Bittner V, Criqui MH, Ginsberg HN, et al. Triglycerides and cardiovascular disease: A scientific statement from the American Heart Association. Circulation 2011;123:2292-2333
22. Daskalova DC, Kolovou GD, Panagiotakos DB, Pilatis ND, Cokkinos DV. Increase in aortic pulse wave velocity is associated with abnormal postprandial triglyceride response. Clin Cardiol 2005;28:577-583
23. Castro Cabezas M, Halkes CJ, Meijssen S, van Oostrom AJ, Erkelens DW. Diurnal triglyceride profiles: A novel approach to study triglyceride changes. Atherosclerosis 2001;155:219-228
24. Geluk CA, Halkes CJ, De Jaegere PP, Plokker TW, Castro Cabezas M. Daytime triglyceridemia in normocholesterolemic patients with premature atherosclerosis and in their first-degree relatives. Metabolism 2004;53:49-53
25. Halkes CJ, Castro Cabezas M, van Wijk JP, Erkelens DW. Gender differences in diurnal triglyceridemia in lean and overweight subjects. Int J Obes Relat Metab Disord 2001;25:1767-1774
26. van Oostrom AJ, Castro Cabezas M, Ribalta J, Masana L, Twickler TB, Remijnse TA, et al. Diurnal triglyceride profiles in healthy normolipidemic male subjects are associated to insulin sensitivity, body composition and diet. Eur J Clin Invest 2000;30:964-971
27. van Wijk JP, Castro Cabezas M, Halkes CJ, Erkelens DW. Effects of different nutrient intakes on daytime triacylglycerolemia in healthy, normolipemic, free-living men. Am J Clin Nutr 2001;74:171-178
28. Brown SA, Boerwinkle E, Kashanian FK, Swanson N, Patsch W. Variation in concentration of lipids, lipoprotein lipids, and apolipoproteins A-I and B in plasma from healthy women. Clin Chem 1990;36:207-210
29. Demacker PN, Schade RW, Jansen RT, Van 't Laar A. Intra-individual variation of serum cholesterol, triglycerides and high density lipoprotein cholesterol in normal humans. Atherosclerosis 1982;45:259-266
30. Godsland IF. Intra-individual variation: Significant changes in parameters of lipid and carbohydrate metabolism in the individual and intra-individual variation in different test populations. Ann Clin Biochem 1985;22:618-624
31. Kafonek SD, Derby CA, Bachorik PS. Biological variability of lipoproteins and apolipoproteins in patients referred to a lipid clinic. Clin Chem 1992;38:864-872
32. Smith SJ, Cooper GR, Myers GL, Sampson EJ. Biological variability in concentrations of serum lipids: Sources of variation among results from published studies and composite predicted values. Clin Chem 1993;39:1012-1022
33. Wasenius A, Stugaard M, Otterstad JE, Froyshov D. Diurnal and monthly intra-individual variability of the concentration of lipids, lipoproteins and apoproteins. Scand J Clin Lab Invest 1990;50:635-642
34. Danesh J, Whincup P, Walker M, Lennon L, Thomson A, Appleby P, et al. Low grade inflammation and coronary heart disease: Prospective study and updated meta-analyses. BMJ 2000;321:199-204
35. Friedman GD, Klatsky AL, Siegelaub AB. The leukocyte count as a predictor of myocardial infarction. N Engl J Med 1974;290:1275-1278
36. Meijssen S, van Dijk H, Verseyden C, Erkelens DW, Castro Cabezas M. Delayed and exaggerated postprandial complement component 3 response in familial combined hyperlipidemia. Arterioscler Thromb Vasc Biol 2002;22:811-816

37. Muscari A, Massarelli G, Bastagli L, Poggiopollini G, Tomassetti V, Drago G, et al. Relationship of serum C3 to fasting insulin, risk factors and previous ischaemic events in middle-aged men. Eur Heart J 2000;21:1081-1090
38. Schmidt MI, Duncan BB, Sharrett AR, Lindberg G, Savage PJ, Offenbacher S, et al. Markers of inflammation and prediction of diabetes mellitus in adults (Atherosclerosis Risk In Communities Study): A cohort study. Lancet 1999;353:1649-1652
39. Verseyden C, Meijssen S, van Dijk H, Jansen H, Castro Cabezas M. Effects of atorvastatin on fasting and postprandial complement component 3 response in familial combined hyperlipidemia. J Lipid Res 2003;44:2100-2108
40. Eriksson EE, Xie X, Werr J, Thoren P, Lindbom L. Direct viewing of atherosclerosis in vivo: Plaque invasion by leukocytes is initiated by the endothelial selectins. FASEB J 2001;15:1149-1157
41. Huo Y, Ley K. Adhesion molecules and atherogenesis. Acta Physiol Scand 2001;173:35-43
42. Murohara T, Buerke M, Lefer AM. Polymorphonuclear leukocyte-induced vasocontraction and endothelial dysfunction. Role of selectins. Arterioscler Thromb 1994;14:1509-1519
43. Worthylake RA, Burridge K. Leukocyte transendothelial migration: Orchestrating the underlying molecular machinery. Curr Opin Cell Biol 2001;13:569-577
44. Van Oostrom AJ, Sijmonsma TP, Rabelink TJ, Van Asbeck BS, Castro Cabezas M. Postprandial leukocyte increase in healthy subjects. Metabolism 2003;52:199-202
45. Wanten G, van Emst-De Vries S, Naber T, Willems P. Nutritional lipid emulsions modulate cellular signaling and activation of human neutrophils. J Lipid Res 2001;42:428-436
46. Bentley C, Hathaway N, Widdows J, Bejta F, De Pascale C, Avella M, et al. Influence of chylomicron remnants on human monocyte activation in vitro. Nutr Metab Cardiovasc Dis 2011;21:871-878
47. Hiramatsu K, Arimori S. Increased superoxide production by mononuclear cells of patients with hypertriglyceridemia and diabetes. Diabetes 1988;37:832-837
48. van Oostrom AJ, Plokker HW, van Asbeck BS, Rabelink TJ, van Kessel KP, Jansen EH, et al. Effects of rosuvastatin on postprandial leukocytes in mildly hyperlipidemic patients with premature coronary sclerosis. Atherosclerosis 2006;185:331-339
49. van Oostrom AJ, Rabelink TJ, Verseyden C, Sijmonsma TP, Plokker HW, De Jaegere PP, et al. Activation of leukocytes by postprandial lipemia in healthy volunteers. Atherosclerosis 2004;177:175-182
50. Tertov VV, Kalenich OS, Orekhov AN. Lipid-laden white blood cells in the circulation of patients with coronary heart disease. Exp Mol Pathol 1992;57:22-28
51. Skrede B, Blomhoff R, Maelandsmo GM, Ose L, Myklebost O, Norum KR. Uptake of chylomicron remnant retinyl esters in human leukocytes in vivo. Eur J Clin Invest 1992;22:229-234
52. Spittler A, Willheim M, Leutmezer F, Ohler R, Krugluger W, Reissner C, et al. Effects of 1 alpha,25-dihydroxyvitamin D3 and cytokines on the expression of MHC antigens, complement receptors and other antigens on human blood monocytes and U937 cells: Role in cell differentiation, activation and phagocytosis. Immunology 1997;90:286-293
53. Oh J, Weng S, Felton SK, Bhandare S, Riek A, Butler B, et al. 1,25(OH)2 vitamin D inhibits foam cell formation and suppresses macrophage cholesterol uptake in patients with type 2 diabetes mellitus. Circulation 2009;120:687-698
54. Lamarche B, Moorjani S, Lupien PJ, Cantin B, Bernard PM, Dagenais GR, et al. Apolipoprotein A-I and B levels and the risk of ischemic heart disease during a five-year follow-up of men in the Quebec Cardiovascular Study. Circulation 1996;94:273-278
55. Boekholdt SM, Arsenault BJ, Mora S, Pedersen TR, LaRosa JC, Nestel PJ, et al. Association of LDL cholesterol, non-HDL cholesterol, and apolipoprotein B levels with risk of cardiovascular events among patients treated with statins: A meta-analysis. JAMA 2012;307:1302-1309

56. Verseyden C, Meijssen S, Castro Cabezas M. Effects of atorvastatin on fasting plasma and marginated apolipoproteins B48 and B100 in large, triglyceride-rich lipoproteins in familial combined hyperlipidemia. J Clin Endocrinol Metab 2004;89:5021-5029
57. Hinderling PH. Red blood cells: A neglected compartment in pharmacokinetics and pharmacodynamics. Pharmacol Rev 1997;49:279-295
58. Hui DY, Noel JG, Harmony JA. Binding of plasma low density lipoproteins to erythrocytes. Biochim Biophys Acta 1981;664:513-526
59. Hui DY, Harmony JA. Interaction of plasma lipoproteins with erythrocytes. I. Alteration of erythrocyte morphology. Biochim Biophys Acta 1979;550:407-424
60. van Oostrom AJ, Alipour A, Plokker TW, Sniderman AD, Castro Cabezas M. The metabolic syndrome in relation to complement component 3 and postprandial lipemia in patients from an outpatient lipid clinic and healthy volunteers. Atherosclerosis 2007;190:167-173
61. Scantlebury T, Sniderman AD, Cianflone K. Regulation by retinoic acid of acylation-stimulating protein and complement C3 in human adipocytes. Biochem J 2001;356:445-452
62. Halkes CJ, van Dijk H, Verseyden C, de Jaegere PP, Plokker HW, Meijssen S, et al. Gender differences in postprandial ketone bodies in normolipidemic subjects and in untreated patients with familial combined hyperlipidemia. Arterioscler Thromb Vasc Biol 2003;23:1875-1880
63. Cornacoff JB, Hebert LA, Smead WL, VanAman ME, Birmingham DJ, Waxman FJ. Primate erythrocyte-immune complex-clearing mechanism. J Clin Invest 1983;71:236-247
64. Li J, Wang JP, Ghiran I, Cerny A, Szalai AJ, Briles DE, et al. Complement receptor 1 expression on mouse erythrocytes mediates clearance of streptococcus pneumoniae by immune adherence. Infect Immun 2010;78:3129-3135
65. Waxman FJ, Hebert LA, Cornacoff JB, VanAman ME, Smead WL, Kraut EH, et al. Complement depletion accelerates the clearance of immune complexes from the circulation of primates. J Clin Invest 1984;74:1329-1340

Chapter 2

A physician's guide for the management of hypertriglyceridemia: the etiology of hypertriglyceridemia determines treatment strategy

Boudewijn Klop
J. Wouter Jukema
Ton J. Rabelink
Manuel Castro Cabezas

Panminerva Med 2012;54:91-103

Abstract

Hypertriglyceridemia is a common lipid disorder associated to different, highly prevalent metabolic derangements like diabetes mellitus, the metabolic syndrome and obesity. The choice of treatment depends on the underlying pathogenesis and the consequences for atherosclerosis or pancreatitis. A family history, physical examination and analysis of the lipid profile including measurement of apolipoprotein B or non-HDL-C are necessary to establish the underlying primary or secondary cause. Due to physiological diurnal variations of triglycerides (TG), the time of measurement (fasting or postprandial) should be taken into account when evaluating TG values. Increased awareness arises concerning the impact of postprandial hypertriglyceridemia on the development of atherosclerosis. Hypertriglyceridemia is strongly associated to postprandial hyperlipidemia, remnant accumulation, increased small dense LDL concentrations, low HDL-C, increased oxidative stress, endothelial dysfunction, leukocyte activation and insulin resistance. All these factors are strongly linked to the development of atherosclerosis. Treatment should be aimed at reducing the secretion of triglyceride-rich lipoproteins, increasing intravascular lipolysis and reducing the number of circulating remnants. The main intervention is a change of lifestyle with decreased alcohol consumption, increased physical activity, dietary changes and, if applicable, adaptation of used medication. Fibrates, fish oil and nicotinic acid are the first choice of treatment in sporadic and familial hypertriglyceridemia to reduce the risk of pancreatitis, whereas high dose statins, sometimes in combination with fibrates, nicotinic acid, or fish oil capsules, are indicated for familial combined hyperlipidemia. Statins are necessary to reach low LDL-C concentrations in patients with type 2 diabetes mellitus and statin dosage should be increased when hypertriglyceridemia is present to reach secondary treatment targets for apolipoprotein B or non-HDL-C. Finally, family screening is mandatory to detect familial lipid disorders for early intervention in other family members.

Introduction

Hypertriglyceridemia is a common lipid disorder associated with an increased risk for cardiovascular disease and pancreatitis, especially at higher triglyceride (TG) concentrations. Approximately one third of the adult US population has TG concentrations above 1.70 mmol/l (150 mg/dl) and a total of 18% of the population showed marked hypertriglyceridemia with TG concentrations above 2.25 mmol/l (200 mg/dl) [1]. Nevertheless, only 3.6% percent of the population with TG concentrations above 2.25 mmol/l used medication for hypertriglyceridemia [1]. Treatment strategies for hypertriglyceridemia strongly depend on the etiology of the hypertriglyceridemia and the relation to complications. Plasma TG concentrations are determined by the production of TG-rich lipoproteins (VLDL, chylomicrons and their respective remnants), the intravascular lipolysis of TG-rich lipoproteins (TRLs) and, finally, the hepatic uptake of TRLs. Hypertriglyceridemia can be subdivided into primary and secondary causes. Therefore, physicians should search for the underlying cause(s) in patients with hypertriglyceridemia, because it influences treatment strategy, cardiovascular risk assessment and, potentially, the necessity to screen families for familial lipid disorders [2]. This review provides an overview of the current evidence how to correctly diagnose hypertriglyceridemia, to search for the underlying etiology in individual cases and how to choose the appropriate treatment strategy.

Triglyceride metabolism

A schematic overview of TG metabolism is shown in Figure 1. TG are a major energy source for the body. After ingestion of food containing fat, TG are hydrolyzed in the intestinal lumen into free fatty acids (FFA) and 2-monoacylglycerols (MAG) and taken up by enterocytes through passive diffusion and specific proteins, like CD36 and various fatty acid transport proteins [3]. Once inside the endoplasmatic reticulum of the enterocyte, FFA and MAG are assembled into TG again and packed with cholesterol, phospholipids and apolipoproteins (apo) B48 and A-IV to form chylomicrons [4,5]. In this process, the microsomal triglyceride transfer protein and the editing ezyme complex are paramount factors [6,7]. Especially the latter is crucial for the synthesis of apo B48, the structural protein of chylomicrons. After assembling, the chylomicrons are secreted into the lymphatics and finally enter the circulation through the thoracic duct. Within the circulation apo A-IV is exchanged for two other apolipoproteins, namely apo C-II and apo E [8].

In fasting physiological conditions, plasma TG are mainly determined by VLDL concentrations synthesized in the liver. VLDL synthesis is a continuous process, which

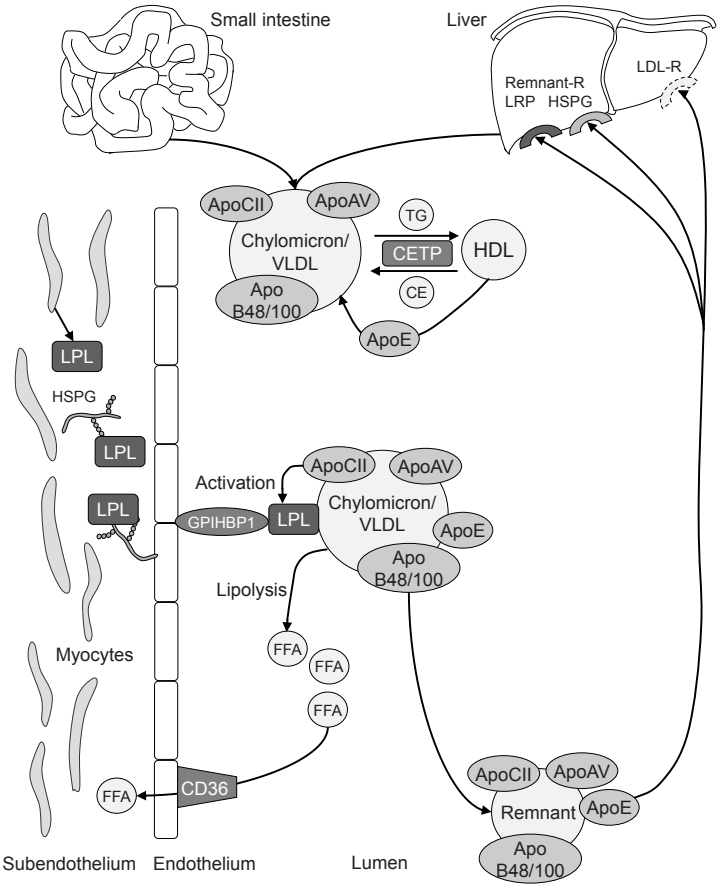

Figure 1: A schematic representation of triglyceride (TG) hydrolysis. Very low density lipoproteins (VLDL) are secreted by the liver and contain apolipoprotein (apo) B100. In contrast, the small intestine secretes chylomicrons, which contain apo B48. Both VLDL and chylomicrons contain high concentrations of TG and hydrolysis is necessary for the delivery of free fatty acids (FFA) to muscular and adipose tissue for energy expenditure or storage. Lipoprotein lipase (LPL) has a central role in the hydrolysis of TG. LPL is mainly produced by myocytes and adipocytes and transported to the subendothelial space by heparan sulphate proteoglycans (HSPG). Finally, LPL is transported towards the capillary lumen by glycosylphosphatidylinositol-anchored high-density-binding protein 1 (GPIHBP1). LPL becomes activated by apo C-II, which is available on both chylomicrons and VLDL. In addition, apo A-V is involved in the TG hydrolysis as well. The TG are hydrolyzed into FFA, which are taken up from the circulation by specific receptors like CD36 or via non-specific proteins like albumin. Chylomicrons and VLDL shrink in size and become denser as hydrolysis continues. The remaining remnants are taken up by the liver via multiple receptors like the LDL-related protein receptor (LRP), several proteoglycans (HSPG) and a small proportion by the LDL-receptor (LDL-R).

Within the circulation, chylomicrons and VLDL interact with HDL by cholesterylester-transfer protein (CETP), which exchanges cholesterylesters (CE) for TG. In case of hypertriglyceridemia, CETP increases cholesterol content of TG-rich lipoproteins in combination with a decrease in HDL-cholesterol. This explains the inverse relationship between TG and HDL-C. Moreover, apo E is transferred from HDL to chylomicrons and VLDL, which is necessary for the hepatic removal of the remnants.

increases postprandially when food derived TG and FFA reach the liver. The VLDL assembly resembles chylomicron synthesis, but in this case, apoB100 is the structural protein of VLDL [7].

Both, chylomicrons and VLDL are essential for the delivery of FFA to mainly the heart, skeletal muscle and adipose tissue for energy expenditure or storage. Hydrolysis of TG in the circulation is the main determinant of FFA delivery to tissues. Many proteins are involved in the hydrolysis of TG and lipoprotein lipase (LPL) and its co-factor apo C-II are the most important. LPL is mainly synthesized by myocytes and adipocytes, which secrete LPL into the surrounding interstitial spaces. LPL is transported from the interstitial space to the capillary lumen by binding to heparan sulphate proteoglycans. Recently it was discovered that LPL anchors on glycosylphosphatidylinositol-anchored high-density-binding protein 1 (GPIHBP1) at the endothelial cell surface of small capillaries, where LPL serves as a docking station for TG-rich lipoproteins [9-11]. Apo C-II, which is carried by chylomicrons and VLDL, serves as a co-factor for LPL activity, whereas apoC-III serves as an inhibitor of LPL [10,12,13]. Furthermore, apo A-V is also a major determinant of TG hydrolysis [14]. FFA are internalized by the endothelium via cell surface receptors like CD36 and via other processes such as non-specific carriers like albumin [15,16]. Lipolysis results in TG depleted chylomicrons and VLDL, which shrink in diameter and become enriched with cholesterol due to the action of cholesterol ester transfer protein (CETP) and other transfer proteins [17], resulting in chylomicron and VLDL remnants, respectively. Finally, the remnants are taken up by the liver via multiple receptor pathways including the LDL-receptor, heparan sulphate proteoglycans and the LDL receptor-related protein [12].

Within the circulation TG-rich lipoproteins interact with HDL by CETP, which exchanges cholesterylesters for TG (Figure 1). Therefore, cholesterol content of TG-rich lipoproteins increases, HDL-C decreases and small dense LDL become more abundant in the case of hypertriglyceridemia [17]. This close metabolic connection between TRLs and HDL, reflects the difficulty to distinguish hypertriglyceridemia as a cardiovascular risk factor independently from low HDL-C concentrations [18]. Apo E is transferred from HDL to chylomicrons and VLDL, where apoE is involved in multiple processes. Apo E has an inhibitory effect on the hydrolysis of TG within the circulation and secondly apo E binds to the LDL receptor, LDL receptor-related protein and heparan sulfate proteoglycans for adequate hepatic removal of remnants [12,19,20].

Fasting versus non-fasting triglycerides

Fasting TG levels below 1.70 mmol/l are considered desirable and hypertriglyceridemia is present when TG levels are above 1.70 mmol/l [18,21]. Current guidelines recommend to

measure TG in the fasting state since TG concentrations increase during the day [18,21]. However, most patients visit the hospital in the non-fasting state and it would be more convenient for both, patients and doctors, to measure non-fasting lipid profiles. Prospective studies have shown that also non-fasting TG predict cardiovascular disease in the non-fasting state [22,23] and it has been suggested that non-fasting TG are even a stronger predictor of cardiovascular disease than fasting TG [24,25]. Moreover, non-fasting TG are strongly correlated to fasting TG concentrations [26] and increase approximately 0.5 mmol/l in females and 1.0 mmol/l in males during the day [27,28]. These values can be used to compose normal ranges for non-fasting TG levels since mean diurnal increase in TG is similar between normotriglyceridemic and hypertriglyceridemic individuals [28]. Intra-individual variability of TG does not significantly increase in females during the day and not in males during daylight hours [28]. In summary, non-fasting TG can be used in clinical practice as long as an average TG increase of 0.5 mmol/l in females and 1.0 mmol/l in males is considered.

One of the inconveniences of measuring non-fasting lipid profiles is the fact that LDL-C calculations need fasting samples to be accurate. Non-fasting samples can lead to lower LDL-C values. It has been suggested that LDL-C is approximately 0.3-0.6 mmol/l lower in the non-fasting state [29]. In our opinion, the falsely decreased LDL-C is probably negligible in non-fasting normotriglyceridemic subjects.

Apo B and non-HDL-C can be used as an alternative for LDL-C. Apo B represents the *number* of circulating atherogenic lipoproteins containing apo B (LDL, VLDL, chylomicrons and their respective remnants) [30]. In contrast, non-HDL-C reflects the *cholesterol concentration* of both LDL and TRLs [31]. A post-hoc analysis of two combined prospective studies has shown that non-HDL-C and apo B are better predictors for future cardiovascular events than LDL-C in subjects receiving statin therapy [32]. High non-HDL-C levels predict cardiovascular disease independently from LDL-C concentrations [33]. In addition, apoB and non-HDL-C levels remain unchanged in the non-fasting state [34]. Normal values for non-HDL-C are 0.8 mmol/l above the normal ranges for LDL-C and the normal value for apoB ranges from 0.8-1.0 g/l [21].

The evaluation of the patient with hypertriglyceridemia

Whether hypertriglyceridemia alone initiates and accelerates atherosclerosis is an ongoing discussion, but hypertriglyceridemia surely is a marker of metabolic abnormalities [13]. Hypertriglyceridemia is often secondary to inappropriate dietary habits, obesity, diabetes, chronic kidney disease, end-stage liver disease or use of certain medications [2,30,35-37]. Besides secondary causes, hypertriglyceridemia can be caused by monogenic or polygenic diseases, which can have different associations with cardiovascular

Table 1: Primary and secondary causes of hypertriglyceridemia

Primary hypertriglyceridemia	Secondary hypertriglyceridemia
Familial combined hyperlipidemia	Obesity
Familial hypertriglyceridemia	Diabetes mellitus
Familial dysbetalipoproteinemia	Dietary patterns
	Smoking
Very rare primary causes:	Excessive alcohol intake
Tangier disease	Hypothyroidism
Lipoprotein lipase deficiency	End stage kidney disease
GP1HBP1 deficiency	End stage liver disease
Apolipoprotein A-V deficiency	Human Immunodeficiency Virus
Apolipoprotein C-II deficiency	Use of medication (estrogens, androgens, glucocorticoids, cyclosporines, tacrolimus, β-blockers, thiazide diuretics, synthetic retinoids, valproate and related drugs)[2,35,37]

GPIHBP1 = glycosylphosphatidylinositol-anchored high-density-binding protein 1

disease. A list of diseases and factors causing hypertriglyceridemia is shown in Table 1. An adequate diagnosis for the cause of hypertriglyceridemia is of importance to determine the exact treatment strategy for each individual patient.

The evaluation of the hypertriglyceridemic patient starts with the family history, specifically aimed to identify lipid disorders and premature cardiovascular disease in first degree relatives. Secondly, potential secondary causes of hypertriglyceridemia should be identified. Therefore, other factors like the patient's medical history, use of medication, dietary pattern and smoking history are also relevant. Physical examination should at least include the body mass index and waist circumference. The presence of xanthomas, which are usually not present in moderate hypertriglyceridemia, does not help to identify the underlying hypertriglyceridemic cause [2]. Laboratory screening should include a lipid profile (fasting or non-fasting) containing total cholesterol, LDL-C, HDL-C, TG and preferably apo B. Non-HDL-C concentrations can be easily calculated without additional costs as an alternative for apo B. In addition, creatinine levels reflecting kidney function, TSH levels, and glucose or glycated haemoglobin A(1c) and "liver function" tests should be measured to search for renal failure, hypothyroidism, diabetes and liver disease, respectively. An apo E genotyping can be performed when the diagnosis of familial dysbetalipoproteinemia is considered.

Primary causes of hypertriglyceridemia

The three most common primary causes of hypertriglyceridemia are familial combined hyperlipidemia (FCH), familial hypertriglyceridemia and familial dysbetalipoproteinemia, which all affect approximately 1% of the population [2,35,38-40]. Besides the three most common primary hypertriglyceridemias, severe hypertriglyceridemia can already present during childhood due to very rare genetic disorders like LPL deficiency, apo A-V deficiency or apo C-II deficiency. Recently, the first human case with a GPIHBP1 deficiency showing marked hypertriglyceridemia was published [41]. However, these rare disorders are beyond the scope of this review.

FCH is characterized by increased TG, LDL-C and apo B in combination with the presence of premature cardiovascular disease in at least one first degree relative [42,43]. Multiple genes are involved in FCH leading to an increased hepatic production of VLDL together with a decreased clearance of circulating remnants [43-50]. Insulin resistance often coincides with FCH [51]. The presence of small, dense LDL is another characteristic of FCH [52]. Cardiovascular risk is substantially increased in FCH [38-40] and it has even been suggested that approximately 10% of the patients with a myocardial infarction below the age of 60 years have FCH [53].

In contrast, familial hypertriglyceridemia (FHTG) is not associated with an increased risk of cardiovascular disease [38] and is characterized by an impaired catabolism of TRLs [35]. The exact genetic disorder is unknown, but several candidate genes have been postulated [54]. Lipid profiles from patients with FHTG typically show increased TG concentrations above 5.0 mmol/l, with decreased LDL-C and normal apo B concentrations. FHTG may not be accompanied by premature atherosclerosis, but chylomicronemia syndrome (TG > 20.00 mmol/l) can develop when FHTG is accompanied by other factors, which can raise TG levels substantially. Highly increased TG levels can cause acute pancreatitis, making treatment imperative [55-57].

Familial dysbetalipoproteinemia (FD), also called "remnant removal disease" or type III hyperlipoproteinemia according to the Fredrickson classification, is most frequently caused by homozygosity for an isoform of apo E, namely apo E2 [12]. Additional genetic or environmental factors such as obesity or diabetes are needed besides the apo E2/E2 genotype for the development of hyperlipidemia, since only 10% of the subjects with the apo E2/E2 genotype develop hyperlipidemia [12]. For example estrogens may protect subjects with the apo E2/E2 genotype, since women do not develop type III hyperlipoproteinemia before the menopause, whereas males are already susceptible after adolescence [58]. Apo E2 has less affinity for the LDL-receptor, heparan sulfate proteoglycans and LDL receptor-related protein compared to the wild type apo E3, which leads to decreased hepatic removal of remnants [12,59]. In addition, the wild type apo E3 enhances the hydrolysis of TG compared to an inhibitory effect on the hydrolysis

by apo E2 [60,61]. FD is typically characterized by TG levels above 3.5 mmol/l and total cholesterol levels above 7.5 mmol/l in combination with normal ranges for apo B and LDL-C and is associated with an increased cardiovascular risk [12,18,62].

Secondary causes of hypertriglyceridemia

Genetic defects can cause hypertriglyceridemia, but dietary habits and other abnormalities can also contribute (Table 1). The most frequent cause of hypertriglyceridemia is obesity, a growing problem in Western countries [63]. Obesity has been associated with an increased mortality, largely by an increased risk of cardiovascular disease, which can be attributed by an increment of obesity related risk factors like hypertriglyceridemia [64]. Mildly obese subjects already have an impaired postprandial TG response, despite having normal fasting TG concentrations [65]. The mechanism of hypertriglyceridemia in the case of obesity has been linked to insulin resistance with an increased flux of FFA from the abdominal fat to the liver leading to increased hepatic VLDL production [51,66]. Triglyceridemia increases even more because competition for clearance between VLDL and chylomicrons occur, since both share the same catabolic processes [65,67]. However, not all obese subjects develop hypertriglyceridemia, which is the main lipid disorder in obesity. A recent study has shown that increased plasma apo C-III, which inhibits hydrolysis, significantly contributes to the development of the hypertriglyceridemic waist [68,69]. Obesity can lead to insulin resistance and subsequently to type 2 diabetes mellitus (T2DM). Lipid abnormalities are common in T2DM, which significantly add to the increased cardiovascular risk of patients with T2DM [70]. Very often TG are increased in patients with T2DM despite adequate treatment of the T2DM, probably because T2DM coexists with other disorders like obesity, smoking, alcohol consumption or the apo E2/E2 genotype [35].

Epidemiologic evidence is inconsistent on the effects of chronic alcohol consumption on TG concentrations. In the fasting state, both unchanged and increased TG have been described in relation to increased alcohol consumption [71-74]. However, in postprandial studies high alcohol intake has been related to elevated TG in contrast to low and moderate consumption [26,74-76]. It has been proposed that alcohol impairs oxidation of FFA by mitochondria, which accelerates TG synthesis leading to an increased hepatic secretion of VLDL [77,78]. Low to moderate alcohol consumption (1-3 consumptions per day) would probably not have major effects on TG, whereas excessive intake can cause hypertriglyceridemia. Besides alcohol, smoking has especially an effect on postprandial lipemia [26]. Smokers showed a postprandial increase in chylomicrons and their respective remnants characterized by increased postprandial apo B48 concentra-

tions. The increased number of chylomicrons and chylomicron remnants is probably due to a decreased clearance of chylomicron remnants [79].

Untreated hypothyroidism is frequently accompanied by hypercholesterolemia and possibly hypertriglyceridemia with increased concentrations of VLDL, LDL and also HDL [80]. Hypothyroidism leads to a reduced number of LDL receptors, which decreases the catabolism and turnover of mainly LDL and in part TRLs [81]. Hormone replacement therapy resulted in normalisation of total cholesterol and LDL-C, but TG concentrations remained unchanged [82,83]. However, it should be noted that the study groups were small and mean TG concentrations were normal at baseline.

Increased TG concentrations also occur in poorly controlled HIV infections together with decreased LDL-C and HDL-C concentrations. HIV-associated hypertriglyceridemia is caused by a combination of hepatic VLDL overproduction and reduced TG clearance [84]. In addition, antiretroviral therapy leads to lipodystrophy in 10 to 80 percent of the treated patients. The lipodystrophy impairs the storage of FFA in adipose tissue, which further increases the HIV-related hypertriglyceridemia [84].

TG concentrations are also frequently elevated in patients with end stage kidney disease, but cholesterol levels are mostly normal or even decreased. Secondly, the composition of the lipoproteins is also altered in end stage kidney disease. For example VLDL contains relatively more cholesterol and less TG in patients with end stage kidney disease. Moreover, clearance and hydrolysis of TRLs is altered by multiple mechanisms, which are reviewed in detail elsewhere [85,86].

Treatment of hypertriglyceridemia by diet and lifestyle

The non-pharmacological treatment with diet and increased physical activity is the most important strategy in reducing hypertriglyceridemia and is applicable on all patients with hypertriglyceridemia regardless the cause. In recent years, it has become clear that lifestyle factors including type of dietary fats, proteins, fibers, micronutrients, alcohol consumption, exercise, obesity and smoking have a strong impact on triglyceridemia [36]. Since all these factors can be altered via improved lifestyle behaviour, hypertriglyceridemia and subsequently reducing cardiovascular risk can be partly reduced by non-pharmacological strategies [87].

The average Western style meal contains 20-40 g of fat and a postprandial rise in TG is already evident after the ingestion of 30 g of fat. Postprandial triglyceridemia is likely to be present most of the day, because people typically eat 3 to 4 meals a day [26]. The amount of dietary fat remains the most important determinant of postprandial lipemia [36], but the type of dietary fat influences triglyceridemia as well. Postprandial lipemia is most pronounced after ingestion of saturated fatty acids (SFA), which are found in high

amounts in animal fat, followed by monounsaturated fatty acids (MUFA), the main fatty acids in olive oil. In contrast, dietary polyunsaturated fatty acids (PUFA), which are mainly found in vegetables (n-6 PUFA) and fish oils (n-3 PUFA), show the smallest postprandial increase in TG [36,88]. Obese subjects probably experience more beneficial effects of a diet rich in PUFA and MUFA and low on SFA compared to lean subjects. Chylomicron remnant concentrations were reduced after a meal with olive oil (22% SFA, 38% MUFA, 4% PUFA) compared to a meal with butter (35% SFA, 22% MUFA, 4% PUFA), but only in obese subjects and not in lean subjects [89].

Besides dietary fats, carbohydrates may influence TG concentrations as well. Reduced postprandial TG responses have been observed with lean red meat, soy protein, casein and whey protein [36,90,91]. In addition, indigestible carbohydrates like oat bran, wheat fiber, wheat germ and psyllium husk also have beneficial effects on TG concentrations [36,92,93]. In contrast, fructose, which is abundant in soft drinks, may enhance postprandial lipemia when consumed more than 50 g per day [94]. Moreover, micronutrients like vitamins and polyphenols found in fruit and beverages like green tea are believed to reduce postprandial lipemia [95,96]. It was recently shown for the first time that a high intake of fruit and vegetables was indeed significantly associated with a reduced risk of cardiovascular disease [87].

Obesity is a strong determinant for hypertriglyceridemia as well and obese patients should be urged to lose weight regardless of the hypertriglyceridemic cause. Moderate weight loss induced by a diet low on carbohydrates and SFA combined with a slight increase in physical activity resulted in a 27%-46% reduction in postprandial TG responses [97]. Moderate weight loss normalized postprandial lipemia, despite persisting obesity. Regular physical activity does reduce weight, but acute exercise bouts alone also reduced postprandial lipemia by 24% to 35% by an increase in LPL activity [36]. However, exercise needs to be done on a regular basis, because the improved clearance of TG-rich lipoproteins already attenuates after 2 to 3 days [98].

In addition to weight loss and exercise, smoking increases TG, postprandial chylomicrons and their remnants suggesting a beneficial effect of smoking cessation on TG. However, a meta-analysis could not confirm a TG lowering effect from cessation of smoking [99]. Nevertheless, smoking cessation should be promoted for improving cardiovascular health and many other reasons [36].

Mechanisms of triglyceride lowering pharmacotherapy

Isolated hypertriglyceridemia can be present, but hypertriglyceridemia is more frequently present in combination with other lipid abnormalities like increased LDL-C or decreased HDL-C depending on the underlying etiology. The four main pharmacological

agents used to treat hypertriglyceridemic disorders, either isolated or in combination with other lipid abnormalities, are fibrates, statins, nicotinic acid and omega-3 fatty acids. Each of them has different mechanisms of action to modify TG synthesis and clearance of TRLs (Figure 2).

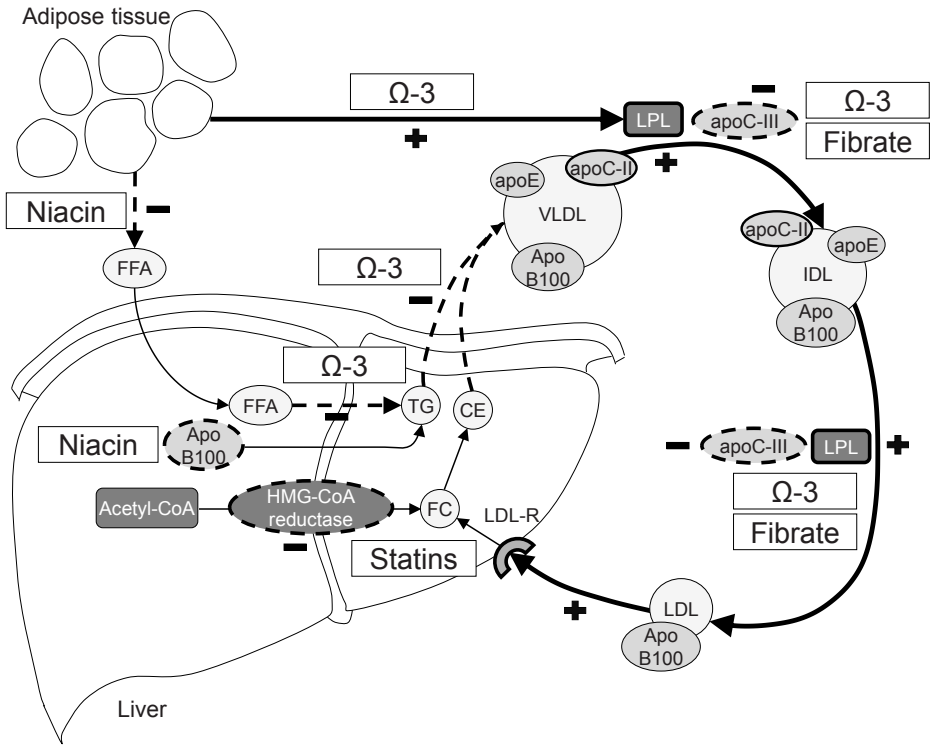

Figure 2: Schematic representation of triglyceride (TG) lowering mechanisms by different pharmacological agents: fibrates, nicotinic acid, statins and omega-3 fatty acids (Ω-3). Fibrates increase transcription of LPL and decrease transcription of apo C-III, which all improves TG clearance from plasma. Nicotinic acid reduces the lipolysis of adipocytes leading to reduced hepatic TG synthesis. Moreover, niacin has the potential to increase apoB100 degradation in hepatocytes. Statins have only a modest effect on TG and inhibit the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which is the rate limiting step of the hepatic cholesterol synthesis. Reduction in the endogenous cholesterol synthesis leads to upregulation of the LDL receptor (LDL-R) and subsequently increased hepatic uptake of LDL. Omega-3 fatty acids have multiple effects on TG metabolism. First of all, omega-3 fatty acids lower gene transcription of sterol regulatory element-binding protein-1c leading to decreased hepatic synthesis of fatty acids and thus TG synthesis. In addition, TG clearance from VLDL and chylomicrons is stimulated by omega-3 fatty acids, because it is a ligand for the farnesoid X receptor, which suppresses apo C-III gene expression and induces apo C-II gene expression. Hydrolysis of VLDL and chylomicrons is also increased by omega-3 fatty acids, potentially via increased lipoprotein lipase (LPL) gene expression in adipose tissue.

Fibrates (fibric acid derivatives) are synthetic ligands that bind to the peroxisome proliferator-activated receptor (PPAR)-α and are known for their TG-lowering and HDL-C increasing effect. Activation of PPAR-α modulates many genes affecting TG metabolism including *LPL*, *APOC3* and *APOA5* [100]. Fibrates increase transcription of LPL and decrease transcription of apo C-III, which favours TG clearance from plasma [101,102]. Most studies investigating the effect of fibrates on apo B metabolism reported increased VLDL apo B100 clearance and in some subjects reduced VLDL apo B100 secretion in patients with hypertriglyceridemia [100]. Treatment with fenofibrate also resulted in reduced apo B48 secretion in two subjects with a rare combination of heterozygous familial hypercholesterolemia combined with dysbetalipoproteinemia [103]. In addition, the catabolic rate of chylomicrons is increased by fenofibrate in hypertriglyceridemic subjects with T2DM [104].

Statins mainly reduce LDL-C, non-HDL-C and apoB concentrations, but statins modestly lower TG levels in hypertriglyceridemic subjects as well [105]. The TG lowering effect of the different statins is comparable with the percentage of cholesterol lowering effect and is probably a reflection of reduced cholesterol content of the TRLs since TG strongly correlate with cholesterol concentrations in the TRLs [25]. Statins inhibit the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which is the rate limiting step of the hepatic cholesterol synthesis. Reduction of the endogenous cholesterol synthesis leads to upregulation of the LDL receptor and subsequently increased hepatic uptake of LDL. When hepatic uptake of LDL is increased, LDL-C and apo B concentrations decrease.

Nicotinic acid has multiple effects on serum lipoprotein levels by raising HDL-C, lowering LDL-C and decreasing TG by 13-50% [106-108]. Nicotinic acid inhibits the lipolysis in adipocytes accompanied by a drop in FFA by binding of nicotinic acid to the nicotinic acid receptor [109]. This reduces the supply of FFA and subsequently hepatic TG synthesis and VLDL formation [110,111]. In addition, an in vitro study has shown the potential of nicotinic acid to degrade intracellular apo B100 [112]. The HDL-C raising property of nicotinic acid is probably a result of decreased TG concentrations. When TG levels are lowered, cholesterylesters are less transferred from VLDL to HDL in exchange for TG by CETP.

Omega-3 fatty acids are PUFA obtained from fatty fish or fish-oil supplements and consist of α-linoleic acid (ALA, 18:3*n*-3), eicosapentaenoic acid (EPA, 20:5*n*-3) and docosahexaenoic acid (DHA, 22:6*n*-3) [113]. Humans are unable to synthesize omega-3 fatty acids and only a small percentage of ALA is converted to EPA [113]. Therefore, supply of EPA and DHA depends on dietary intake. Fatty acids with multiple double bonds, like omega-3 fatty acids, are essential to increase cell membrane fluidity for improved receptor functions and signalling pathways [113]. Studies have shown that omega-3 fatty acids lower TG by reducing hepatic TG synthesis and VLDL secretion and enhancing TG

clearance from circulating chylomicrons and VLDL. However, animal studies investigating the exact mechanisms were not always consistent or physiologically relevant [114]. It has been shown that omega-3 fatty acids lower gene transcription of sterol regulatory element-binding protein-1c (SREBP-1c), which stimulates the hepatic synthesis of fatty acids and thus TG synthesis [115]. In addition, TG clearance from VLDL and chylomicrons is stimulated by DHA, because it is a ligand for the farnesoid X receptor (FXR), which suppresses apo C-III gene expression and induces apo C-II gene expression [113,116,117]. Hydrolysis of TRLs is even more stimulated by omega-3 fatty acids via increased LPL gene expression in adipose tissue [118,119].

Treatment targets

Treatment of hypertriglyceridemia is complex due to the multifactorial basis of this lipid abnormality. Lifestyle changes including weight loss, physical exercise and a healthy diet should be advised to all patients with hypertriglyceridemia. The importance of lifestyle changes is clear, but different opinions exist about the pharmacological treatment of hypertriglyceridemia. Some pharmacological agents are more appropriate than others in certain cases of hypertriglyceridemia but evidence is often lacking (Table 2). The recent ESC/EAS guidelines advise treatment targets of LDL-C < 1.8 mmol/l and < 2.5 mmol/l in subjects with very high and high cardiovascular risk, respectively [21]. Non-HDL-C is advised as a secondary treatment target in case of hypertriglyceridemia, which treatment target is 0.8 mmol/l higher than the corresponding LDL-C target [21]. Moreover, apo B can be used as a treatment target, because it reflects the number of

Table 2: Choosing the right pharmacological agent depends on the underlying hypertriglyceridemic cause. Lifestyle changes are always important besides pharmacological therapy.

Etiology of hypertriglyceridemia	Choice of pharmacological treatment in specific situations	Lifestyle changes applicable to all conditions
Familial combined hyperlipidemia	High dose statins, sometimes in combination with fibrates, niacin or fish oil	· Weight loss · Physical exercise · Diet low on fat and SFA and rich in dietary fibres, soy, casein, whey and lean red meat and PUFA. · Reducing alcohol · Cessation of smoking
Familial hypertriglyceridemia	Fibrates, nicotinic acid	
Familial dysbetalipoproteinemia	Statins + fibrate when lifestyle changes are insufficient	
Obesity	None	
Type 2 diabetes mellitus	High dose statins	
Hypothyroidism	Adequate treatment with thyroid hormone	

Note: do not combine gemfibrozil with a statin. Instead ciprofibrate, bezafibrate or fenofibrate can be combined with a statin

atherogenic particles. Treatment targets of apo B are 0.8 g/l and 1.0 g/l for subjects with very high and high cardiovascular risk, respectively [21]. No specific treatment targets are available for TG or for the addition of a secondary agent on top of statin therapy to treat the residual cardiovascular risk associated with hypertriglyceridemia. However, more evidence is becoming available on efficacy and safety concerning combination therapies [120].

Pharmacological treatment for different causes of hypertriglyceridemia

Statins are the first choice of treatment for FCH since it is characterised by increased hepatic VLDL production, small dense LDL and substantially increased cardiovascular risk [52,121]. Although no specific studies exist on the long-term effects of treatment of FCH, results can be extrapolated from subjects with combined hyperlipidemia. High dosages of statins are advised to further reduce apo B and non-HDL-C, because of the increased remnant cholesterol in subjects with FCH. Lowering non-HDL-C results in a linear reduction of cardiovascular risk [122] and non-HDL-C is the strongest predictor of the residual cardiovascular risk in subjects already on statin therapy [32]. When statin related myopathy occurs, a combination of fenofibrate and ezetimibe, an intestinal cholesterol absorption inhibitor, can be prescribed. This combination decreases cholesterol in both VLDL and LDL and reduces the amount of small dense LDL particles [123].

Treatment of FHTG should not be aimed at reducing cardiovascular risk, but to reduce the risk of hypertriglyceridemia associated pancreatitis [120]. Medical treatment with a fibrate should be initiated when TG are > 10.00 mmol/l to reduce the risk of pancreatitis [2], but the threshold for developing pancreatitis could be much higher [2,57]. A common side effect of fibrates is an increase in serum creatinine, which is often reversible [124]. Nicotinic acid can be used as an alternative when fibrates can not be prescribed, because nicotinic acid has a strong TG lowering capacity of 30-50% as well [125]. Patients receiving nicotinic acid should be warned for the frequent occurring side effect of severe flushing, which lasts for approximately 30 to 90 minutes after intake [109].

The most optimal pharmacological treatment of FD is controversial since FD seems to improve by all available interventions. It should be noted that most subjects with the apo E2/E2 genotype have normal lipid parameters. Pharmacological treatment is indicated when lifestyle changes and treatment of other secondary causes have not improved the combined hyperlipidemia [126]. Both statins and fibrates have been investigated in relation to FD, but studies on clinical outcomes are unavailable. Statins reduce mainly LDL-C, whereas fibrates lower mainly TG in subjects with FD [62,127]. In

addition, both bezafibrate 400 mg daily and atorvastatin 10 mg show both a reduction in non-HDL-C of approximately 45% [62]. Combination therapy with a statin and fibrate may be considered in therapy refractory subjects [126,128,129].

Post hoc subgroup analyses showed that fibrates reduce cardiovascular risk substantially in subjects with characteristics of the metabolic syndrome [130-132]. However, results could not always be replicated. Treatment of patients with T2DM with fenofibrate alone or on top of statin therapy did not result in a reduction of cardiovascular events [133,134]. It seems that gemfibrozil has more potential to reduce cardiovascular risk in subjects with the metabolic syndrome compared to bezafibrate, possibly by decreased concentrations of small dense LDL particles [135,136]. However, statins are the first choice of treatment for diabetic dyslipidemia and gemfibrozil should not be combined with a statin, because of an increased risk of rhabdomyolysis [102,120]. Targets for patients with T2DM are LDL-C concentrations < 1.8 mmol/l, but often diabetic patients have elevated TG as well [21]. In the case of hypertriglyceridemia, statin therapy should be intensified to further reduce non-HDL-C concentrations < 2.6 mmol/l [21].

Omega-3 fatty acids reduce TG levels but are insufficient as monotherapy. However, they can be safely combined with other drugs to further decrease TG concentrations [113]. Drugs like cholesterol absorption inhibitors (ezetimibe) or bile acid sequestrants, have no place in the specific treatment of hypertriglyceridemia and the latter are even contraindicated since these agents can increase TG concentrations.

Conclusion

Hypertriglyceridemia is common and can be caused by primary lipid disorders like familial combined hyperlipidemia, familial hypertriglyceridemia and familial dysbetalipoproteinemia or by secondary causes, mainly obesity and T2DM. It is often possible to diagnose the underlying hypertriglyceridemic cause with a family history, physical examination and lipid profile including apo B. Treatment should always include lifestyle changes and different pharmacological treatment options exist aiming at different pathways within the vast lipid metabolism. Pharmacological agents reduce the TG-rich lipoprotein secretion, increase lipolysis of TG-rich lipoproteins or reduce remnant cholesterol levels. Multiple agents can be used when monotherapy is insufficient to reach treatment targets. In addition, families should be screened for lipid abnormalities in case of a familial lipid disorder.

References

1. Ford ES, Li C, Zhao G, Pearson WS, Mokdad AH. Hypertriglyceridemia and its pharmacologic treatment among us adults. Arch Intern Med 2009;169:572-578
2. Brunzell JD. Clinical practice. Hypertriglyceridemia. N Engl J Med 2007;357:1009-1017
3. Pan X, Hussain MM. Gut triglyceride production. Biochim Biophys Acta 2011;1821:727-735
4. Mansbach CM, Siddiqi SA. The biogenesis of chylomicrons. Annu Rev Physiol 2010;72:315-333
5. Iqbal J, Hussain MM. Intestinal lipid absorption. Am J Physiol Endocrinol Metab 2009;296:E1183-1194
6. Leung GK, Veniant MM, Kim SK, Zlot CH, Raabe M, Bjorkegren J, et al. A deficiency of microsomal triglyceride transfer protein reduces apolipoprotein B secretion. J Biol Chem 2000;275:7515-7520
7. Klop B, Castro Cabezas M. Chylomicrons: A key biomarker and risk factor for cardiovascular disease and for the understanding of obesity. Curr Cardiovasc Risk Rep 2012;6:27-34
8. Schaefer EJ, Wetzel MG, Bengtsson G, Scow RO, Brewer HB, Jr., Olivecrona T. Transfer of human lymph chylomicron constituents to other lipoprotein density fractions during in vitro lipolysis. J Lipid Res 1982;23:1259-1273
9. Dallinga-Thie GM, Franssen R, Mooij HL, Visser ME, Hassing HC, Peelman F, et al. The metabolism of triglyceride-rich lipoproteins revisited: New players, new insight. Atherosclerosis 2010;211:1-8
10. Davies BS, Beigneux AP, Barnes RH, 2nd, Tu Y, Gin P, Weinstein MM, et al. GPIHBP1 is responsible for the entry of lipoprotein lipase into capillaries. Cell Metab 2010;12:42-52
11. Davies BS, Beigneux AP, Fong LG, Young SG. New wrinkles in lipoprotein lipase biology. Curr Opin Lipidol 2012;23:35-42
12. Mahley RW, Huang Y, Rall SC, Jr. Pathogenesis of type III hyperlipoproteinemia (dysbetalipoproteinemia). Questions, quandaries, and paradoxes. J Lipid Res 1999;40:1933-1949
13. Goldberg IJ, Eckel RH, McPherson R. Triglycerides and heart disease: Still a hypothesis? Arterioscler Thromb Vasc Biol 2011;31:1716-1725
14. Jang Y, Kim JY, Kim OY, Lee JE, Cho H, Ordovas JM, et al. The -1131T-->C polymorphism in the apolipoprotein A5 gene is associated with postprandial hypertriacylglycerolemia; elevated small, dense LDL concentrations; and oxidative stress in nonobese korean men. Am J Clin Nutr 2004;80:832-840
15. Bharadwaj KG, Hiyama Y, Hu Y, Huggins LA, Ramakrishnan R, Abumrad NA, et al. Chylomicron- and VLDL-derived lipids enter the heart through different pathways: In vivo evidence for receptor- and non-receptor-mediated fatty acid uptake. J Biol Chem 2010;285:37976-37986
16. Goldberg IJ, Eckel RH, Abumrad NA. Regulation of fatty acid uptake into tissues: Lipoprotein lipase- and CD36-mediated pathways. J Lipid Res 2009;50 Suppl:S86-90
17. Kolovou GD, Anagnostopoulou KK, Kostakou PM, Mikhailidis DP. Cholesterol ester transfer protein (CETP), postprandial lipemia and hypolipidemic drugs. Curr Med Chem 2009;16:4345-4360
18. Miller M, Stone NJ, Ballantyne C, Bittner V, Criqui MH, Ginsberg HN, et al. Triglycerides and cardiovascular disease: A scientific statement from the American Heart Association. Circulation 2011;123:2292-2333
19. de Beer F, van Dijk KW, Jong MC, van Vark LC, van der Zee A, Hofker MH, et al. Apolipoprotein E2 (lys146-->gln) causes hypertriglyceridemia due to an apolipoprotein E variant-specific inhibition of lipolysis of very low density lipoproteins-triglycerides. Arterioscler Thromb Vasc Biol 2000;20:1800-1806

20. Hara M, Iso ON, Satoh H, Noto H, Togo M, Ishibashi S, et al. Differential effects of apolipoprotein E isoforms on lipolysis of very low-density lipoprotein triglycerides. Metabolism 2006;55:1129-1134
21. Catapano AL, Reiner Z, De Backer G, Graham I, Taskinen MR, Wiklund O, et al. ESC/EAS guidelines for the management of dyslipidaemias the task force for the management of dyslipidaemias of the European Society of Cardiology (ESC) and the European Atherosclerosis Society (EAS). Atherosclerosis 2011;217 Suppl 1:1-44
22. Hokanson JE, Austin MA. Plasma triglyceride level is a risk factor for cardiovascular disease independent of high-density lipoprotein cholesterol level: A meta-analysis of population-based prospective studies. J Cardiovasc Risk 1996;3:213-219
23. Mora S, Rifai N, Buring JE, Ridker PM. Fasting compared with nonfasting lipids and apolipoproteins for predicting incident cardiovascular events. Circulation 2008;118:993-1001
24. Bansal S, Buring JE, Rifai N, Mora S, Sacks FM, Ridker PM. Fasting compared with nonfasting triglycerides and risk of cardiovascular events in women. JAMA 2007;298:309-316
25. Nordestgaard BG, Benn M, Schnohr P, Tybjaerg-Hansen A. Nonfasting triglycerides and risk of myocardial infarction, ischemic heart disease, and death in men and women. JAMA 2007;298:299-308
26. Sharrett AR, Heiss G, Chambless LE, Boerwinkle E, Coady SA, Folsom AR, et al. Metabolic and lifestyle determinants of postprandial lipemia differ from those of fasting triglycerides: The Atherosclerosis Risk In Communities (ARIC) Study. Arterioscler Thromb Vasc Biol 2001;21:275-281
27. van Wijk JP, van Oostrom AJ, Castro Cabezas M. Normal ranges of non-fasting triglycerides in healthy Dutch males and females. Clin Chim Acta 2003;337:49-57
28. Klop B, Cohn JS, van Oostrom AJ, van Wijk JP, Birnie E, Castro Cabezas M. Daytime triglyceride variability in men and women with different levels of triglyceridemia. Clin Chim Acta 2011;412:2183-2189
29. Langsted A, Nordestgaard BG. Nonfasting lipids, lipoproteins, and apolipoproteins in individuals with and without diabetes: 58 434 individuals from the Copenhagen General Population Study. Clin Chem 2011;57:482-489
30. Klop B, Proctor SD, Mamo JC, Botham KM, Castro Cabezas M. Understanding postprandial inflammation and its relationship to lifestyle behaviour and metabolic diseases. Int J Vasc Med 2012;2012:947417
31. Sniderman A, McQueen M, Contois J, Williams K, Furberg CD. Why is non-high-density lipoprotein cholesterol a better marker of the risk of vascular disease than low-density lipoprotein cholesterol? J Clin Lipidol 2010;4:152-155
32. Kastelein JJ, van der Steeg WA, Holme I, Gaffney M, Cater NB, Barter P, et al. Lipids, apolipoproteins, and their ratios in relation to cardiovascular events with statin treatment. Circulation 2008;117:3002-3009
33. Arsenault BJ, Rana JS, Stroes ES, Despres JP, Shah PK, Kastelein JJ, et al. Beyond low-density lipoprotein cholesterol: Respective contributions of non-high-density lipoprotein cholesterol levels, triglycerides, and the total cholesterol/high-density lipoprotein cholesterol ratio to coronary heart disease risk in apparently healthy men and women. J Am Coll Cardiol 2009;55:35-41
34. Langsted A, Freiberg JJ, Nordestgaard BG. Fasting and nonfasting lipid levels: Influence of normal food intake on lipids, lipoproteins, apolipoproteins, and cardiovascular risk prediction. Circulation 2008;118:2047-2056
35. Kolovou GD, Anagnostopoulou KK, Kostakou PM, Bilianou H, Mikhailidis DP. Primary and secondary hypertriglyceridaemia. Curr Drug Targets 2009;10:336-343

36. Lopez-Miranda J, Williams C, Lairon D. Dietary, physiological, genetic and pathological influences on postprandial lipid metabolism. Br J Nutr 2007;98:458-473
37. Mantel-Teeuwisse AK, Kloosterman JM, Maitland-van der Zee AH, Klungel OH, Porsius AJ, de Boer A. Drug-induced lipid changes: A review of the unintended effects of some commonly used drugs on serum lipid levels. Drug Saf 2001;24:443-456
38. Austin MA, McKnight B, Edwards KL, Bradley CM, McNeely MJ, Psaty BM, et al. Cardiovascular disease mortality in familial forms of hypertriglyceridemia: A 20-year prospective study. Circulation 2000;101:2777-2782
39. Goldstein JL, Schrott HG, Hazzard WR, Bierman EL, Motulsky AG. Hyperlipidemia in coronary heart disease. II. Genetic analysis of lipid levels in 176 families and delineation of a new inherited disorder, combined hyperlipidemia. J Clin Invest 1973;52:1544-1568
40. Hopkins PN, Heiss G, Ellison RC, Province MA, Pankow JS, Eckfeldt JH, et al. Coronary artery disease risk in familial combined hyperlipidemia and familial hypertriglyceridemia: A case-control comparison from the national heart, lung, and blood institute family heart study. Circulation 2003;108:519-523
41. Beigneux AP, Franssen R, Bensadoun A, Gin P, Melford K, Peter J, et al. Chylomicronemia with a mutant GPIHBP1 (q115p) that cannot bind lipoprotein lipase. Arterioscler Thromb Vasc Biol 2009;29:956-962
42. Brunzell JD, Albers JJ, Chait A, Grundy SM, Groszek E, McDonald GB. Plasma lipoproteins in familial combined hyperlipidemia and monogenic familial hypertriglyceridemia. J Lipid Res 1983;24:147-155
43. Castro Cabezas M, de Bruin TW, Erkelens DW. Familial combined hyperlipidaemia: 1973-1991. Neth J Med 1992;40:83-95
44. Castro Cabezas M, de Bruin TW, Jansen H, Kock LA, Kortlandt W, Erkelens DW. Impaired chylomicron remnant clearance in familial combined hyperlipidemia. Arterioscler Thromb 1993;13:804-814
45. Hegele RA, Ban MR, Hsueh N, Kennedy BA, Cao H, Zou GY, et al. A polygenic basis for four classical fredrickson hyperlipoproteinemia phenotypes that are characterized by hypertriglyceridemia. Hum Mol Genet 2009;18:4189-4194
46. Sabatti C, Service SK, Hartikainen AL, Pouta A, Ripatti S, Brodsky J, et al. Genome-wide association analysis of metabolic traits in a birth cohort from a founder population. Nat Genet 2009;41:35-46
47. Saxena R, Voight BF, Lyssenko V, Burtt NP, de Bakker PI, Chen H, et al. Genome-wide association analysis identifies loci for type 2 diabetes and triglyceride levels. Science 2007;316:1331-1336
48. Teslovich TM, Musunuru K, Smith AV, Edmondson AC, Stylianou IM, Koseki M, et al. Biological, clinical and population relevance of 95 loci for blood lipids. Nature 2010;466:707-713
49. Wallace C, Newhouse SJ, Braund P, Zhang F, Tobin M, Falchi M, et al. Genome-wide association study identifies genes for biomarkers of cardiovascular disease: Serum urate and dyslipidemia. Am J Hum Genet 2008;82:139-149
50. Willer CJ, Sanna S, Jackson AU, Scuteri A, Bonnycastle LL, Clarke R, et al. Newly identified loci that influence lipid concentrations and risk of coronary artery disease. Nat Genet 2008;40:161-169
51. Castro Cabezas M, de Bruin TW, de Valk HW, Shoulders CC, Jansen H, Willem Erkelens D. Impaired fatty acid metabolism in familial combined hyperlipidemia. A mechanism associating hepatic apolipoprotein B overproduction and insulin resistance. J Clin Invest 1993;92:160-168
52. Arai H, Ishibashi S, Bujo H, Hayashi T, Yokoyama S, Oikawa S, et al. Management of type IIb dyslipidemia. J Atheroscler Thromb 2012;19:105-114

53. Brunzell JD, Schrott HG, Motulsky AG, Bierman EL. Myocardial infarction in the familial forms of hypertriglyceridemia. Metabolism 1976;25:313-320
54. Lopez-Miranda J, Perez-Martinez P, Marin C, Moreno JA, Gomez P, Perez-Jimenez F. Postprandial lipoprotein metabolism, genes and risk of cardiovascular disease. Curr Opin Lipidol 2006;17:132-138
55. Deng LH, Xue P, Xia Q, Yang XN, Wan MH. Effect of admission hypertriglyceridemia on the episodes of severe acute pancreatitis. World J Gastroenterol 2008;14:4558-4561
56. Cappell MS. Acute pancreatitis: Etiology, clinical presentation, diagnosis, and therapy. Med Clin North Am 2008;92:889-923, ix-x
57. Lloret Linares C, Pelletier AL, Czernichow S, Vergnaud AC, Bonnefont-Rousselot D, Levy P, et al. Acute pancreatitis in a cohort of 129 patients referred for severe hypertriglyceridemia. Pancreas 2008;37:13-12
58. Huang Y, Schwendner SW, Rall SC, Jr., Sanan DA, Mahley RW. Apolipoprotein E2 transgenic rabbits. Modulation of the type III hyperlipoproteinemic phenotype by estrogen and occurrence of spontaneous atherosclerosis. J Biol Chem 1997;272:22685-22694
59. Mahley RW, Ji ZS. Remnant lipoprotein metabolism: Key pathways involving cell-surface heparan sulfate proteoglycans and apolipoprotein E. J Lipid Res 1999;40:1-16
60. Thuren T, Weisgraber KH, Sisson P, Waite M. Role of apolipoprotein E in hepatic lipase catalyzed hydrolysis of phospholipid in high-density lipoproteins. Biochemistry 1992;31:2332-2338
61. Thuren T, Wilcox RW, Sisson P, Waite M. Hepatic lipase hydrolysis of lipid monolayers. Regulation by apolipoproteins. J Biol Chem 1991;266:4853-4861
62. Kawashiri MA, Kobayashi J, Nohara A, Noguchi T, Tada H, Nakanishi C, et al. Impact of bezafibrate and atorvastatin on lipoprotein subclass in patients with type III hyperlipoproteinemia: Result from a crossover study. Clin Chim Acta 2011;412:1068-1075
63. Van Gaal LF, Mertens IL, De Block CE. Mechanisms linking obesity with cardiovascular disease. Nature 2006;444:875-880
64. Calle EE, Thun MJ, Petrelli JM, Rodriguez C, Heath CW, Jr. Body-mass index and mortality in a prospective cohort of U.S. Adults. N Engl J Med 1999;341:1097-1105
65. Halkes CJ, Van Wijk JP, Ribalta J, Masana L, Castro Cabezas M. Diurnal triglyceridaemia and insulin resistance in mildly obese subjects with normal fasting plasma lipids. J Intern Med 2004;255:74-81
66. Yki-Jarvinen H, Taskinen MR. Interrelationships among insulin's antilipolytic and glucoregulatory effects and plasma triglycerides in nondiabetic and diabetic patients with endogenous hypertriglyceridemia. Diabetes 1988;37:1271-1278
67. Karpe F, Bickerton AS, Hodson L, Fielding BA, Tan GD, Frayn KN. Removal of triacylglycerols from chylomicrons and VLDL by capillary beds: The basis of lipoprotein remnant formation. Biochem Soc Trans 2007;35:472-476
68. Taskinen MR, Adiels M, Westerbacka J, Soderlund S, Kahri J, Lundbom N, et al. Dual metabolic defects are required to produce hypertriglyceridemia in obese subjects. Arterioscler Thromb Vasc Biol 2011;31:2144-2150
69. Eckel RH. The complex metabolic mechanisms relating obesity to hypertriglyceridemia. Arterioscler Thromb Vasc Biol 2011;31:1946-1948
70. Goldberg RB, Capuzzi D. Lipid disorders in type 1 and type 2 diabetes. Clin Lab Med 2001;21:147-172
71. Marques-Vidal P, Cambou JP, Nicaud V, Luc G, Evans A, Arveiler D, et al. Cardiovascular risk factors and alcohol consumption in France and Northern Ireland. Atherosclerosis 1995;115:225-232

72. Brien SE, Ronksley PE, Turner BJ, Mukamal KJ, Ghali WA. Effect of alcohol consumption on biological markers associated with risk of coronary heart disease: Systematic review and meta-analysis of interventional studies. BMJ 2011;342:D636
73. Ruidavets JB, Ducimetiere P, Evans A, Montaye M, Haas B, Bingham A, et al. Patterns of alcohol consumption and ischaemic heart disease in culturally divergent countries: The prospective epidemiological study of myocardial infarction (PRIME). BMJ 2010;341:c6077
74. Gaziano JM, Buring JE, Breslow JL, Goldhaber SZ, Rosner B, VanDenburgh M, et al. Moderate alcohol intake, increased levels of high-density lipoprotein and its subfractions, and decreased risk of myocardial infarction. N Engl J Med 1993;329:1829-1834
75. Naissides M, Mamo JC, James AP, Pal S. The effect of acute red wine polyphenol consumption on postprandial lipaemia in postmenopausal women. Atherosclerosis 2004;177:401-408
76. van Tol A, van der Gaag MS, Scheek LM, van Gent T, Hendriks HF. Changes in postprandial lipoproteins of low and high density caused by moderate alcohol consumption with dinner. Atherosclerosis 1998;141 Suppl 1:S101-103
77. Baraona E, Lieber CS. Alcohol and lipids. Recent Dev Alcohol 1998;14:97-134
78. Fielding BA, Reid G, Grady M, Humphreys SM, Evans K, Frayn KN. Ethanol with a mixed meal increases postprandial triacylglycerol but decreases postprandial non-esterified fatty acid concentrations. Br J Nutr 2000;83:597-604
79. Mero N, Syvanne M, Eliasson B, Smith U, Taskinen MR. Postprandial elevation of apoB-48-containing triglyceride-rich particles and retinyl esters in normolipemic males who smoke. Arterioscler Thromb Vasc Biol 1997;17:2096-2102
80. Pearce EN. Hypothyroidism and dyslipidemia: Modern concepts and approaches. Curr Cardiol Rep 2004;6:451-456
81. Duntas LH. Thyroid disease and lipids. Thyroid 2002;12:287-293
82. Teixeira PF, Reuters VS, Ferreira MM, AImeida CP, Reis FA, Buescu A, et al. Lipid profile in different degrees of hypothyroidism and effects of levothyroxine replacement in mild thyroid failure. Transl Res 2008;151:224-231
83. Friis T, Pedersen LR. Serum lipids in hyper- and hypothyroidism before and after treatment. Clin Chim Acta 1987;162:155-163
84. van Wijk JP, Castro Cabezas M. Hypertriglyceridemia, metabolic syndrome, and cardiovascular disease in HIV-infected patients: Effects of antiretroviral therapy and adipose tissue distribution. Int J Vasc Med 2012;2012:201027
85. Vaziri ND. Causes of dysregulation of lipid metabolism in chronic renal failure. Semin Dial 2009;22:644-651
86. Vaziri ND. Dyslipidemia of chronic renal failure: The nature, mechanisms, and potential consequences. Am J Physiol Renal Physiol 2006;290:F262-272
87. Crowe FL, Roddam AW, Key TJ, Appleby PN, Overvad K, Jakobsen MU, et al. Fruit and vegetable intake and mortality from ischaemic heart disease: Results from the European Prospective Investigation into Cancer and Nutrition (EPIC)-Heart Study. Eur Heart J 2011;32:1235-1243
88. Zampelas A, Peel AS, Gould BJ, Wright J, Williams CM. Polyunsaturated fatty acids of the n-6 and n-3 series: Effects on postprandial lipid and apolipoprotein levels in healthy men. Eur J Clin Nutr 1994;48:842-848
89. Lozano A, Perez-Martinez P, Delgado-Lista J, Marin C, Cortes B, Rodriguez-Cantalejo F, et al. Body mass interacts with fat quality to determine the postprandial lipoprotein response in healthy young adults. Nutr Metab Cardiovasc Dis 2012;22:355-361

90. Lairon D. Macronutrient intake and modulation on chylomicron production and clearance. Atheroscler Suppl 2008;9:45-48
91. Mortensen LS, Hartvigsen ML, Brader LJ, Astrup A, Schrezenmeir J, Holst JJ, et al. Differential effects of protein quality on postprandial lipemia in response to a fat-rich meal in type 2 diabetes: Comparison of whey, casein, gluten, and cod protein. Am J Clin Nutr 2009;90:41-48
92. Khossousi A, Binns CW, Dhaliwal SS, Pal S. The acute effects of psyllium on postprandial lipaemia and thermogenesis in overweight and obese men. Br J Nutr 2008;99:1068-1075
93. Lairon D, Play B, Jourdheuil-Rahmani D. Digestible and indigestible carbohydrates: Interactions with postprandial lipid metabolism. J Nutr Biochem 2007;18:217-227
94. Harbis A, Perdreau S, Vincent-Baudry S, Charbonnier M, Bernard MC, Raccah D, et al. Glycemic and insulinemic meal responses modulate postprandial hepatic and intestinal lipoprotein accumulation in obese, insulin-resistant subjects. Am J Clin Nutr 2004;80:896-902
95. Basu A, Lucas EA. Mechanisms and effects of green tea on cardiovascular health. Nutr Rev 2007;65:361-375
96. Burton-Freeman B, Linares A, Hyson D, Kappagoda T. Strawberry modulates ldl oxidation and postprandial lipemia in response to high-fat meal in overweight hyperlipidemic men and women. J Am Coll Nutr 2010;29:46-54
97. Maraki MI, Aggelopoulou N, Christodoulou N, Anastasiou CA, Toutouza M, Panagiotakos DB, et al. Lifestyle intervention leading to moderate weight loss normalizes postprandial triacylglycerolemia despite persisting obesity. Obesity (Silver Spring) 2011;19:968-976
98. Mestek ML. Physical activity, blood lipids, and lipoproteins. Am J Lifestyle Med 2009;3:279-283
99. Maeda K, Noguchi Y, Fukui T. The effects of cessation from cigarette smoking on the lipid and lipoprotein profiles: A meta-analysis. Prev Med 2003;37:283-290
100. Shah A, Rader DJ, Millar JS. The effect of PPAR-alpha agonism on apolipoprotein metabolism in humans. Atherosclerosis 2010;210:35-40
101. Staels B, Vu-Dac N, Kosykh VA, Saladin R, Fruchart JC, Dallongeville J, et al. Fibrates downregulate apolipoprotein C-III expression independent of induction of Peroxisomal Acyl Coenzyme A Oxidase. A potential mechanism for the hypolipidemic action of fibrates. J Clin Invest 1995;95:705-712
102. Barter PJ, Rye KA. Is there a role for fibrates in the management of dyslipidemia in the metabolic syndrome? Arterioscler Thromb Vasc Biol 2008;28:39-46
103. Tremblay AJ, Lamarche B, Ruel IL, Hogue JC, Deshaies Y, Gagne C, et al. Effects of fenofibrate on apolipoprotein kinetics in patients with coexisting dysbetalipoproteinemia and heterozygous familial hypercholesterolemia. Atherosclerosis 2006;188:203-212
104. Hogue JC, Lamarche B, Tremblay AJ, Bergeron J, Gagne C, Couture P. Differential effect of atorvastatin and fenofibrate on plasma oxidized low-density lipoprotein, inflammation markers, and cell adhesion molecules in patients with type 2 diabetes mellitus. Metabolism 2008;57:380-386
105. Stein EA, Lane M, Laskarzewski P. Comparison of statins in hypertriglyceridemia. Am J Cardiol 1998;81:66B-69B
106. Group TCDPR. Clofibrate and niacin in coronary heart disease. JAMA 1975;231:360-381
107. Whitney EJ, Krasuski RA, Personius BE, Michalek JE, Maranian AM, Kolasa MW, et al. A randomized trial of a strategy for increasing high-density lipoprotein cholesterol levels: Effects on progression of coronary heart disease and clinical events. Ann Intern Med 2005;142:95-104
108. Cashin-Hemphill L, Mack WJ, Pogoda JM, Sanmarco ME, Azen SP, Blankenhorn DH. Beneficial effects of colestipol-niacin on coronary atherosclerosis. A 4-year follow-up. JAMA 1990;264:3013-3017

109. Gille A, Bodor ET, Ahmed K, Offermanns S. Nicotinic acid: Pharmacological effects and mechanisms of action. Annu Rev Pharmacol Toxicol 2008;48:79-106
110. Vosper H. Niacin: A re-emerging pharmaceutical for the treatment of dyslipidaemia. Br J Pharmacol 2009;158:429-441
111. Mahboubi K, Witman-Jones T, Adamus JE, Letsinger JT, Whitehouse D, Moorman AR, et al. Triglyceride modulation by acifran analogs: Activity towards the niacin high and low affinity G protein-coupled receptors HM74a and HM74. Biochem Biophys Res Commun 2006;340:482-490
112. Jin FY, Kamanna VS, Kashyap ML. Niacin accelerates intracellular apoB degradation by inhibiting triacylglycerol synthesis in human hepatoblastoma (HEPG2) cells. Arterioscler Thromb Vasc Biol 1999;19:1051-1059
113. Harris WS, Miller M, Tighe AP, Davidson MH, Schaefer EJ. Omega-3 fatty acids and coronary heart disease risk: Clinical and mechanistic perspectives. Atherosclerosis 2008;197:12-24
114. Harris WS, Bulchandani D. Why do omega-3 fatty acids lower serum triglycerides? Curr Opin Lipidol 2006;17:387-393
115. Yoshikawa T, Shimano H, Yahagi N, Ide T, Amemiya-Kudo M, Matsuzaka T, et al. Polyunsaturated fatty acids suppress sterol regulatory element-binding protein 1c promoter activity by inhibition of liver X receptor (LXR) binding to LXR response elements. J Biol Chem 2002;277:1705-1711
116. Kast HR, Nguyen CM, Sinal CJ, Jones SA, Laffitte BA, Reue K, et al. Farnesoid X-activated receptor induces apolipoprotein C-II transcription: A molecular mechanism linking plasma triglyceride levels to bile acids. Mol Endocrinol 2001;15:1720-1728
117. Claudel T, Inoue Y, Barbier O, Duran-Sandoval D, Kosykh V, Fruchart J, et al. Farnesoid X receptor agonists suppress hepatic apolipoprotein CIII expression. Gastroenterology 2003;125:544-555
118. Park Y, Harris WS. Omega-3 fatty acid supplementation accelerates chylomicron triglyceride clearance. J Lipid Res 2003;44:455-463
119. Khan S, Minihane AM, Talmud PJ, Wright JW, Murphy MC, Williams CM, et al. Dietary long-chain n-3 PUFAs increase LPL gene expression in adipose tissue of subjects with an atherogenic lipoprotein phenotype. J Lipid Res 2002;43:979-985
120. Dujovne CA, Williams CD, Ito MK. What combination therapy with a statin, if any, would you recommend? Curr Atheroscler Rep 2011;13:12-22
121. Rubenfire M, Brook RD, Rosenson RS. Treating mixed hyperlipidemia and the atherogenic lipid phenotype for prevention of cardiovascular events. Am J Med 2010;123:892-898
122. Robinson JG, Wang S, Smith BJ, Jacobson TA. Meta-analysis of the relationship between non-high-density lipoprotein cholesterol reduction and coronary heart disease risk. J Am Coll Cardiol 2009;53:316-322
123. Tribble DL, Farnier M, Macdonell G, Perevozskaya I, Davies MJ, Gumbiner B, et al. Effects of fenofibrate and ezetimibe, both as monotherapy and in coadministration, on cholesterol mass within lipoprotein subfractions and low-density lipoprotein peak particle size in patients with mixed hyperlipidemia. Metabolism 2008;57:796-801
124. Jun M, Foote C, Lv J, Neal B, Patel A, Nicholls SJ, et al. Effects of fibrates on cardiovascular outcomes: A systematic review and meta-analysis. Lancet 2010;375:1875-1884
125. Bodor ET, Offermanns S. Nicotinic acid: An old drug with a promising future. Br J Pharmacol 2008;153 Suppl 1:S68-75
126. Guyton JR. Treatment of type III hyperlipoproteinemia. Am Heart J 1999;138:17-18
127. Civeira F, Cenarro A, Ferrando J, Puzo J, Garcia-Otin AL, Mozas P, et al. Comparison of the hypolipidemic effect of gemfibrozil versus simvastatin in patients with type III hyperlipoproteinemia. Am Heart J 1999;138:156-162

128. Illingworth DR, O'Malley JP. The hypolipidemic effects of lovastatin and clofibrate alone and in combination in patients with type III hyperlipoproteinemia. Metabolism 1990;39:403-409
129. Cho EJ, Min YJ, Oh MS, Kwon JE, Kim JE, Kim CJ. Disappearance of angina pectoris by lipid-lowering in type III hyperlipoproteinemia. Am J Cardiol 2011;107:793-796
130. Tenkanen L, Manttari M, Manninen V. Some coronary risk factors related to the insulin resistance syndrome and treatment with gemfibrozil. Experience from the Helsinki Heart Study. Circulation 1995;92:1779-1785
131. Rubins HB, Robins SJ, Collins D, Nelson DB, Elam MB, Schaefer EJ, et al. Diabetes, plasma insulin, and cardiovascular disease: Subgroup analysis from the department of Veterans Affairs High-Density Lipoprotein Intervention Trial (VA-HIT). Arch Intern Med 2002;162:2597-2604
132. Tenenbaum A, Motro M, Fisman EZ, Tanne D, Boyko V, Behar S. Bezafibrate for the secondary prevention of myocardial infarction in patients with metabolic syndrome. Arch Intern Med 2005;165:1154-1160
133. Keech A, Simes RJ, Barter P, Best J, Scott R, Taskinen MR, et al. Effects of long-term fenofibrate therapy on cardiovascular events in 9795 people with type 2 diabetes mellitus (the Field Study): Randomised controlled trial. Lancet 2005;366:1849-1861
134. Ginsberg HN, Elam MB, Lovato LC, Crouse JR, 3rd, Leiter LA, Linz P, et al. Effects of combination lipid therapy in type 2 diabetes mellitus. N Engl J Med 2010;362:1563-1574
135. Otvos JD, Collins D, Freedman DS, Shalaurova I, Schaefer EJ, McNamara JR, et al. Low-density lipoprotein and high-density lipoprotein particle subclasses predict coronary events and are favorably changed by gemfibrozil therapy in the veterans affairs high-density lipoprotein intervention trial. Circulation 2006;113:1556-1563
136. Barter PJ, Rye KA. Cardioprotective properties of fibrates: Which fibrate, which patients, what mechanism? Circulation 2006;113:1553-1555

Chapter 3

Daytime triglyceride variability in men and women with different levels of triglyceridemia

Boudewijn Klop
Jeffrey S. Cohn
Antonie J.H.H.M. van Oostrom
Jeroen P.H. van Wijk
Erwin Birnie
Manuel Castro Cabezas

Clin Chim Acta 2011;412:2183-2189

Abstract

Background: Triglyceride (TG) levels measured in either the fasting or non-fasting state predict the risk of cardiovascular disease (CVD). Since CVD risk assessment is affected by variability in TG, the aim of the study was to investigate intra-individual variability of non-fasting TG.

Methods: Capillary triglyceride (cTG) levels were measured in 246 free-living individuals at six time-points during the day on three separate occasions. Intra-individual variability in cTG was assessed by calculating the standard deviation of three measures at each time-point. Subjects were analyzed by gender and by fasting TG level.

Results: In the fasting state, intra-individual variability was similar in males and females (0.28 and 0.35 mmol/l, respectively), but increased significantly in male but not in female subjects during the day, i.e., 0.28 to 0.69, and 0.35 to 0.36 mmol/l, resp. Subjects with higher fasting TG levels had greater absolute variability in both fasting and non-fasting TG.

Conclusions: The variability in non-fasting TG is greater in males and in individuals with higher levels of TG. Since greatest variability in non-fasting TG occurs very late in the day, it is unlikely to affect the assessment of CVD risk, which is based on a blood sample taken during daylight hours.

Introduction

Blood triglyceride (TG) concentration increases during the day and decreases overnight in human subjects [1-4]. Men have higher fasting and postprandial levels of TG than women [5]. Their TG levels increase by about 75% during the day, while women have a more modest increase, i.e., around 25% [6]. Due to the diurnal changes in blood TG levels, TG measurements are usually carried out in the fasting state for cardiovascular risk assessment [5]. However, most patients visit the hospital in the non-fasting state and are therefore compelled to have their blood taken on a different occasion. Furthermore, prospective studies have shown that non-fasting TG levels are associated with increased risk of cardiovascular disease (CVD) [6,7] and it has been suggested that non-fasting TG levels are a stronger predictor of cardiovascular disease than fasting TG levels [8,9].

Even without the influence of sequential meals, fasting TG levels have a high intra-individual day-to-day variability of 15% to 30% [10-15]. Only a limited number of studies have investigated the variability of non-fasting TG levels and this has been restricted to a relatively small number of individuals [11,15,16]. The purpose of the present study was to determine differences in intra-individual variability in fasting compared to non-fasting TG levels. Our aim was: 1) to quantitate the extent to which TG increase in different subjects during the day, 2) to study the extent to which intra-individual variability in TG vary between males and females, and 3) to determine if intra-individual variability in TG differ between normotriglyceridemic and hypertriglyceridemic subjects. These questions were addressed by analyzing data obtained from previous studies always using the same protocol, in which a hand-held point-of-care testing device was used to determine capillary TG concentrations (cTG) during the day, in free-living male and female individuals [16-21].

Material and methods

Subjects and capillary measurements

Subjects were healthy volunteers or patients with hyperlipidemia or a known medical history of CVD or type 2 diabetes mellitus (T2DM) that had participated in clinical studies aimed to investigate factors influencing postprandial lipemia [16-21]. Males and females were aged 18-80 years. Exclusion criteria were renal, liver or thyroid disease and/or the use of lipid lowering drugs. Participants using lipid lowering drugs were investigated after withdrawal of these drugs during 4 weeks. Briefly, subjects visited the department after a 12-hour overnight fast. Height, weight, blood pressure and waist circumference were measured. Self-measurement of cTG was performed with a triglyceride-specific point-of-care testing device (Accutrend GCT®, Roche Diagnostics, Germany) [22,23]. Sub-

jects were instructed to measure their cTG on three different days (preferably Monday, Wednesday and Friday - not weekend days) at the following six time-points: fasting, before and three hours after lunch, before and three hours after dinner, and at bedtime. The three-hour postprandial measurements were performed exactly three hours after the meals, regardless of intake of snacks. Subjects were requested to refrain from heavy physical activity, although normal daily activities such as riding a bike to work were allowed. Subjects did not receive recommendations concerning the frequency and composition of meals and were requested to follow their normal diet during the study. Alcohol consumption was recorded in a diary. The diaries were evaluated by a trained physician together with each subject.

The measurement range for cTG was 0.80 to 6.86 mmol/l. When cTG levels were below 0.80 mmol/l or above 6.86 mmol/l the Accutrend registered a cTG level of 0.80 and 6.86 mmol/l respectively. Besides this limitation, the TG-analyser detects TG reliably, regardless of the type of lipoprotein carrying the TG in the blood sample (e.g., chylomicron or VLDL) [22]. In a previous study, variation coefficients for different cTG concentrations were 3.3% for high TG (6.12 mmol/l) and 5.3% for low TG (1.81 mmol/l) [23]. The correlation coefficient between cTG measurements with the TG-analyser and plasma measurements according to enzymatic methods is 0.94 [23]. Similar results have been obtained in our laboratory [16,17]. Capillary TG concentrations are generally 0.2-0.3 mmol/l higher than TG concentrations in venous plasma [16-20,22,23], which may reflect differences between capillary and venous samples or differences in methodology.

Analytical determinations

Fasting blood was collected at inclusion after a 12 h fast for measurement of lipids, apolipoproteins and insulin from plasma. Cholesterol and TG in plasma and HDL cholesterol (obtained after precipitation with dextransulphate/$MgCl_2$), were determined using a Vitros 250 analyzer (Johnson & Johnson Rochester, NY, USA). Plasma apolipoprotein (apo) B was measured by nephelometry using apo B monoclonal antibodies (Behring Diagnostics NV, OSAN 14/15). Plasma apo AI was measured by nephelometry using apo AI monoclonal antibodies (Behring Diagnostics NV, OUED 14/15). Insulin was measured by competitive radio immunoassay with polyclonal antibodies. LDL cholesterol was calculated using the Friedewald calculations (if fasting TG were below 4.6 mmol/l). For estimation of the insulin sensitivity, the HOMA-IR index (= glucose (mmol/l) * insulin (mU/l) / 22.5) was calculated.

Statistics

Data are given as mean ± standard deviations. Intra-individual variability in cTG are given as medians with interquartile ranges (IQR), because of their skewed distribution. Capillary TG measurements of 0.80 mmol/l were excluded from the analysis of intra-

individual cTG variability, since these measures reflected the lower limit of the point-of-care testing device. Absolute intra-individual variation in cTG (SD) was assessed by calculating the standard deviation for the three cTG measurements obtained at each specific time-point. The relative intra-individual variation in cTG was assessed for each individual at every time-point by calculating the coefficient of variation (CV). The formula to calculate the CV for cTG was the standard deviation divided by mean cTG at a specific time-point multiplied by 100 [16]. Subjects were also divided into tertiles based on their three-day average fasting cTG levels. Ranges were calculated for males and females separately, because TG levels are lower in females [18,21]. The TG ranges for tertiles were respectively 1.03 – 1.62 mmol/l for males and 0.98 – 1.49 mmol/l for females. Differences between two groups were tested by Student's t-test or Chi-square test where appropriate. Comparisons between multiple groups were performed using one-way ANOVA with Bonferroni correction for numerical data and Chi-square test or Fisher's exact test for categorical data. For ANOVA analysis cTG, SD and CV were logarithmically transformed to obtain a normal distribution. The Pearson's correlation was used to test for associations between the SD and different variables. Statistical analyses were performed using PASW statistics version 18.0. Statistical significance was defined as $P < 0.05$.

Results

Baseline characteristics of male (n = 125) and female subjects (n = 121) are shown in Table 1. The participants had an average age of 40 years and a mean BMI of 25. About one-fifth of the subjects had a history of CVD and only six subjects had been diagnosed with T2DM. Nearly one-third of the subjects were smokers. Average lipid and apolipoprotein levels are shown in Table 1, and significant differences between males and females are indicated.

The capillary TG concentration in the fasting state was not significantly different between males and females (Table 2). However, after lunch and for the rest of the day, cTG was significantly higher in males. In both male and female subjects, cTG rose significantly during the day, with a maximum increase of 1.05 ± 0.87 mmol/l in males, which occurred at bedtime, and 0.53 ± 0.69 mmol/l in females, which occurred after dinner. cTG levels were significantly higher in males compared to females after lunch until bedtime ($P < 0.001$).

The absolute and relative intra-individual variability in cTG for males and females in the fasting state and at time-points during the day are shown in Table 3. Median values are given since the data were skewed. In the fasting state, the intra-individual variability was similar in males and females (0.28 and 0.35 mmol/l, respectively), representing on average a relative variability of 21%. This variability increased as the day progressed in

Table 1: Baseline characteristics of female and male subjects.

	Females (n = 121)	Males (n = 125)	P-value
Age (years)	41.3 ± 13.3	39.4 ± 13.4	NS
BMI (weight/height^2)	25.5 ± 5.2	24.5 ± 3.5	NS
History of CVD (n, %)	17 (18.1%)	25 (23.6%)	NS
History of T2DM (n, %)	3 (2.7%)	3 (2.5%)	NS
HOMA-IR	2.97 ± 3.07	2.13 ± 1.79	P = 0.001
Smokers (n, %)	36 (31.3%)	32 (26.4%)	NS
Alcohol intake (g/day)	7.6 ± 11.5	17.8 ± 20.9	P < 0.001
Use of aspirin (n, %)	5 (6.7%)	11 (10.8%)	NS
Use of ß-blockers (n, %)	9 (7.9%)	13 (10.8%)	NS
Use of ACE-inhibitors (n, %)	3 (2.6%)	7 (5.8%)	NS
Use of diuretics (n, %)	3 (2.6%)	3 (2.5%)	NS
Use of calcium-antagonists (n, %)	5 (4.1%)	5 (4.2%)	NS
Use of oral contraceptives (n, %)	30 (51.7%)	0 (0.0%)	P < 0.001
Fasting plasma TG (mmol/l)	1.23 ± 0.74	1.33 ± 0.78	NS
Total cholesterol (mmol/l)	5.0 ± 1.2	5.2 ± 1.0	NS
LDL-C (mmol/l)	3.4 ± 1.1	3.8 ± 1.0	P = 0.009
HDL-C (mmol/l)	1.42 ± 0.40	1.21 ± 0.30	P < 0.001
Apo B (g/l)	0.85 ± 0.26	0.99 ± 0.25	P < 0.001
Apo A-I (g/l)	1.50 ± 0.32	1.32 ± 0.21	P < 0.001

Data are given as mean ± standard deviation unless stated otherwise.

Table 2: Mean capillary triglyceride concentration (cTG) and mean increase in cTG at time-points throughout the day in female and male subjects

	Female Mean cTG (mmol/l)	Female Mean increase in cTG from fasting (mmol/l)	Male Mean cTG (mmol/l)	Male Mean increase in cTG from fasting (mmol/l)
Fasting	1.48 ± 0.80	-	1.54 ± 0.77	-
Before lunch	1.61 ± 0.85	0.13 ± 0.44	1.68 ± 0.82	0.14 ± 0.63
After lunch	1.82 ± 1.05	0.34 ± 0.63	2.08 ± 0.95 * [a]	0.54 ± 0.67 *
Before dinner	1.75 ± 1.01	0.28 ± 0.50	1.96 ± 0.93 * [a]	0.43 ± 0.62 *
After dinner	2.00 ± 1.14 [a]	0.53 ± 0.69	2.53 ± 1.16 ** [a]	0.99 ± 0.87 **
Bedtime	1.92 ± 1.08 [a]	0.45 ± 0.75	2.59 ± 1.16 ** [a]	1.05 ± 0.87 **

Data represent means ± SD for female (n = 121) and male (n = 125) subjects.

* Significantly different from corresponding value in females (P < 0.05).

** Significantly different from corresponding value in females (P < 0.001).

[a] Significantly different from fasting (P < 0.01).

Table 3: Intra-individual variability in capillary triglyceride levels at time-points during the day in female and male subjects.

	Female Intra-individual Variability (mmol/l)	Female Intra-individual Variability (%)	Male Intra-individual Variability (mmol/l)	Male Intra-individual Variability (%)
Fasting	0.35 (0.19-0.54)	20.5 (12.9-27.8)	0.28 (0.13-0.67)	21.0 (9.7-35.3)
Before lunch	0.28 (0.13-0.53)	17.6 (8.6-25.6)	0.35 (0.18-0.61)	19.7 (10.8-33.6)
After lunch	0.40 (0.18-0.67)	22.3 (12.8-32.4)	0.41 (0.19-0.75)	21.4 (9.9-33.2)
Before dinner	0.33 (0.16-0.56)	18.1 (12.4-32.4)	0.41 (0.23-0.69) *	23.2 (13.3-34.9)
After dinner	0.44 (0.20-0.71)	21.3 (12.4-27.7)	0.64 (0.32-0.98) ** [a]	25.5 (14.4-36.8)
Bedtime	0.36 (0.18-0.79)	21.6 (11.0-37.4)	0.69 (0.39-1.00) ** [b]	27.3 (17.3-41.7) *

Intra-individual variability is given in absolute terms (mmol/l) as a standard deviation for cTG (SD) measured on three separate occasions, and in relative terms (%) as a coefficient of variation for cTG measured on three occasions. Data are given as medians with interquartile ranges (IQR) for females (n = 121) and males (n = 125).

* Significantly different from corresponding value in female subjects ($P < 0.05$).

** Significantly different from corresponding value in female subjects ($P < 0.01$).

[a] Significantly different from fasting ($P < 0.01$).

[b] Significantly different from fasting ($P < 0.001$).

males but not in females. The absolute variability was significantly greater after dinner and at bedtime (0.64 and 0.69 mmol/l) compared to the beginning of the day (0.28 mmol/l) in males and it was also significantly greater at the end-of-day time-points compared to female subjects.

Subjects were divided into tertiles based on their three-day average fasting cTG concentration. Baseline characteristics from tertile data by gender are shown in Table 4. Both females and males with the highest levels of triglyceridemia were older and showed increased BMI, HOMA-IR, total cholesterol and apo B. Moreover, males with the highest level of triglyceridemia were more frequently smokers, had a higher prevalence of CVD, and presented with increased fasting plasma TG and decreased HDL-C concentrations.

At all time-points, cTG was significantly higher in females and males in the second tertile compared to subjects in the first tertile, and as expected subjects in the third tertile showed the highest cTG concentrations. Surprisingly, no differences were found in mean increase in diurnal cTG between the tertiles in both females and males (Tables 5 and 6). Fasting and non-fasting absolute intra-individual variability was increased in females and males with the highest level of triglyceridemia compared to subjects from the lowest tertile (Tables 7 and 8). However, statistical significance was not reached for males at time-points before dinner and at bedtime. In contrast, the relative intra-individual variability was mostly unchanged between the tertiles, except for the first male tertile in the fasting state, which was significantly lower compared to the second and third

Table 4: Baseline characteristics for females and males with different levels of triglyceridemia.

	Females			Males		
	First tertile (n = 40)	**Second tertile (n = 41)**	**Third tertile (n = 40)**	**First tertile (n = 42)**	**Second tertile (n = 41)**	**Third tertile (n = 42)**
Age (years)	37.1 ± 12.5	41.1 ±10.9	45.9 ± 15.6 *	34.7 ± 13.1	39.0 ± 13.6	44.7 ± 11.8 *
BMI (kg/m^2)	23.1 ± 3.1	24.6 ± 3.4	28.8 ± 6.6 ***	22.6 ± 2.3	24.0 ± 3.1	26.8 ± 3.5 ***
History of CVD (n, %)	2 (7.1%)	7 (20.6%)	8 (25%)	2 (6.1%)	7 (17.1%)	16 (47.1%) ***
History of T2DM (n, %)	0 (0%)	0 (0%)	3 (8.6%)	0 (0%)	0 (0%)	3 (7.5%)
HOMA-IR	1.89 ± 0.75	2.47 ±1.18	4.56 ± 4.82 ***	1.31 ± 0.30	1.97 ± 1.23 *	2.95 ± 2.61 *
Smokers (n, %)	9 (23.1)	12 (30.8%)	15 (40.5%)	7 (17.5%)	9 (22.0%)	16 (39%) *
Alcohol intake (g/day)	8.8 ± 9.8	6.7 ± 7.4	7.5 ± 16.1	13.8 ± 17.8	20.7 ± 22.5	18.8 ± 22.0
Use of aspirin (n,%)	0 (0.0%)	2 (9.1%)	3 (13.0%)	1 (2.4%)	4 (11.1%)	6 (24.0%) *
Use of ß-blockers (n, %)	1 (2.6%)	5 (12.5%)	3 (8.3%)	1 (2.4%)	4 (10.0%)	8 (20.5%) *
Use of ACE-inhibitors (n, %)	1 (2.6%)	1 (2.5%)	1 (2.8%)	0 (0.0%)	1 (2.5%)	6 (15.4%) *
Use of diuretics (n, %)	1 (2.6%)	1 (2.4%)	2 (5.4%)	0 (0.0%)	1 (2.5%)	2 (5.1%)
Use of calcium-antagonists (n, %)	2 (5.0%)	3 (7.5%)	2 (5.4%)	0 (0.0%)	3 (7.5%)	2 (4.8%)
Use of oral contraceptives (n, %)	8 (40.0%)	13 (56.5%)	6 (60%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Fasting plasma TG (mmol/l)	0.75 ± 0.31	1.06 ± 0.53	1.92 ± 0.76	1.01 ± 0.41	1.06 ± 0.35	1.91 ± 1.02 ***
Total cholesterol (mmol/l)	4.5 ± 0.9	5.0 ± 1.1	5.7 ± 1.2 ***	4.8 ± 0.9	5.3 ± 1.07	5.5 ± 1.08 *
LDL-C (mmol/l)	2.9 ± 0.8	3.4 ± 1.0	4.1 ± 1.1	3.3 ± 0.9	3.8 ± 1.09	4.2 ± 1.0
HDL-C (mmol/l)	1.50 ± 0.34	1.48 ± 0.37	1.28 ± 0.47	1.31 ± 0.30	1.28 ± 0.24	1.03 ± 0.27 ***
Apo B (g/l)	0.70 ±0.16	0.85 ± 0.25 *	1.01 ± 0.25 ***	0.87 ± 0.21	1.01 ± 0.24 *	1.09 ± 0.24 *
Apo A-I (g/l)	1.50 ± 0.37	1.56 ± 0.25	1.44 ± 0.37	1.35 ± 0.21	1.36 ± 0.18	1.24 ± 0.24 **

Data represent means ± SD for females (n = 121) and males (n = 125) divided into tertiles based on their three-day average fasting cTG.

* Significantly different from corresponding value in subjects from first tertile ($P < 0.05$).

** Significantly different from corresponding value in subjects from second tertile ($P < 0.05$).

*** Significantly different from corresponding value in subjects from first and second tertile ($P < 0.05$).

Table 5: Mean capillary triglyceride concentration (cTG) and mean increase in cTG at time-points during the day in female subjects with different levels of triglyceridemia.

	Females					
	First tertile Mean cTG (mmol/l)	**First tertile Mean increase in cTG from fasting (mmol/l)**	**Second tertile Mean cTG (mmol/l)**	**Second tertile Mean increase in cTG from fasting (mmol/l)**	**Third tertile Mean cTG (mmol/l)**	**Third tertile Mean increase in cTG from fasting (mmol/l)**
Fasting	0.84 ± 0.06 *	-	1.21 ± 0.16 *	-	2.39 ± 0.77 *	-
Before lunch	0.99 ± 0.21 *	0.15 ± 0.21	1.35 ± 0.36 *	0.14 ± 0.36	2.51 ± 0.88 *	0.11 ± 0.65
After lunch	1.08 ± 0.28 * [b]	0.24 ± 0.27	1.61 ± 0.70 *	0.40 ± 0.72	2.78 ± 1.11 *	0.39 ± 0.79
Before dinner	1.04 ± 0.23 * [a]	0.21 ± 0.23	1.54 ± 0.57 *	0.33 ± 0.56	2.69 ± 1.13 *	0.29 ± 0.62
After dinner	1.20 ± 0.41 * [b]	0.36 ± 0.41	1.80 ± 0.76 * [b]	0.59 ± 0.76	3.02 ± 1.23 *	0.63 ± 0.80
Bedtime	1.27 ± 0.37 * [b]	0.43 ± 0.38	1.67 ± 0.62 * [a]	0.46 ± 0.63	2.84 ± 1.30 *	0.45 ± 1.09

Data represent means ± SD for female subjects (n = 121) divided into tertiles based on their three-day average fasting cTG.

* Significantly different between corresponding values of all three tertiles (all $P < 0.001$)

[a] Significantly different from fasting ($P < 0.05$)

[b] Significantly different from fasting ($P < 0.001$)

Table 6: Mean capillary triglyceride concentration (cTG) and mean increase in cTG at time-points during the day in male subjects with different levels of triglyceridemia.

	Males					
	First tertile Mean cTG (mmol/l)	**First tertile Mean increase in cTG from fasting (mmol/l)**	**Second tertile Mean cTG (mmol/l)**	**Second tertile Mean increase in cTG from fasting (mmol/l)**	**Third tertile Mean cTG (mmol/l)**	**Third tertile Mean increase in cTG from fasting (mmol/l)**
Fasting	0.90 ± 0.07 *	-	1.30 ± 0.19 *	-	2.40 ± 0.72 *	-
Before lunch	1.13 ± 0.37 * [a]	0.23 ± 0.36	1.54 ± 0.54 *	0.24 ± 0.54	2.35 ± 0.90 *	-0.05 ± 0.86
After lunch	1.35 ± 0.45 * [b]	0.45 ± 0.46	2.00 ± 0.57 * [b]	0.69 ± 0.55	2.88 ± 1.00 *	0.48 ± 0.90
Before dinner	1.31 ± 0.34 * [b]	0.41 ± 0.34	1.81 ± 0.53 * [b]	0.51 ± 0.52	2.77 ± 1.04 *	0.36 ± 0.89
After dinner	1.74 ± 0.60 * [b]	0.84 ± 0.57	2.46 ± 0.86 * [b]	1.16 ± 0.86	3.39 ± 1.26 * [a]	0.99 ± 1.10
Bedtime	1.84 ± 0.66 * [b]	0.94 ± 0.64	2.46 ± 0.84 * [b]	1.16 ± 0.80	3.47 ± 1.25 * [b]	1.06 ± 1.12

Data represent means ± SD for male subjects (n = 125) divided into tertiles based on their three-day average fasting cTG.

* Significantly different between corresponding values of all three tertiles (all $P < 0.01$)

[a] Significantly different from fasting ($P < 0.05$)

[b] Significantly different from fasting ($P < 0.001$)

Table 7: Intra-individual variability in capillary triglyceride levels at time-points during the day in female subjects with different levels of triglyceridemia.

	Females					
	First tertile Intra-individual Variability (mmol/l)	**First tertile Intra-individual Variability (%)**	**Second tertile Intra-individual Variability (mmol/l)**	**Second tertile Intra-individual Variability (%)**	**Third tertile Intra-individual Variability (mmol/l)**	**Third tertile Intra-individual Variability (%)**
Fasting	0.08 (0.03-0.25)	8.9 (3.5-24.3)	0.29 (0.18-0.43)	21.4 (16.0-33.0)	0.46 (0.30-0.65) **	18.8 (13.3-25.3)
Before lunch	0.10 (0.04-0.31)	9.2 (3.5-28.1)	0.26 (0.14-0.42)	21.1 (11.1-27.7)	0.44 (0.24-0.70) **	15.9 (11.3-24.6)
After lunch	0.21 (0.13-0.45)	17.1 (9.7-33.7)	0.37 (0.18-0.57)	23.8 (11.9-33.9)	0.55 (0.39-0.79) *	22.7 (13.4-26.8)
Before dinner	0.21 (0.14-0.43)	17.8 (13.5-28.8)	0.29 (0.15-0.44)	17.3 (10.8-28.4)	0.46 (0.31-0.74) **	18.3 (12.9-24.2)
After dinner	0.19 (0.10-0.33)	13.2 (7.8-24.5)	0.47 (0.28-0.88) * [a]	26.9 (17.7-40.2) *	0.64 (0.38-0.88) *	20.6 (13.2-30.1)
Bedtime	0.27 (0.12-0.59)	20.2 (11.3-38.7)	0.32 (0.15-0.62)	21.8 (10.2-35.4)	0.53 (0.27-1.22) **	22.3 (14.4-41.8)

Data are given as medians with interquartile ranges for female subjects (n = 121) divided into tertiles based on their three-day average fasting cTG. Intra-individual variability is given in absolute terms (mmol/l) as a standard deviation for cTG (SD) measured on three separate occasions, and in relative terms (%) as a coefficient of variation for cTG measured on three occasions.

* Significantly different from corresponding value in subjects from first tertile ($P < 0.05$).

** Significantly different from corresponding value in subjects from first and second tertile ($P < 0.05$).

[a] Significantly different from fasting ($P < 0.05$)

Table 8: Intra-individual variability in capillary triglyceride levels at time-points during the day in male subjects with different levels of triglyceridemia.

	Males					
	First tertile Intra-individual Variability (mmol/l)	**First tertile Intra-individual Variability (%)**	**Second tertile Intra-individual Variability (mmol/l)**	**Second tertile Intra-individual Variability (%)**	**Third tertile Intra-individual Variability (mmol/l)**	**Third tertile Intra-individual Variability (%)**
Fasting	0.09 (0.06-0.22)	9.4 (6.4-20.1)	0.25 (0.15-0.43)	21.4 (12.2-31.1) *	0.67 (0.26-1.08) **	29.7 (14.2-42.2) *
Before lunch	0.19 (0.09-0.44)	18.5 (9.0-32.9)	0.39 (0.20-0.65)	27.1 (13.6-33.7)	0.46 (0.29-0.66) *	19.7 (9.7-25.6)
After lunch	0.25 (0.11-0.56)	21.9 (8.7-32.9)	0.34 (0.16-0.68)	19.2 (7.6-30.8)	0.60 (0.34-0.90) *	19.8 (12.0-38.5)
Before dinner	0.34 (0.22-0.51) [a]	26.1 (17.9-34.4) [a]	0.45 (0.16-0.73)	24.8 (12.4-36.1)	0.50 (0.29-0.87)	18.4 (11.1-31.5)
After dinner	0.47 (0.28-0.76) [c]	29.5 (15.5-46.8) [b]	0.64 (0.27-0.82) [a]	22.7 (14.2-33.9)	0.96 (0.41-1.36) **	28.0 (13.0-35.1)
Bedtime	0.52 (0.28-0.76) [c]	31.1 (20.1-48.3) [c]	0.68 (0.46-1.00) [c]	32.6 (19.2-43.8)	0.79 (0.42-1.10)	23.7 (16.1-31.8)

Data are given as medians with interquartile ranges for male subjects (n = 125) divided into tertiles based on their three-day average fasting cTG. Intra-individual variability is given in absolute terms (mmol/l) as a standard deviation for cTG (SD) measured on three separate occasions, and in relative terms (%) as a coefficient of variation for cTG measured on three occasions.

* Significantly different from corresponding value in subjects from first tertile ($P < 0.05$).

** Significantly different from corresponding value in subjects from first and second tertile ($P < 0.05$).

[a] Significantly different from fasting ($P < 0.05$)

[b] Significantly different from fasting ($P < 0.01$)

[c] Significantly different from fasting ($P < 0.001$)

tertiles and for the second tertile in females after dinner, which was significantly higher compared to the first tertile (Tables 7 and 8).

Similarly to the total female group, the variability did not increase significantly as the day progressed when females were divided into tertiles, except for the absolute intra-individual variability after dinner, which was significantly higher in the second tertile compared to the first tertile (Table 7). In males, the variability increased as the day progressed, except in the highest tertile. In male subjects from the first tertile, the absolute and relative variability were increased before dinner, after dinner and at bedtime. In males from the second tertile, only the absolute variability was increased after dinner and at bedtime, but the relative variability remained unchanged (Table 8).

Significant correlations were found between fasting cTG and fasting absolute intra-individual variability in both females and males (r: 0.459; $P < 0.001$ and r: 0.759; $P < 0.001$, respectively) and between cTG at bedtime and the absolute intra-individual variability at bedtime in both females and males (r: 0.523; $P < 0.001$ and r: 0.362; $P < 0.001$, respectively). No significant correlations were found between the absolute intra-individual TG variability and age, BMI or HOMA-IR during fasting or at bedtime.

Discussion

The present study confirms our previous work showing that: 1) cTG increases during the course of the day, and 2) daytime increase in cTG is greater, and thus non-fasting cTG levels are higher, in male compared to female subjects [24]. While intra-individual variability in fasting cTG levels was found to be similar in males and females (0.28 and 0.35 mmol/l, i.e., 21.0% and 20.5%, respectively), the intra-individual variability in non-fasting cTG levels was found to increase during the day (i.e., 2-fold) in males but not in females. When subjects were divided into tertiles on the basis of their fasting cTG levels, the daytime increase in cTG was similar in all three groups, and subjects with higher levels of cTG had a greater absolute intra-individual variability in both fasting and most non-fasting time points. Our data are consistent with the concept that TG concentrations in the blood tend to increase throughout the day and are dependent on: 1) the size, composition and frequency of fat-containing meals, 2) the levels of stress and exercise, and 3) hormonal and metabolic factors. Numerous environmental and metabolic parameters thus contribute to the variability of non-fasting TG levels, which tends to be greater in male subjects and in individuals with higher levels of circulating TG.

The ability of blood TG levels to predict or diagnose the presence of cardiovascular disease depends on the stability of the TG concentration over time. Current guidelines thus recommend the measurement of TG levels after an eight to twelve hour fast in order to reduce day-to-day variability [5]. However, even in the fasted state, blood TG

levels vary considerably. For example, in 44 healthy women, aged 18-35 years, plasma TG had an intra-individual variance of 22% [12]. In a group of 83 healthy men and women, the biological variability in fasting capillary TG was found to be 14.7% and did not differ from venous samples [25]. In patients with T2DM treated with either simvastatin or atorvastatin, the biological variability in fasting serum TG was 12.1% and 19.7%, respectively [26]. A meta-analysis of 30 studies published from 1970 to 1992 found the overall average intra-individual biological variability to be 22.6% [11]. This coefficient of variation is relatively similar to the CV of 21.0% in males and 20.5% in females in the fasted state observed in the current study, confirming that our mixed group of healthy volunteers and patients is representative of other cohorts.

We had assumed that the intra-individual variability in cTG would be greater in non-fasting than in fasting samples, however somewhat surprisingly, this was only found to be the case in males but not in females. The intra-individual variability in daytime non-fasting cTG levels thus increased significantly from 0.28 to 0.69 mmol/l in males, and was 0.35 and 0.36 mmol/l in females. Greater intra-individual variability in cTG in males in the non-fasting state could in part be due to the beneficial effects of estrogens in females which tends to reduce fluctuations in TG levels [17,21,27]. In a separate study, diurnal variation in serum TG was determined by measuring blood samples taken at 9:00 a.m. (fasting), 11.00 a.m., 3.00 p.m. and at 8.00 p.m. (after dinner). Women were found to have a diurnal variability of 23.8% and men had 35.7% [15]. Generally these data are consistent with the concept that male individuals who tend to have higher levels of blood TG in the non-fasting state, have greater diurnal as well as day-to-day variability in their circulating TG levels.

The absolute intra-individual TG variability was higher in females and males with elevated TG levels (third tertile). This is most probably a physiological phenomenon. It must be acknowledged, however, that the intra-individual variability in subjects with low levels of triglyceridemia may have been partly affected by technical limitations of the point-of-care testing device. This device had a limit of sensitivity of 0.80 mmol/l, and therefore gave a reading of 0.80 mmol/l whenever cTG was 0.80 mmol/l or below. Corrections were attempted by disregarding measurements that were equal to 0.80 mmol/l, however this could still have reduced the variability of cTG measurements artificially at this lower end of detection. Of greater clinical relevance is the finding that subjects with higher levels of cTG (i.e., the third tertile) had the highest intra-individual TG variability. These individuals could potentially be diagnosed as being normotriglyceridemic, and subsequently be categorized in a lower cardiovascular risk category. This concern pertains to both fasting and non-fasting blood samples since intra-individual variability in cTG was high at all time-points. As expected, subjects with the highest levels of triglyceridemia had a significantly higher age, BMI, HOMA-IR and higher prevalence of CVD. The variability correlated strongly with cTG concentrations but not with age, BMI or

HOMA-IR. Therefore, it seems likely that TG variability is more influenced by the degree of triglyceridemia than factors as age, BMI or HOMA-IR.

Langsted et al. have recently shown using cross-sectional data that in both diabetic and non-diabetic individuals [28], plasma triglyceride concentration differs very little (i.e., 0.2-0.3 mmol/l) when measured in fasting and non-fasting samples. They have thus concluded that plasma TG levels change minimally in response to normal food intake in individuals in the general population. This is however an erroneous extrapolation of the population data, as evidenced by the present results in male and female individuals, showing that non-fasting TG levels actually increase by 0.5 to 2.0 mmol/l during the day. The majority of this increase is at the end of the day however, as is the increase in intra-individual variability, leading us to suggest that the accuracy of TG measurements for cardiovascular risk assessment is likely to be minimally affected if non-fasting blood samples are drawn during daylight hours.

It is generally believed that the postprandial period is more atherogenic, and it therefore seems logical to try to establish new standards for non-fasting lipid measurements. Large epidemiological studies have shown that increased non-fasting TG are a risk factor for cardiovascular disease independent from other well established risk factors [8,9,29] and evidence has been presented suggesting that non-fasting TG is an even stronger predictor of cardiovascular disease than fasting TG [6,8]. In the future, measurement of non-fasting TG could replace fasting TG once reference values for non-fasting TG have been established [30]. In a pilot study with limited numbers of healthy males and females, an attempt was made to describe reference values for diurnal TG in Dutch subjects [21]. Similar studies need to be done in other populations taking into consideration, age, gender and race.

In conclusion, TG levels increase throughout the day by an average of 0.53 mmol/l in females and 1.05 mmol/l in males. Non-fasting TG show the greatest variability in males, especially in those with hypertriglyceridemia. Since the greatest postprandial variability occurs very late in the day (around dinner time and bedtime), this is unlikely to affect the assessment of cardiovascular risk, which is usually made with a daytime blood sample. In view of the fact that non-fasting TG levels are likely to be used in the future to assess the individual risk of cardiovascular disease, future studies are required to determine normal ranges for non-fasting TG levels.

References

1. Nordestgaard BG, Langsted A, Freiberg JJ. Nonfasting hyperlipidemia and cardiovascular disease. Curr Drug Targets 2009;10:328-335
2. Patsch W, Esterbauer H, Foger B, Patsch JR. Postprandial lipemia and coronary risk. Curr Atheroscler Rep 2000;2:232-242
3. Langsted A, Freiberg JJ, Nordestgaard BG. Fasting and nonfasting lipid levels: Influence of normal food intake on lipids, lipoproteins, apolipoproteins, and cardiovascular risk prediction. Circulation 2008;118:2047-2056
4. Cohn JS. Postprandial lipemia and remnant lipoproteins. Clin Lab Med 2006;26:773-786
5. Third report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel III) final report. Circulation 2002;106:3143-3421
6. Mora S, Rifai N, Buring JE, Ridker PM. Fasting compared with nonfasting lipids and apolipoproteins for predicting incident cardiovascular events. Circulation 2008;118:993-1001
7. Hokanson JE, Austin MA. Plasma triglyceride level is a risk factor for cardiovascular disease independent of high-density lipoprotein cholesterol level: A meta-analysis of population-based prospective studies. J Cardiovasc Risk 1996;3:213-219
8. Bansal S, Buring JE, Rifai N, Mora S, Sacks FM, Ridker PM. Fasting compared with nonfasting triglycerides and risk of cardiovascular events in women. JAMA 2007;298:309-316
9. Nordestgaard BG, Benn M, Schnohr P, Tybjaerg-Hansen A. Nonfasting triglycerides and risk of myocardial infarction, ischemic heart disease, and death in men and women. JAMA 2007;298:299-308
10. Wasenius A, Stugaard M, Otterstad JE, Froyshov D. Diurnal and monthly intra-individual variability of the concentration of lipids, lipoproteins and apoproteins. Scand J Clin Lab Invest 1990;50:635-642
11. Smith SJ, Cooper GR, Myers GL, Sampson EJ. Biological variability in concentrations of serum lipids: Sources of variation among results from published studies and composite predicted values. Clin Chem 1993;39:1012-1022
12. Brown SA, Boerwinkle E, Kashanian FK, Swanson N, Patsch W. Variation in concentration of lipids, lipoprotein lipids, and apolipoproteins A-I and B in plasma from healthy women. Clin Chem 1990;36:207-210
13. Godsland IF. Intra-individual variation: Significant changes in parameters of lipid and carbohydrate metabolism in the individual and intra-individual variation in different test populations. Ann Clin Biochem 1985;22:618-624
14. Kafonek SD, Derby CA, Bachorik PS. Biological variability of lipoproteins and apolipoproteins in patients referred to a lipid clinic. Clin Chem 1992;38:864-872
15. Demacker PN, Schade RW, Jansen RT, Van 't Laar A. Intra-individual variation of serum cholesterol, triglycerides and high density lipoprotein cholesterol in normal humans. Atherosclerosis 1982;45:259-266
16. Delawi D, Meijssen S, Castro Cabezas M. Intra-individual variations of fasting plasma lipids, apolipoproteins and postprandial lipemia in familial combined hyperlipidemia compared to controls. Clin Chim Acta 2003;328:139-145
17. Castro Cabezas M, Halkes CJ, Meijssen S, van Oostrom AJ, Erkelens DW. Diurnal triglyceride profiles: A novel approach to study triglyceride changes. Atherosclerosis 2001;155:219-228

18. Halkes CJ, Castro Cabezas M, van Wijk JP, Erkelens DW. Gender differences in diurnal triglyceridemia in lean and overweight subjects. Int J Obes Relat Metab Disord 2001;25:1767-1774
19. van Oostrom AJ, Castro Cabezas M, Ribalta J, Masana L, Twickler TB, Remijnse TA, et al. Diurnal triglyceride profiles in healthy normolipidemic male subjects are associated to insulin sensitivity, body composition and diet. Eur J Clin Invest 2000;30:964-971
20. van Wijk JP, Castro Cabezas M, Halkes CJ, Erkelens DW. Effects of different nutrient intakes on daytime triacylglycerolemia in healthy, normolipemic, free-living men. Am J Clin Nutr 2001;74:171-178
21. van Wijk JP, van Oostrom AJ, Castro Cabezas M. Normal ranges of non-fasting triglycerides in healthy Dutch males and females. Clin Chim Acta 2003;337:49-57
22. Luley C, Ronquist G, Reuter W, Paal V, Gottschling HD, Westphal S, et al. Point-of-care testing of triglycerides: Evaluation of the Accutrend triglycerides system. Clin Chem 2000;46:287-291
23. Moses RG, Calvert D, Storlien LH. Evaluation of the accutrend GCT with respect to triglyceride monitoring. Diabetes Care 1996;19:1305-1306
24. Cohn JS, McNamara JR, Cohn SD, Ordovas JM, Schaefer EJ. Postprandial plasma lipoprotein changes in human subjects of different ages. J Lipid Res 1988;29:469-479
25. Kafonek SD, Donovan L, Lovejoy KL, Bachorik PS. Biological variation of lipids and lipoproteins in fingerstick blood. Clin Chem 1996;42:2002-2007
26. Sathyapalan T, Atkin SL, Kilpatrick ES. Variability of lipids in patients with type 2 diabetes taking statin treatment: Implications for target setting. Diabet Med 2008;25:909-915
27. Jackson KG, Abraham EC, Smith AM, Murray P, O'Malley B, Williams CM, et al. Impact of age and menopausal status on the postprandial triacylglycerol response in healthy women. Atherosclerosis 2010;208:246-252
28. Langsted A, Nordestgaard BG. Nonfasting lipids, lipoproteins, and apolipoproteins in individuals with and without diabetes: 58 434 individuals from the Copenhagen General Population Study. Clin Chem 2011;57:482-489
29. Eberly LE, Stamler J, Neaton JD. Relation of triglyceride levels, fasting and nonfasting, to fatal and nonfatal coronary heart disease. Arch Intern Med 2003;163:1077-1083
30. Stalenhoef AF, de Graaf J. Association of fasting and nonfasting serum triglycerides with cardiovascular disease and the role of remnant-like lipoproteins and small dense LDL. Curr Opin Lipidol 2008;19:355-361

Chapter 4

Leukocyte cell population data (VCS) in postprandial leukocyte activation

Boudewijn Klop
Gert-Jan M. van de Geijn
Tjin L. Njo
Hans W. Janssen
Arie P. Rietveld
Addy van Miltenburg
Laura Fernández-Sender
Jan Willem F. Elte
Manuel Castro Cabezas

Int J Lab Hematol 2013;35:644-51

Abstract

Introduction: Changes in leukocyte cell population data have been reported in various infectious diseases, but little is known in other inflammatory conditions such as the postprandial state. We investigated whether leukocyte cell population data change during postprandial leukocyte activation.

Methods: Healthy volunteers underwent a standardized oral fat loading test (OFLT). Flowcytometric quantification of leukocyte activation markers CD11b, CD66b, CD35 and CD36, together with leukocyte cell population data from LH750 hematology analyzers were measured fasting and at 4 and 8 hours postprandially.

Results: Twelve volunteers were included. Postprandial leukocyte activation was confirmed by increased expression of CD11b by monocytes (+11.7%) and neutrophils (+15.0%) and by increased expression of CD66b (+14.7%) and CD35 (+16.6%) by neutrophils at T = 4h. The mean scatter from neutrophils, reflecting granularity, significantly decreased at T = 4h ($P < 0.05$) and returned to baseline at T = 8h (P-ANOVA = 0.048). The mean volume of monocytes increased significantly at T = 4h ($P < 0.001$) and returned to baseline at T = 8h (P-ANOVA = 0.0008). At T = 4h CD11b expression on neutrophils was associated with a reduction in mean scatter of neutrophils (Pearson's r: -0.677, P = 0.016).

Conclusion: Postprandial leukocyte activation is accompanied by temporary changes in leukocyte cell population data, similar to changes observed during various infections, but to a lesser extent.

Introduction

Triglyceride-rich lipoproteins are abundant in the postprandial phase and are able to induce an inflammatory response, which includes postprandial leukocyte activation [1]. Neutrophils are recruited within two hours after the ingestion of fat and/or glucose together with concomitant impairment of vessel dilatation [2,3]. In vitro experiments have shown that chylomicron remnants activate monocytes and neutrophils [4,5]. Moreover, postprandial leukocyte activation is observed in vivo after the ingestion of a fat-containing meal, which directly correlates with the postprandial increase in triglycerides (TG) [5-7]. Postprandial activated monocytes can adhere on endothelial cells by increased expression of adhesion molecules, mainly CD11c/CD18 [7], which may facilitate subendothelial migration of monocytes, with risk of foam cell formation and subsequently the development of atherosclerosis [1].

Changes in leukocyte cell population data have been reported in various infectious diseases. Monocyte and neutrophil volume temporarily increases combined with decreased light scatter by neutrophils during bacterial infections or sepsis [8-10]. In contrast, little is known about changes in leukocyte cell population data during other non-infectious inflammatory conditions like the inflammatory postprandial state. Leukocyte cell population data are acquired during the 5-part leukocyte differentiation by automatic cell counters in clinical laboratories. The LH750 hematology analyzer (Beckman Coulter, Miami, USA) uses Volume Conductivity Scatter (VCS) technology to perform the 5-part leukocyte differential count. Different parameters of VCS-data reflect the granularity and activation state of leukocytes. Over 8000 cells are measured using direct current impedance to measure cell volume (V). A radio frequency probe measures conductivity (C) that reflects nuclear shape, lobularity and nuclear/cytoplasmic ratio. Finally, laser light scatter (S) quantifies cellular granularity [11,12]. Together, these leukocyte cell population data are used to identify the different leukocytes for the 5-part differential count. Recently, it has been suggested that leukocyte cell population data may also be a clinical useful indicator for acute bacterial infections [8,10], postoperative infections [13], malaria [14] and as a discriminator between fungemia and bacteremia [15].

Since postprandial lipemia is one of the most physiological stimuli for leukocyte activation, our aim was to study whether changes in leukocyte cell population data occur postprandially and whether there is a concomitant relationship with leukocyte activation markers.

Materials and methods

Subjects and study design

Male and female volunteers were recruited by advertisement. Subjects had to be healthy and aged between 18 and 50 years. Exclusion criteria were the use of any medication or the presence of hypertriglyceridemia (fasting TG > 2.00 mmol/l). Participants visited the hospital after a 10h overnight fast. Anthropometric characteristics like length, weight, body mass index, waist circumference and blood pressure were measured. A fasting venous blood sample was drawn (T = 0h) and subjects were given an oral fat load using fresh cream (Fresh cream, Albert Heijn, Zaandam, the Netherlands) in a dosage of 50g of fat per square meter body surface at 9:00 AM. During the oral fat loading test (OFLT) participants were not allowed to smoke, eat or drink (except water) and participants refrained from physical activity. Venous blood samples were taken at 4 (T = 4h) and 8 hours (T = 8h) after the ingestion of the oral fat load. The time-points T = 4h and T = 8h were chosen since postprandial triglyceridemia in healthy subjects is maximal at T = 4h and TG return to fasting levels at T = 8h. In addition, it has been shown that postprandial leukocyte activation is highest at T = 4h [5]. The study was approved by the independent Regional Medical Ethical Committee Rotterdam (Maasstad Hospital, the Netherlands) and all subjects gave written informed consent.

Clinical chemistry

All clinical chemistry measurements were carried out on freshly drawn blood in the department of Clinical Chemistry, Sint Franciscus Gasthuis, Rotterdam. Baseline renal function, C-reactive protein, glucose, plasma cholesterol, HDL-C and triglycerides were measured using the LX20 or DxC analyzers (Beckman Coulter, Miami, USA). LDL-C values were calculated using the Friedewald formula. Apolipoprotein A-I and apo B were determined by nephelometry using an IMAGE instrument with commercially available kits (Beckman Coulter).

Leukocyte VCS-data

Blood cell counts and 5-part leukocyte differentiation were determined automatically using LH750 analyzers (Beckman Coulter) within 45 minutes after venipuncture. VCS-data were obtained from lymphocytes, monocytes and neutrophils. These data were generated by optical and electronic measurements of individual cells by the LH750 analyzers during generation of the automated leukocyte 5-part differential count. The parameters include the mean channel and standard deviation of volume (V), conductivity (C) and light scatter (S) for lymphocytes, monocytes and neutrophils. Using a normal blood sample that was measured 10 consecutive times on the LH750 we established the coefficient of variation (CV) for each of these parameters. With the exception of lympho-

cyte conductivity (CV: 1.41%) all CV's were below 1%. This indicates that VCS-data are robust and have very little analytical variation, as was also reported by others [10].

CD11b, CD66b, CD35 and CD36 as markers for *in vivo* leukocyte activation

Blood samples for the determination of leukocyte activation markers CD11b, CD66b, CD35 and CD36 were collected in tubes containing 5.4mg K2 EDTA (Becton Dickinson, Plymouth, United Kingdoms) at baseline and at four and eight hours after ingestion of the oral fat load. The method has been described in detail before [5,6]. Briefly, the staining procedure was started within 15 minutes after venipuncture. All measurements were carried out in triplicate. Separate tubes were prepared: 1) a combination of fluorescein isothiocynate (FITC) conjugated CD66b, phycoerythrin (PE) conjugated CD11b and phycoerythrin-Texas Red-X (ECD) conjugated CD45 and 2) a combination of FITC conjugated CD36, PE conjugated CD35 and ECD conjugated CD45. All antibodies were from Beckman Coulter, except for CD35-PE (BD Biosciences, Franklin Lakes, NJ, USA). A total of 20 µl of whole blood was added to each tube and incubated for 15 minutes in the dark on room temperature. Erythrocytes were lysed by addition of 500µl lysis solution (1.5 M ammonium chloride, 100 mM potassium hydrogen carbonate, 0.82 mM EDTA, pH7.4) followed by 15 min of incubation in the dark. The samples were measured on a Navios flow cytometer (Beckman Coulter). Samples were measured for a maximum of five minutes or until at least 2000 monocytes were acquired. Lymphocytes, monocytes and neutrophils were identified in the side scatter versus CD45 dot plot. The fluorescence intensity of each cell type was expressed as the mean fluorescence intensity (MFI) of the triplicate measurements. Before each use, the optics and settings of the flow cytometer were checked with Flow-Check Pro and Flow-Set Pro beads (Beckman Coulter). Identi-

Table 1: Antibody information concerning the measurements of *in vivo* leukocyte activation by flow cytometry.

FL-channel	Marker	Intra-assay variation (%, range)		Dilution	Antibody clone	Catalog number	Company
		Monocytes	Neutrophils				
FITC	CD66b	NA	1.8% (0.4-9.5)	20x	80H3	0531	BC
FITC	CD36	5.6% (1.0-17.0)	NA	5x	FA6.152	PN IM07664	BC
PE	CD35	4.4% (0.6-11.1)	4.0% (1.9-7.0)	1x	E11	559872	BD
PE	CD11b	2.5% (0.2-6.3)	2.5% (0.8-6.3)	10x	Bear1	PN IM2581	BC
ECD	CD45	NA	NA	5x	J33	A07784	BC

The intra-assay variation is shown in relative terms (%) as the coefficient of variation of the triplicate measurements of the respective antibody and cell type in the fasting state. The mean and range of all subjects is given.

Abbreviations: fluorescein isothiocynate (FITC), phycoerythrin (PE), phycoerythrin-Texas Red-X (ECD), not applicable (NA), Beckman Coulter (BC), Becton Dickinson Biosciences (BD).

cal instrument settings were used for the complete study. An overview of the antibody information and the intra-assay variation of the respective *in vivo* leukocyte activation markers is given in Table 1.

Statistical analysis

Data are given as mean ± SD in the text and tables and as mean ± SEM in the figures unless stated otherwise. The relative intra-assay variation in markers for *in vivo* leukocyte activation by flow cytometry was assessed by calculating the coefficient of variation (CV) of the triplicate measurements in the fasting state of the respective marker for each subject. The CV was calculated for each specific marker and cell type as: (standard deviation / mean MFI) * 100. Postprandial time effects were analyzed using repeated measures ANOVA with Dunnett's Multiple Comparison Test as post hoc analysis. VCS-data of one time-point (t = 8h) was missing in two volunteers and these data were imputed by using the mean of the respective individual. Correlations were calculated using the Pearson's correlation coefficient. PRISM version 5.0 (Graph Pad Software, San Diego, USA) and PASW 18.0 (IBM, New York, USA) were used for statistical analyses. Statistical significance was set at $P < 0.05$ (two-sided).

Table 2: Characteristics of the subjects. Data are given as mean ± standard deviation, unless stated otherwise.

	n = 12
Age (years)	35.3 ± 12.7
Male gender (n, %)	6 (50)
Body mass index (kg/m^2)	21.9 ± 2.1
Systolic blood pressure (mmHg)	110.5 ± 9.1
Diastolic blood pressure (mmHg)	65.1 ± 8.8
Leucocyte count (*10^9/l)	6.1 ± 1.1
Absolute lymphocyte count (*10^9/l)	1.7 ± 0.3
Absolute monocyte count (*10^9/l)	0.46 ± 0.13
Absolute neutrophil count (*10^9/l)	3.8 ± 0.9
C-reactive protein (mg/l)	1.4 ± 0.7
Total cholesterol (mmol/l)	4.6 ± 0.6
LDL-C (mmol/l)	2.7 ± 0.6
HDL-C (mmol/l)	1.63 ± 0.39
Triglycerides (mmol/l)	0.71 ± 0.24
Apolipoprotein B (g/l)	0.78 ± 0.14
Apolipoprotein A-I (g/l)	1.88 ± 0.35

Table 3: Postprandial changes in triglycerides and absolute counts for total leukocytes, lymphocytes, monocytes and neutrophils after a standardized oral fat load (n = 12). Data are given as mean ± standard deviation.

	T = 0h	T = 4h	T = 8h	P-ANOVA
Triglycerides (mmol/l)	0.71 ± 0.24	1.62 ± 0.70***	0.81 ± 0.57	<0.001
Total leucocyte count (*10^9/l)	6.1 ± 1.1	6.7 ± 1.5	7.5 ± 2.0***	0.0017
Absolute lymphocyte count (*10^9/l)	1.8 ± 0.3	2.0 ± 0.4*	2.4 ± 0.6***	<0.001
Absolute monocyte count (*10^9/l)	0.46 ± 0.13	0.45 ± 0.14	0.54 ± 0.15***	0.002
Absolute neutrophil count (*10^9/l)	3.8 ± 0.9	4.3 ± 1.4	4.4 ± 1.9	0.26
Relative lymphocyte count (%)	28.3 ± 4.8	29.5 ± 6.5	33.1 ± 8.6*	0.04
Relative monocyte count (%)	7.6 ± 1.9	7.0 ± 1.8	7.3 ± 1.8	0.17
Relative neutrophil count (%)	62.5 ± 6.3	61.8 ± 8.3	57.6 ± 10.4	0.06

* Significantly different from corresponding value at T = 0h (P < 0.05)
*** Significantly different from corresponding value at T = 0h (P < 0.001)

Table 4: Postprandial changes in VCS-data after a standardized oral fat loading test (n = 12). Data represent the mean ± standard deviation.

	T = 0h	T = 4h	T = 8h	P-ANOVA
Lymphocytes				
V-MN	81.2 ± 2.7	81.5 ± 2.3	80.5 ± 2.5	0.35
V-SD	13.7 ± 0.8	13.5 ± 1.2	13.2 ± 0.8	0.24
C-MN	114.0 ± 1.6	114.1 ± 1.9	116.0 ± 2.3*	0.009
C-SD	10.5 ± 1.6	9.8 ± 1.1	9.9 ± 1.2	0.22
S-MN	67.3 ± 3.1	67.7 ± 3.2	67.6 ± 4.3	0.87
S-SD	16.3 ± 1.4	16.1 ± 1.8	15.9 ± 1.7	0.69
Monocytes				
V-MN	162.4 ± 4.3	165.0 ± 4.8***	163.5 ± 4.2	0.0008
V-SD	17.2 ± 2.0	17.1 ± 1.6	17.3 ± 1.6	0.96
C-MN	123.4 ± 3.0	122.5 ± 3.2	124.8 ± 3.0	0.022
C-SD	4.5 ± 0.7	4.4 ± 0.4	4.5 ± 0.5	0.76
S-MN	90.0 ± 3.5	88.7 ± 2.7	89.5 ± 2.2	0.22
S-SD	9.6 ± 0.7	9.5 ± 0.7	9.4 ± 1.1	0.65
Neutrophils				
V-MN	140.8 ± 2.7	142.3 ± 3.8	140.9 ± 4.6	0.27
V-SD	18.4 ± 0.6	19.0 ± 0.9	18.9 ± 1.4	0.30
C-MN	146.4 ± 2.8	146.1 ± 3.5	148.8 ± 3.6*	0.0064
C-SD	5.6 ± 0.9	5.7 ± 0.7	5.6 ± 0.6	0.79
S-MN	147.5 ± 4.7	145.4 ± 5.0*	146.4 ± 6.3	0.048
S-SD	10.7 ± 1.2	11.0 ± 1.0	10.8 ± 1.2	0.53

* Significantly different from corresponding value at T = 0h (P < 0.05)
*** Significantly different from corresponding value at T = 0h (P < 0.001)
Abbreviations: mean (MN), standard deviation (SD), volume (V), conductivity (C), scatter (S).

Results

A total of 12 healthy subjects, 6 males and 6 females, were included in the study. Subjects were lean, normotensive and showed a normal lipid profile, leukocyte concentration and automated 5-part leukocyte differentiation (Table 2). Plasma TG increased by 0.91 ± 0.53 mmol/l (P < 0.001) four hours postprandially and returned to baseline at T = 8h. Total leukocyte count and absolute monocyte count were significantly increased

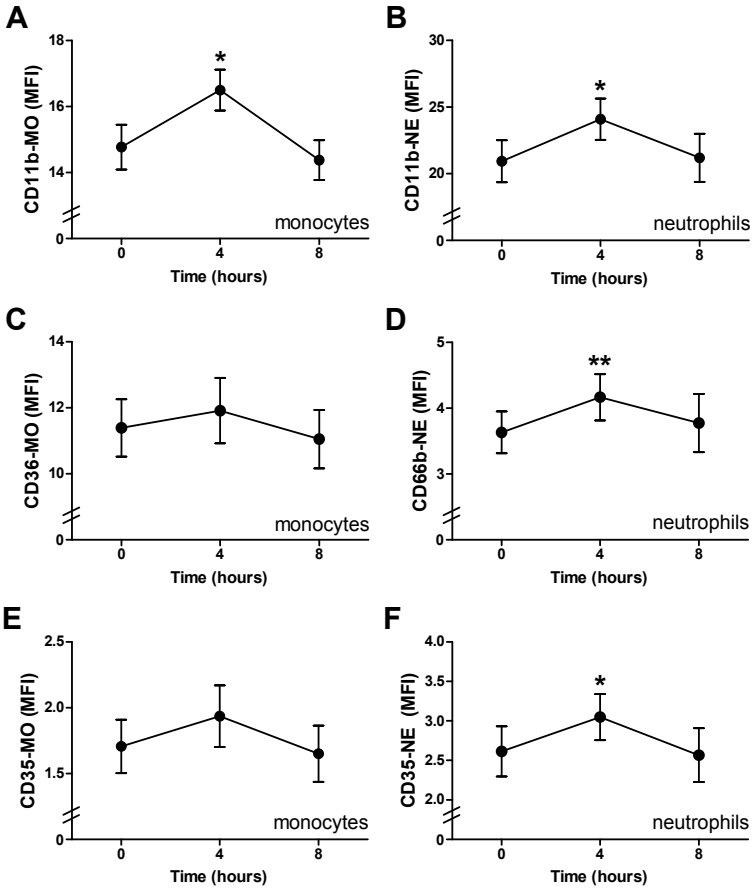

Figure 1: The mean (± SEM) changes in activation markers of monocytes and neutrophils after the ingestion of a standardized oral fat loading test (n = 12): Expression of CD11b by monocytes (A) and neutrophils (B) was significantly increased postprandially (P-ANOVA 0.004 and 0.024, respectively). CD36 expressed by monocytes (C) remained unchanged, but the degranulation marker CD66b increased significantly on neutrophils (D) postprandially (P-ANOVA 0.007). Furthermore, CD35 was significantly more expressed by monocytes (E) and neutrophils (F) postprandially (P-ANOVA 0.021 and 0.016, respectively). Abbreviations: cluster of differentiation (CD), monocytes (MO), neutrophils (NE). *P < 0.05 and **P < 0.01 when compared to T = 0h.

at the end of the OFLT, whereas absolute lymphocyte count gradually increased during the OFLT. The absolute neutrophil count increased gradually during the OFLT, but did not reach statistical significance (Table 3). The expression of CD11b on monocytes and CD11b, CD66b and CD35 on neutrophils increased significantly at T = 4h and declined thereafter. In addition, monocytes showed a trend of a postprandial increase in CD35 expression, whereas the expression of CD36 by monocytes remained unchanged (Figure 1).

The mean volume of monocytes increased significantly from 162.4 ± 4.3 a.u. to 165.0 ± 4.8 a.u. at t = 4h ($P < 0.001$) and returned to 163.5 ± 4.2 a.u. at T = 8h (P-ANOVA 0.0008). The mean volume of lymphocytes and neutrophils remained unchanged postprandially. The mean conductivity of lymphocytes, monocytes and neutrophils increased at the end of the OFLT (T = 8h) (Table 4). The mean scatter of neutrophils decreased significantly from 147.5 ± 4.7 to 145.4 ± 5.0 ($P < 0.05$) at T = 4h and returned to 146.4 ± 6.3 at T = 8h (P-ANOVA 0.048). The mean scatter from lymphocytes and monocytes remained unchanged postprandially. No significant postprandial changes were observed in the standard deviations of the VCS-data.

At T = 4h, CD11b expression on neutrophils was negatively correlated with the mean scatter of neutrophils (Pearson's r: -0.677, $P = 0.016$) and CD36 expression on monocytes was positively correlated with the mean conductivity of monocytes (Pearson's r: 0.606, $P = 0.037$). No other correlations were found between activation markers on monocytes and neutrophils with corresponding VCS-parameters at the postprandial peak.

Discussion

To our knowledge this is the first report on postprandial changes in leukocyte cell population data. Our study illustrates that leukocyte cell population data are influenced by non-infectious inflammatory conditions like physiological postprandial leukocyte activation. As reported earlier [5,6], postprandial leukocyte activation was confirmed by an increased expression of CD35, CD11b and CD66b on neutrophils and CD11b on monocytes at the postprandial peak. Postprandial TG increased by 0.91 mmol/l, which is comparable with a physiological diurnal increase in TG, since TG normally increase approximately 0.5-1.0 mmol/l during the day [16].

The mean monocyte volume was increased four hours postprandially. This result is in concordance with others who reported increased mean volume of monocytes in bacteremia [10,15], fungemia [15], hepatitis B infection [17] and malaria infection [14]. Our study also observed a postprandial decrease in mean scatter of neutrophils, which correlated with the postprandial expression of CD11b on neutrophils. These results were expected since neutrophils become activated postprandially. Similar changes in mean

scatter of neutrophils, albeit with a stronger magnitude, have been observed during systemic infection or sepsis [8,15] and after activation of neutrophils with granulocyte colony-stimulating factor (G-CSF) [18]. Neutrophil activation leads to degranulation and thus to a decrease in mean scatter. Upon activation, the secretory vesicles of neutrophils are fused with the plasma membrane and are exocytosed. These secretory vesicles are the main reservoir for CD35 and membrane proteins CD11b and CD66b [19]. Therefore, leukocyte activation leads to increased membrane expression of CD11b, CD66b and CD35.

The observed postprandial changes in mean volume of monocytes and mean scatter of neutrophils we observed were 1.7% and 1.4% compared to reported changes of 4.1 - 11.2% in infectious conditions [9,10,14,15]. The postprandial state may theoretically influence the usability of leukocyte cell population data as a diagnostic tool for infection detection. However, the postprandial changes in leukocyte cell population were rather small, which probably limits the clinical relevance of these postprandial changes in mean volume of monocytes and mean scatter of neutrophils. Nevertheless, our results confirm that leukocyte cell population data are already affected by a physiological postprandial inflammatory reaction. We hypothesize that these morphological changes are due to internalization of TG-rich lipoproteins by monocytes and neutrophils [5,7,20]. The question remains whether subjects with hypertriglyceridemia or diabetes will show similar changes in postprandial leukocytes.

A left shift of neutrophils may occur with the presence of band forms and immature neutrophils during an acute bacterial infection, which can lead to an increased standard deviation in volume of neutrophils [9,15]. However, here we did not observe any postprandial changes in the standard deviation of VCS parameters, nor did the LH750 flag any of the samples for immature neutrophils. We did observe a significant increase in total leukocyte count, lymphocyte and monocyte count during the OFLT, whereas neutrophils tended to increase postprandially without reaching statistical significance. These results are in concordance with previous reports, which showed increases in leukocyte count and absolute lymphocyte and neutrophil counts during comparable experiments [2,3,21]. Recently lymphocyte counts were shown to decrease one to two hours after a light mixed meal with normalization at four hours postprandially [22]. Here we have studied four and eight hours postprandially aimed at detecting leukocyte activation postprandially. We observed an increase in lymphocyte count, especially eight hours postprandially. Therefore, we could have missed an initial decrease in lymphocyte counts during the first two to four hours of the OFLT. Comparing both studies is difficult since different meals were used (a mixed meal versus a fat load) and the addition of glucose to a fat load has been shown to reduce the postprandial increase in TG [23].

In conclusion, monocytes and neutrophils of healthy adults become activated after the ingestion of a fat-containing meal, which temporarily affects leukocyte cell popula-

tion data in a similar way as in certain infectious diseases. However, the magnitude of these changes is limited. Therefore, the postprandial changes in leukocyte cell population data probably do not affect the clinical use of leukocyte cell population data as a diagnostic tool for infection detection.

References

1. Klop B, Proctor SD, Mamo JC, Botham KM, Castro Cabezas M. Understanding postprandial inflammation and its relationship to lifestyle behaviour and metabolic diseases. Int J Vasc Med 2012;2012:947417
2. Van Oostrom AJ, Sijmonsma TP, Rabelink TJ, Van Asbeck BS, Castro Cabezas M. Postprandial leukocyte increase in healthy subjects. Metabolism 2003;52:199-202
3. van Oostrom AJ, Sijmonsma TP, Verseyden C, Jansen EH, de Koning EJ, Rabelink TJ, et al. Postprandial recruitment of neutrophils may contribute to endothelial dysfunction. J Lipid Res 2003;44:576-583
4. Bentley C, Hathaway N, Widdows J, Bejta F, De Pascale C, Avella M, et al. Influence of chylomicron remnants on human monocyte activation in vitro. Nutr Metab Cardiovasc Dis 2011;21:871-878
5. Alipour A, van Oostrom AJ, Izraeljan A, Verseyden C, Collins JM, Frayn KN, et al. Leukocyte activation by triglyceride-rich lipoproteins. Arterioscler Thromb Vasc Biol 2008;28:792-797
6. van Oostrom AJ, Rabelink TJ, Verseyden C, Sijmonsma TP, Plokker HW, De Jaegere PP, et al. Activation of leukocytes by postprandial lipemia in healthy volunteers. Atherosclerosis 2004;177:175-182
7. Gower RM, Wu H, Foster GA, Devaraj S, Jialal I, Ballantyne CM, et al. CD11c/CD18 expression is upregulated on blood monocytes during hypertriglyceridemia and enhances adhesion to vascular cell adhesion molecule-1. Arterioscler Thromb Vasc Biol 2011;31:160-166
8. Chaves F, Tierno B, Xu D. Quantitative determination of neutrophil VCS parameters by the Coulter automated hematology analyzer: New and reliable indicators for acute bacterial infection. Am J Clin Pathol 2005;124:440-444
9. Chaves F, Tierno B, Xu D. Neutrophil volume distribution width: A new automated hematologic parameter for acute infection. Arch Pathol Lab Med 2006;130:378-380
10. Mardi D, Fwity B, Lobmann R, Ambrosch A. Mean cell volume of neutrophils and monocytes compared with C-reactive protein, interleukin-6 and white blood cell count for prediction of sepsis and nonsystemic bacterial infections. Int J Lab Hematol 2010;32:410-418
11. Richardson-Jones A. An automated hematology instrument for comprehensive WBC, RBC, and platelet analysis. Am Clin Lab 1990;9:18-22
12. Krause JR. Automated differentials in the hematology laboratory. Am J Clin Pathol 1990;93:S11-16
13. Zhu Y, Cao X, Chen Y, Zhang K, Wang Y, Yuan K, et al. Neutrophil cell population data: Useful indicators for postsurgical bacterial infection. Int J Lab Hematol 2012;34:295-299
14. Briggs C, Da Costa A, Freeman L, Aucamp I, Ngubeni B, Machin SJ. Development of an automated malaria discriminant factor using VCS technology. Am J Clin Pathol 2006;126:691-698
15. Park DH, Park K, Park J, Park HH, Chae H, Lim J, et al. Screening of sepsis using leukocyte cell population data from the coulter automatic blood cell analyzer DXH800. Int J Lab Hematol 2011;33:391-399
16. Klop B, Cohn JS, van Oostrom AJ, van Wijk JP, Birnie E, Castro Cabezas M. Daytime triglyceride variability in men and women with different levels of triglyceridemia. Clin Chim Acta 2011;412:2183-2189
17. Zhu Y, Cao X, Xu D. Detection of morphologic changes in peripheral mononuclear cells in hepatitis B virus infection using the Beckman Coulter LH 750. Lab Hematol 2011;17:22-26
18. Lee SE, Lim J, Kim Y, Min WS, Han K. Leukocyte cell population analysis from the coulter automatic blood cell analyzer DXH800 to monitor the effect of G-CSF. J Clin Lab Anal 2012;26:194-199

19. Borregaard N, Kjeldsen L, Lollike K, Sengelov H. Granules and secretory vesicles of the human neutrophil. Clin Exp Immunol 1995;101 Suppl 1:6-9
20. Varela LM, Ortega A, Bermudez B, Lopez S, Pacheco YM, Villar J, et al. A high-fat meal promotes lipid-load and apolipoprotein B-48 receptor transcriptional activity in circulating monocytes. Am J Clin Nutr 2011;93:918-925
21. Hansen K, Sickelmann F, Pietrowsky R, Fehm HL, Born J. Systemic immune changes following meal intake in humans. Am J Physiol 1997;273:R548-553
22. Lippi G, Lima-Oliveira G, Salvagno GL, Montagnana M, Gelati M, Picheth G, et al. Influence of a light meal on routine haematological tests. Blood Transfus 2010;8:94-99
23. van Oostrom AJ, van Dijk H, Verseyden C, Sniderman AD, Cianflone K, Rabelink TJ, et al. Addition of glucose to an oral fat load reduces postprandial free fatty acids and prevents the postprandial increase in complement component 3. Am J Clin Nutr 2004;79:510-515

Chapter 5

Gender differences in vitamin D3 mediated effects on postprandial leukocyte activation and arterial stiffness

Boudewijn Klop
Gert-Jan M. van de Geijn
Erwin Birnie
Tjin L. Njo
Hans W. Janssen
Hermi G. Jansen
J. Wouter Jukema
Jan Willem F. Elte
Manuel Castro Cabezas

Eur J Clin Nutr 2014;68:635-7

Abstract

Background: The postprandial phase is considered pro-atherogenic, in part due to triglyceride-rich lipoprotein mediated inflammation. Vitamin D has been proposed to reduce inflammation and to improve arterial elasticity. We hypothesized that vitamin D3 could improve postprandial arterial elasticity by modulation of leukocyte activation.
Methods: Healthy volunteers underwent two oral fat loading tests (OFLT). Arterial elasticity by augmentation index (AIx) and flowcytometric quantification of leukocyte activation markers CD11b, CD66b, CD35 and CD36 were measured. After the first OFLT a dose of 100000 IU of vitamin D3 was administered and a second OFLT was carried out seven days later.
Results: Six men and 6 women were included. 25-Hydroxyvitamin D3 levels rose from 63.0 ± 28.8 nmol/l to 98.5 ± 26.8 nmol/l after vitamin D3 supplementation ($P < 0.001$). Baseline arterial elasticity did not change postprandially, but a significant favourable reduction in AIx was found after vitamin D3 supplementation ($P = 0.042$) in both men and women. After vitamin D3, exclusively in women a reduction in the area under the postprandial curve for monocyte CD11b and CD35 by 10.5% ($P = 0.016$) and 12.5% ($P = 0.04$) and neutrophil CD11b by 17.0% ($P = 0.014$) was observed.
Conclusions: Vitamin D3 probably increased postprandial arterial elasticity, but vitamin D3 reduced postprandial leukocyte activation exclusively in women.

Introduction

In the past 20 years, the diagnosis of vitamin D deficiency has increased to epidemic proportions, mainly due to obesity, lack of sun exposure and increased screening for vitamin D deficiency [1]. Vitamin D has not only been related to bone mineralization and the metabolism of calcium and phosphate, but also to numerous other physiological processes [1,2]. The vitamin D receptor has been identified on multiple cell types, including immunological and endothelial cells [3-5]. Several studies have shown that low vitamin D concentrations are associated with endothelial dysfunction, increased arterial stiffness, increased carotid intima media thickness, hypertension, the metabolic syndrome and an increased risk for cardiovascular disease [6-13]. Endothelial function and arterial elasticity can improve after vitamin D supplementation in vitamin D deficient subjects as shown in two different studies using cut-off points for vitamin D deficiency of 25 nmol/l and 75 nmol/l, respectively [14,15]. The exact mechanisms of the beneficial effects of vitamin D on cardiovascular disease and arterial stiffness are still unknown.

The immunological effects of vitamin D may be the key link between vitamin D and cardiovascular disease, since inflammation is highly involved in the development of impaired vascular function and atherosclerosis [16]. *In vitro* experiments have shown that the active metabolite of vitamin D, 1,25-dihydroxyvitamin D3, decreased the expression of CD11b on monocytes and their phagocytic capacity [17]. CD11b is an integrin expressed on monocytes and neutrophils, which improves the binding of monocytes and neutrophils to the endothelium, thereby facilitating subendothelial migration [18,19]. 1,25-Dihydroxyvitamin D3 reduces the uptake of cholesterol by macrophages with inhibition of macrophage differentiation into atherogenic foam cells [20]. Gender differences have been described concerning the activation of leukocytes mediated by sex steroids with a potential functional synergy between 1,25-dihydroxyvitamin D3 and estradiol [21,22]. Since acute inflammatory changes as well as vascular dysfunction occur in the postprandial state [18,19,23,24], the postprandial state can be considered an adequate physiological model to further investigate the relationships between vitamin D, postprandial leukocyte activation and arterial stiffness in vivo. Therefore, the aim of the present study was to investigate if vitamin D3 can reduce leukocyte activation and improve arterial elasticity postprandially in both genders.

Materials and methods

Subjects and study design

The study was designed as a non-randomized cross-over study of healthy males and females. Measurements were performed before and after the intervention i.e. supple-

mentation of vitamin D3; participants served as their own controls. Male and female volunteers were recruited by advertisement between March 2011 and October 2011. Included were healthy subjects between 18 and 50 years of age. Exclusion criterion was the use of any medication except oral contraceptives. At day one participants visited the hospital after a 10h overnight fast. Anthropometric characteristics (length, weight, body mass index, waist circumference) and blood pressure were measured. At baseline, arterial elasticity was determined by augmentation index (AIx), which was measured at the radial artery according to standard operating procedures (Sphygmocor, AtCor Medical, Sydney, Australia) in duplicate after a 5 minute rest in a supine position [25]. The AIx was corrected for a heart rate of 75/min. A fasting venous blood sample was drawn and subjects received an oral fat load using fresh cream (Albert Heijn, Zaandam, the Netherlands) in a dose of 50g of fat per square meter body surface. This method reflects the physiological diurnal increase in triglycerides. During the oral fat loading test participants were not allowed to eat or drink except water and they were asked to refrain from physical activity. Blood pressure, AIx and venous blood sampling were repeated at two hourly intervals until eight hours. At the end of the first oral fat loading test participants received 100000 IU of vitamin D3 dissolved in water (cholecalciferol, 50000 IU/ml). Since 25-hydroxyvitamin D3 reaches its highest serum concentration after 7 days [26,27], the second oral fat loading test was performed exactly seven days later. The procedure and measurements of the second oral fat loading test were identical to the first. All subjects gave written informed consent and the study was approved by the independent Regional Medical Ethical Committee Rotterdam, Maasstad Hospital, the Netherlands (NL3372110110). The study protocol was prospectively registered at EudraCT (2010-024182-44).

Laboratory measurements

All clinical chemistry and hematology measurements were carried out on freshly drawn blood at the Department of Clinical Chemistry, Sint Franciscus Gasthuis, Rotterdam. Baseline C-reactive protein, plasma cholesterol, HDL-C and triglycerides were measured using the LX20 or DxC analyzers (Beckman Coulter, Miami, USA). LDL-C values were calculated using the Friedewald formula. Apolipoprotein A-I and apo B were determined by nephelometry using an IMMAGE instrument with commercially available kits (Beckman Coulter). Blood cell counts and 5-part leucocyte differentiation were determined automatically using LH750 analyzers (Beckman Coulter). The vitamin D status was determined by measuring 25-hydroxyvitamin D3 on serum that had been stored at -70°C (Liaison, DiaSorin, Saluggia, Italy).

CD11b, CD66b, CD35 and CD36 as markers for in vivo leukocyte activation

Blood samples for the determination of leukocyte activation markers CD11b, CD66b, CD35 and CD36 were collected in tubes containing 5.4mg K2 EDTA (Becton Dickinson, Plymouth, United Kingdoms) at baseline and at four and eight hours postprandially. The method has been described in detail before [18,28]. Briefly, the staining procedure was started within 15 minutes after venipuncture. All measurements were carried out in triplicate to reduce variability. Two separate tubes were prepared: 1) a combination of fluorescein isothiocynate (FITC) conjugated CD66b, phycoerythrin (PE) conjugated CD11b and phycoerythrin-Texas Red-X (ECD) conjugated CD45 and 2) a combination of FITC conjugated CD36, PE conjugated CD35 and ECD conjugated CD45. All antibodies were from Beckman Coulter, except for CD35-PE (BD Biosciences, Franklin Lakes, NJ, USA). A total of 20 µl of whole blood was added to each tube and incubated for 15 minutes in the dark at room temperature. Erythrocytes were lysed by addition of 500µl lysis solution (1.5 M ammonium chloride, 100 mM potassium hydrogen carbonate, 0.82 mM EDTA, pH 7.4) followed by 15 min of incubation in the dark. The samples were measured on a Navios flow cytometer (Beckman Coulter). Samples were measured for a maximum of five minutes or until at least 2000 monocytes were acquired. Lymphocytes, monocytes and neutrophils were identified in the side scatter versus CD45 dot plot. The fluorescence intensity of each cell type was expressed as the mean fluorescence intensity (MFI) of the triplicate measurements. Before each use, the optics and settings of the flow cytometer were checked with Flow-Check Pro and Flow-Set Pro beads (Beckman Coulter). Identical flow cytometric settings were used for the complete study.

Statistical analysis

Data are given as mean ± SD in the text and tables and as mean ± SEM in the figures. Differences between the first oral fat load and the second were tested using the paired Student's t-test in case of normally distributed variables or by the non-parametric Wilcoxon matched pairs signed ranks test in case of skewed variables. Differences were tested for the total group and for men and women seperately. All statistical analyses were performed using PASW statistics version 18.0 (IBM SPSS Statistics, New York, United States). The total area under the postprandial curve (AUC) was calculated with PRISM version 5.0 (Graph Pad Software, San Diego). Statistical significance was set at $P < 0.05$ (two tailed).

Results

Cholesterol, apolipoproteins and postprandial lipemia after vitamin D3

A total of 6 men and 6 women were included. Oral contraceptives were used by three women. The baseline characteristics for the total group and for each gender are shown in Table 1. No significant differences were observed between genders. Seven days after the administration of 100000 IU of vitamin D3, serum 25-hydroxyvitamin D3 concentrations increased significantly from 63.1 ± 28.8 nmol/l to 98.5 ± 26.8 nmol/l for the total group ($P < 0.001$), from 49.9 ± 25.2 nmol/l to 88.2 ± 22.1 nmol/l for men ($P < 0.001$) and from 76.2 ± 27.9 nmol/l to 108.7 ± 28.9 nmol/l for women ($P = 0.001$). Only one male participant had an initial 25-hydroxyvitamin D3 serum concentration below 30.0 nmol/l in contrast

Table 1: Characteristics in the fasting state at baseline (control) and after vitamin D3 supplementation. Results are shown for the total group and for men and women separately.

	Total group (n = 12)		Men (n = 6)		Women (n = 6)	
	Control	**Vitamin D3**	**Control**	**Vitamin D3**	**Control**	**Vitamin D3**
Age (years)	35.3 ± 12.7	-	34.5 ± 12.3	-	36.2 ± 14.1	-
Body mass index (kg/m^2)	21.9 ± 2.1	-	21.1 ± 2.6	-	22.7 ± 0.9	-
Systolic blood pressure (mmHg)	110.5 ± 9.1	108.2 ± 10.6	111.3 ± 10.1	108 ± 13.8	109.7 ± 8.9	108.3 ± 7.4
Diastolic blood pressure (mmHg)	65.1 ± 8.8	65.8 ± 7.1	67.2 ± 11.3	65.3 ± 9.0	63.0 ± 5.8	66.3 ± 5.4
Augmentation Index (%)	14.8 ± 11.7	14.0 ± 12.4	7.9 ± 12.0	10.3 ± 9.2	20.1 ± 11.9	17.5 ± 15.5
25-hydroxyvitamin D3 (nmol/l)	63.1 ± 28.8	98.5 ± 26.8**	49.9 ± 25.2	88.2 ± 22.1**	76.2 ± 27.9	108.7 ± 28.9*
Leukocyte count (*10^9/l)	6.1 ± 1.1	5.8 ± 1.4	5.8 ± 1.2	5.9 ± 1.1	6.3 ± 1.0	5.8 ± 1.8
Absolute lymphocyte count (*10^9/l)	1.68 ± 0.33	1.76 ± 0.38	1.65 ± 0.40	1.82 ± 0.48	1.70 ± 0.27	1.70 ± 0.28
Absolute monocyte count (*10^9/l)	0.46 ± 0.13	0.43 ± 0.12	0.52 ± 0.15	0.45 ± 0.14	0.40 ± 0.09	0.40 ± 0.11
Absolute neutrophil count (*10^9/l)	3.83 ± 0.87	3.49 ± 1.15	3.50 ± 0.80	3.45 ± 0.87	4.15 ± 0.88	3.53 ± 1.46
C-reactive protein (mg/l)	1.42 ± 0.67	1.67 ± 1.72	1.17 ± 0.41	1.17 ± 0.41	1.67 ± 0.82	2.2 ± 2.4
Total cholesterol (mmol/l)	4.6 ± 0.6	4.6 ± 0.8	4.5 ± 0.6	4.9 ± 0.8	4.8 ± 0.7	4.3 ± 0.7
LDL-C (mmol/l)	2.7 ± 0.6	2.7 ± 0.6	2.7 ± 0.5	2.6 ± 0.5	2.7 ± 0.7	2.7 ± 0.7
HDL-C (mmol/l)	1.63 ± 0.39	1.58 ± 0.45	1.52 ± 0.32	1.65 ± 0.31	1.75 ± 0.46	1.52 ± 0.59
Triglycerides (mmol/l)	0.71 ± 0.24	0.70 ± 0.30	0.71 ± 0.22	0.73 ± 0.28	0.72 ± 0.28	0.67 ± 0.34
Apolipoprotein B (g/l)	0.78 ± 0.14	0.77 ± 0.18	0.76 ± 0.14	0.75 ± 0.17	0.79 ± 0.14	0.78 ± 0.20
Apolipoprotein A-I (g/l)	1.88 ± 0.35	1.75 ± 0.28	1.79 ± 0.34	1.86 ± 0.28	1.97 ± 0.38	1.63 ± 0.26

* Significantly different from corresponding control value ($P = 0.001$)

** Significantly different from corresponding control value ($P < 0.001$)

to none in the females. No significant changes were observed in leukocyte counts, absolute lymphocyte, monocyte and neutrophil counts, lipids or arterial stiffness in the fasting state after vitamin D3 supplementation (Table 1). The postprandial triglyceride response was similar before and seven days after supplementation of vitamin D3 for the total group (Figure 1A) and for men and women separately (Figure 1B). As expected, women tended to have lower fasting and postprandial triglycerides when compared to men, but statistical significance was not reached. The AUC for triglycerides for the total group and for men and women separately remained unchanged after vitamin D3 (Table 2).

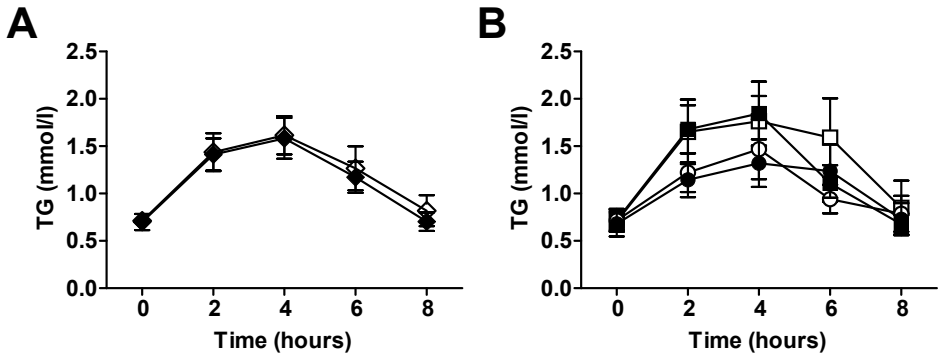

Figure 1: Mean ± SEM postprandial changes in plasma triglycerides (TG) for the total group (n = 12) before (open diamonds, ◊) and seven days after the administration of 100000 IU of vitamin D3 (closed diamonds, ♦) (A). Postprandial changes in plasma TG were separated by gender (B). Men (n = 6) before (open square, □) and after vitamin D3 administration (closed square, ■) and women (n = 6) before (open circle, ○) and after vitamin D3 administration (closed circle, ●) are shown.

Postprandial leukocyte activation after vitamin D3 supplementation

Postprandial leukocyte activation before and after supplementation of vitamin D3 is shown for men and women in Figure 2. In the fasting state, integrin expression on monocytes tended to be higher in women in contrast to the expression on neutrophils. Both in men and in women, the expression of integrins on monocytes and on neutrophils increased postprandially at T = 4h and returned to baseline at T = 8h.

In the fasting state, no changes in integrin expression for both monocytes and neutrophils in males and females were observed after vitamin D3 supplementation. After vitamin D3 supplementation the reduction of the AUC for CD11b expression on monocytes was 6.9% (P = 0.043) and on neutrophils 8.9% (P = 0.019) for the total group (Table 2). A similar trend was observed for the CD35 expression on neutrophils. In females, the AUC for CD11b expression on monocytes and neutrophils and for CD35 expression on monocytes was significantly reduced together with a similar trend for the

CD35 and CD66b expression on neutrophils and CD36 expression on monocytes, while no changes were observed in men (Table 2).

Arterial stiffness after vitamin D3 supplementation

Women tended to have a higher AIx when compared to men. The AIx did not change postprandially during the first oral fat load in contrast to the situation after supplementation of vitamin D3 showing a postprandial decrease. This pattern was observed in the

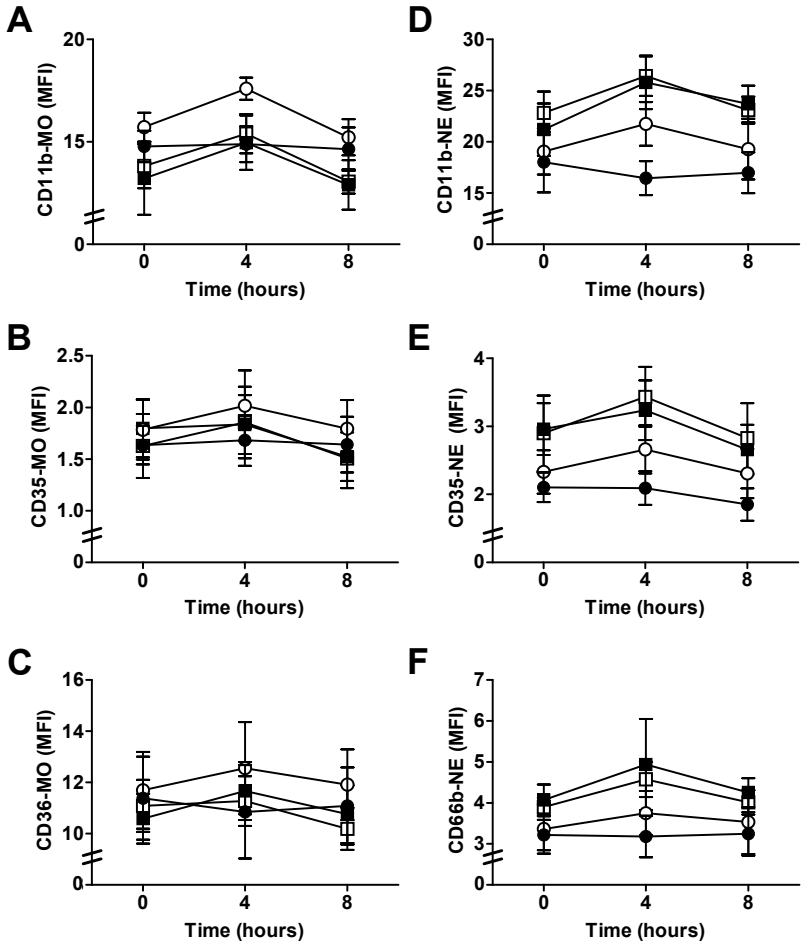

Figure 2: Mean ± SEM postprandial changes in mean fluorescence intensity (MFI) of CD11b expression on monocytes (MO) (A) and neutrophils (NE) (D), CD35 expression on monocytes (B) and neutrophils (E), CD36 expression on monocytes (E) and CD66b expression on neutrophils (F) before (open squares, □, for men and open bullets, ○, for women) and seven days after the administration of 100000 IU of vitamin D3 (closed squares, ■, for men and closed bullets, ●, for women).

Table 2: Postprandial changes in triglycerides, leukocyte activation markers and arterial stiffness expressed as the total area under the postprandial curve at baseline (Control) and seven days after the administration of 100000 IU of vitamin D3.

Area under the curve	Total group (n = 12)		P-value	Men (n = 6)		P-value	Women (n = 6)		P-value
	Control	Vitamin D3		Control	Vitamin D3		Control	Vitamin D3	
Triglycerides (mmol*h/l)	10.2±4.3	9.7±3.9	0.23	11.6±4.5	10.7±4.0	0.16	8.8±4.0	8.8±3.9	0.84
Monocyte CD11b (MFI*h)	123.8±14.2	115.2±16.3	0.043	115.4±15.2	112.0±18.9	0.60	132.3±6.2	118.4±14.2	0.016
Monocyte CD35 (MFI*h)	14.5±5.8	13.6±4.6	0.20	13.7±6.2	14.0±5.1	0.72	15.2±5.8	13.3±4.5	0.04
Monocyte CD36 (MFI*h)	92.6±24.9	88.9±25.8	0.33	87.7±18.2	89.3± 9.8	0.78	97.4±31.2	88.3±32.8	0.051
Neutrophil CD11b (MFI*h)	180.6±39.4	164.5±42.9	0.019	197.6±33.0	193.0±28.3	0.51	163.7±40.5	135.9±36.0	0.014
Neutrophil CD35 (MFI*h)	22.6±8.0	20.2±7.0	0.069	25.2±9.4	24.2±7.4	0.54	19.9±5.8	16.3±3.8	0.08
Neutrophil CD66b (MFI*h)	31.5±9.6	31.0±11.7	0.82	34.1±6.6	36.4±12.1	0.50	28.8±11.8	25.7±9.3	0.07
Augmentation index (%*h)	107.5±93.6	88.7±98.3	0.042	57.7±47.2	37.8±41.2	0.13	157.3±105.4	139.5±115.6	0.31

Abbreviations: MFI = mean fluorescent intensity; CD = cluster of differentiation

total group (Figure 3A) and in both genders separately (Figure 3B). This resulted in a significant reduction of the AUC for AIx for the total group after vitamin D3 supplementation (107.5 ± 93.6 %*h vs 88.7 ± 98.3 %*h, P = 0.042), which was observed in both genders (Table 2).

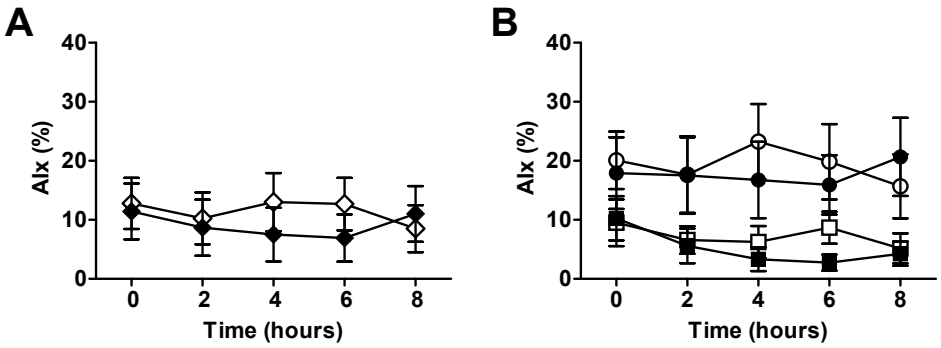

Figure 3: Mean ± SEM postprandial changes in augmentation index (AIx) for the total group (n = 12) before (open diamonds, ◊) and seven days after the administration of 100000 IU of vitamin D3 (closed diamonds, ♦) (A). Postprandial changes in AIx were separated by gender (B). Men (n = 6) before (open square, □) and after vitamin D3 administration (closed square, ■) and women (n = 6) before (open circle, ○) and after vitamin D3 supplementation (closed circle, ●) are shown.

Discussion

Multiple studies have demonstrated the association between vitamin D deficiency and a subsequent increased cardiovascular risk [6,12,13], but the exact mechanism is not clearly understood. The atheroprotective effect of vitamin D may be attributed to beneficial modulation of the immune system [29]. The present study shows that a single high dose of vitamin D3 reduced postprandial leukocyte activation only in women, whereas the improvement in postprandial arterial elasticity was observed in both men and women. Serum 25-hydroxyvitamin D3 concentrations increased substantially in all our subjects seven days after a single dose of 100000 IU of vitamin D3. However, it should be noted that higher serum 25-hydroxyvitamin D3 concentrations do not necessarily translate into increased 1.25-dihydroxyvitamin D3 serum concentrations [30]. Nevertheless, it is generally accepted that 1.25-dihydroxyvitamin D3 will be higher when 25-hydroxyvitamin D3 serum concentrations rise.

The postprandial CD11b expression on monocytes and neutrophils was significantly reduced after vitamin D3 supplementation. However, this effect was mainly observed in women. Others have shown that 1,25-dihydroxyvitamin D3 reduced the CD11b expression on monocytes with a concomitant reduction in the phagocytic capacity *in vitro* [17]. On the contrary, 1,25-dihydroxyvitamin D3 upregulates the CD11b gene in

leukocytes [31]. Since *in vitro* 1,25-dihydroxyvitamin D3 reduced the CD11b expression on monocytes despite a contradictory upregulation of the CD11b gene, vitamin D may have differential effects on leukocyte CD11b regulation.

We hypothesize that sex steroids may have influenced the vitamin D3 effects on the expression of integrins like CD11b. It has been demonstrated that estradiol reduces the CD11b expression on monocytes *in vitro* [21], whereas testosterone potentiates neutrophil activation [32]. Animal studies showed that the CD11b expression on neutrophils is also regulated by estradiol, which limits the entry of calcium into cells, thereby blunting neutrophil activation [32,33]. This may explain the observed trend to lower fasting and postprandial CD11b, CD35 and CD66b expressions on neutrophils in women compared to men in the present study.

Another explanation for the gender differences observed here may be related to an interaction between estrogen and vitamin D. Others have shown that women have fewer CYP24A1 transcripts, encoding the 1,25-dihydroxyvitamin D3-inactivating enzyme, when compared to men [22]. Therefore, binding and cellular accumulation of 1,25-dihydroxyvitamin D3 is increased in women with a subsequent increased anti-inflammatory effect on T-lymphocytes and macrophages [22]. The *ex vivo* addition of estradiol to male T-lymphocytes and macrophages reproduced these effects, suggesting a synergistic effect between 1,25-dihydroxyvitamin D3 and estradiol [22]. Therefore, vitamin D induced reductions in postprandial leukocyte activation may be dependent on estrogen.

We observed that the relative change in 25-hydroxyvitamin D3 was greater in men compared to women, but all subjects reached levels that were well above generally accepted normal concentrations. In contrast, females showed higher absolute serum 25-hydroxyvitamin D3 concentrations after supplementation, probably because of higher initial levels. The results may have been influenced by the wide variation in 25-hydroxyvitamin D3 concentrations between subjects, especially in males, which may also have contributed to the observed gender differences. *In vitro* a clear dose response relationship between a reduction in CD11b expression on monocytes with increasing concentrations of 1,25-dihydroxyvitamin D3 has been demonstrated [17]. Future studies including primarily vitamin D deficient subjects are needed to evaluate whether vitamin D3 supplementation in vitamin D deficiency is beneficial in reducing postprandial leukocyte activation.

The postprandial CD36 expression on monocytes was reduced by 9.3% in women after vitamin D3 supplementation. CD36 is a scavenger receptor facilitating the uptake of free fatty acids and oxidized LDL by macrophages with subsequent risk of foam cell formation. *In vitro*, macrophages showed less cholesterol uptake and foam cell formation, with suppression of the CD36 expression when 1,25-dihydroxyvitamin D3 was present in the culture medium [20,34]. Recently, comparable results were obtained in a vitamin D-deficient mouse model [35]. However, it should be noted that this mouse

model included both male and female animals. Therefore, our results showing a blunted postprandial response in leukocyte activation by vitamin D3 in females are partly in line with previous publications.

We did not observe any effect of vitamin D3 on postprandial lipemia and these results are comparable to previous reports [36,37]. Therefore, the observed effects on leukocyte activation and arterial stiffness we found could not be attributed to postprandial lipid changes. Although it should be noted that triglycerides reflect total postprandial response of both very low density lipoproteins and chylomicrons and that we did not differentiate between them using specific antibodies against apo B-100 and apo B-48.

Low 25-hydroxyvitamin D3 levels have been associated with increased arterial stiffness [38] and vitamin D supplementation improved endothelial function, which was determined with flow mediated vasodilatation, in asymptomatic subjects [14] and in patients with type 2 diabetes mellitus [39]. In addition, vitamin D3 supplementation can reduce arterial stiffness during childhood (14-18 years of age) [15]. All these interventional studies were performed in both genders and the beneficial effects of vitamin D3 supplementation on arterial elasticity were observed in both [14,15,39]. In addition, the association between 25-hydroxyvitamin D3 and arterial stiffness was independent from gender status [38]. Our data extend those observations showing similar effects in both genders. Since we observed a reduction in postprandial leukocyte activation solely in women, the improved postprandial arterial elasticity after vitamin D3 in both genders can not be explained by the reduction in postprandial leukocyte activation after vitamin D3 supplementation. Therefore, a direct effect of vitamin D on the arterial wall may have played a role. *In vitro* 1,25-dihydroxyvitamin D3 inhibited vascular cellular adhesion molecule-1 and interleukin-8 production in human arterial endothelial cells [40]. However, a placebo controlled randomized trial was unable to show any changes in circulating vascular cell adhesion molecules after vitamin D2 supplementation [41].

In our study women tended to have a higher AIx, which is in line with a recent community-based cohort study with 983 participants showing that women have a higher degree of arterial stiffness and concomitant AIx compared to men [42]. We did not observe any changes in AIx postprandially during the first oral fat load, whereas the AIx was temporarily reduced in the postprandial state after vitamin D3 supplementation. A postprandial improvement in arterial elasticity has been observed by others during the first 120 minutes after the ingestion of a mixed meal [43]. These authors suggested that postprandial changes in autonomic function could be responsible for temporary postprandial improvements in arterial elasticity. We did not measure parameters like heart rate variability to investigate changes in autonomic function in the postprandial state or after vitamin D3 supplementation. It has been reported that vitamin D3 may alter the autonomic nervous system [44]. *In vitro* 1,25-dihydroxyvitamin D3 stimulates myocytes

in a similar way as the beta-adrenergic agonist isoproterenol [45]. Moreover, vitamin D3 maintained the expression of cholinergic receptors in rats with diabetes mellitus [46].

In conclusion, a single dose of 100000 IU of vitamin D3 increased postprandial arterial elasticity in healthy men and women probably by a direct effect on the arterial wall. Vitamin D3 reduced postprandial leukocyte activation exclusively in women and therefore, could not fully explain the increased postprandial arterial elasticity after vitamin D supplementation.

References

1. Holick MF. Vitamin D deficiency. N Engl J Med 2007;357:266-281
2. Bouillon R, Carmeliet G, Verlinden L, van Etten E, Verstuyf A, Luderer HF, et al. Vitamin D and human health: Lessons from vitamin D receptor null mice. Endocr Rev 2008;29:726-776
3. Bell DS. Protean manifestations of vitamin D deficiency, part 3: Association with cardiovascular disease and disorders of the central and peripheral nervous systems. South Med J 2011;104:340-344
4. Bell DS. Protean manifestations of vitamin D deficiency, part 1: The epidemic of deficiency. South Med J 2011;104:331-334
5. Lavie CJ, Lee JH, Milani RV. Vitamin D and cardiovascular disease will it live up to its hype? J Am Coll Cardiol 2011;58:1547-1556
6. Hosseinpanah F, Yarjanli M, Sheikholeslami F, Heibatollahi M, Eskandary PS, Azizi F. Associations between vitamin D and cardiovascular outcomes; Tehran Lipid and Glucose Study. Atherosclerosis 2011;218:238-242
7. Carrelli AL, Walker MD, Lowe H, McMahon DJ, Rundek T, Sacco RL, et al. Vitamin D deficiency is associated with subclinical carotid atherosclerosis: The Northern Manhattan Study. Stroke 2011;42:2240-2245
8. Pacifico L, Anania C, Osborn JF, Ferraro F, Bonci E, Olivero E, et al. Low 25(OH)D3 levels are associated with total adiposity, metabolic syndrome, and hypertension in caucasian children and adolescents. Eur J Endocrinol 2011;165:603-611
9. Zagura M, Serg M, Kampus P, Zilmer M, Eha J, Unt E, et al. Aortic stiffness and vitamin D are independent markers of aortic calcification in patients with peripheral arterial disease and in healthy subjects. Eur J Vasc Endovasc Surg 2011;42:689-695
10. Al Mheid I, Patel R, Murrow J, Morris A, Rahman A, Fike L, et al. Vitamin D status is associated with arterial stiffness and vascular dysfunction in healthy humans. J Am Coll Cardiol 2011;58:186-192
11. Lee JI, Oh SJ, Ha WC, Kwon HS, Sohn TS, Son HS, et al. Serum 25-hydroxyvitamin D concentration and arterial stiffness among type 2 diabetes. Diabetes Res Clin Pract 2011
12. Schottker B, Haug U, Schomburg L, Kohrle J, Perna L, Muller H, et al. Strong associations of 25-hydroxyvitamin D concentrations with all-cause, cardiovascular, cancer, and respiratory disease mortality in a large cohort study. Am J Clin Nutr 2013;97:782-793
13. Wang L, Song Y, Manson JE, Pilz S, Marz W, Michaelsson K, et al. Circulating 25-hydroxy-vitamin D and risk of cardiovascular disease: A meta-analysis of prospective studies. Circ Cardiovasc Qual Outcomes 2012;5:819-829
14. Tarcin O, Yavuz DG, Ozben B, Telli A, Ogunc AV, Yuksel M, et al. Effect of vitamin D deficiency and replacement on endothelial function in asymptomatic subjects. J Clin Endocrinol Metab 2009;94:4023-4030
15. Dong Y, Stallmann-Jorgensen IS, Pollock NK, Harris RA, Keeton D, Huang Y, et al. A 16-week randomized clinical trial of 2000 international units daily vitamin D3 supplementation in black youth: 25-hydroxyvitamin D, adiposity, and arterial stiffness. J Clin Endocrinol Metab 2010;95:4584-4591
16. Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med 1999;340:115-126
17. Spittler A, Willheim M, Leutmezer F, Ohler R, Krugluger W, Reissner C, et al. Effects of 1 alpha,25-dihydroxyvitamin D3 and cytokines on the expression of MHC antigens, complement receptors and other antigens on human blood monocytes and U937 cells: Role in cell differentiation, activation and phagocytosis. Immunology 1997;90:286-293

18. Alipour A, van Oostrom AJ, Izraeljan A, Verseyden C, Collins JM, Frayn KN, et al. Leukocyte activation by triglyceride-rich lipoproteins. Arterioscler Thromb Vasc Biol 2008;28:792-797
19. Gower RM, Wu H, Foster GA, Devaraj S, Jialal I, Ballantyne CM, et al. CD11c/CD18 expression is upregulated on blood monocytes during hypertriglyceridemia and enhances adhesion to vascular cell adhesion molecule-1. Arterioscler Thromb Vasc Biol 2011;31:160-166
20. Oh J, Weng S, Felton SK, Bhandare S, Riek A, Butler B, et al. 1,25(OH)2 vitamin D inhibits foam cell formation and suppresses macrophage cholesterol uptake in patients with type 2 diabetes mellitus. Circulation 2009;120:687-698
21. Cutini PH, Campelo AE, Agriello E, Sandoval MJ, Rauschemberger MB, Massheimer VL. The role of sex steroids on cellular events involved in vascular disease. J Steroid Biochem Mol Biol 2012;132:322-330
22. Correale J, Ysrraelit MC, Gaitan MI. Gender differences in 1,25 dihydroxyvitamin D3 immunomodulatory effects in multiple sclerosis patients and healthy subjects. J Immunol 2010;185:4948-4958
23. Klop B, Castro Cabezas M. Chylomicrons: A key biomarker and risk factor for cardiovascular disease and for the understanding of obesity. Curr Cardiovasc Risk Rep 2012;6:27-34
24. van Oostrom AJ, Sijmonsma TP, Verseyden C, Jansen EH, de Koning EJ, Rabelink TJ, et al. Postprandial recruitment of neutrophils may contribute to endothelial dysfunction. J Lipid Res 2003;44:576-583
25. Berry SE, Tucker S, Banerji R, Jiang B, Chowienczyk PJ, Charles SM, et al. Impaired postprandial endothelial function depends on the type of fat consumed by healthy men. J Nutr 2008;138:1910-1914
26. Heaney RP, Armas LA, Shary JR, Bell NH, Binkley N, Hollis BW. 25-hydroxylation of vitamin D3: Relation to circulating vitamin D3 under various input conditions. Am J Clin Nutr 2008;87:1738-1742
27. Ilahi M, Armas LA, Heaney RP. Pharmacokinetics of a single, large dose of cholecalciferol. Am J Clin Nutr 2008;87:688-691
28. van Oostrom AJ, Rabelink TJ, Verseyden C, Sijmonsma TP, Plokker HW, De Jaegere PP, et al. Activation of leukocytes by postprandial lipemia in healthy volunteers. Atherosclerosis 2004;177:175-182
29. Takeda M, Yamashita T, Sasaki N, Nakajima K, Kita T, Shinohara M, et al. Oral administration of an active form of vitamin D3 (calcitriol) decreases atherosclerosis in mice by inducing regulatory T cells and immature dendritic cells with tolerogenic functions. Arterioscler Thromb Vasc Biol 2010;30:2495-2503
30. Biancuzzo RM, Clarke N, Reitz RE, Travison TG, Holick MF. Serum concentrations of 1,25-dihydroxyvitamin D2 and 1,25-dihydroxyvitamin D3 in response to vitamin D2 and vitamin D3 supplementation. J Clin Endocrinol Metab 2013;98:973-979
31. Moeenrezakhanlou A, Shephard L, Lam L, Reiner NE. Myeloid cell differentiation in response to calcitriol for expression CD11b and CD14 is regulated by myeloid zinc finger-1 protein downstream of phosphatidylinositol 3-kinase. J Leukoc Biol 2008;84:519-528
32. Deitch EA, Ananthakrishnan P, Cohen DB, Xu da Z, Feketeova E, Hauser CJ. Neutrophil activation is modulated by sex hormones after trauma-hemorrhagic shock and burn injuries. Am J Physiol Heart Circ Physiol 2006;291:H1456-1465
33. Faas MM, Moes H, van der Schaaf G, Heineman MJ, de Vos P. Monocyte activation, but not granulocyte activation, is inhibited in the presence of developing ovarian follicles. J Reprod Immunol 2006;70:21-32

Chapter 6

Erythrocyte-associated apolipoprotein B and its relationship with clinical and subclinical atherosclerosis

Sarah A. Bovenberg
Boudewijn Klop
Arash Alipour
Sergio Martinez-Hervas
Andrew Westzaan
Gert-Jan M. van de Geijn
Hans W.J. Janssen
Tjin L. Njo
Erwin Birnie
Rob van Mechelen
Arie P. Rietveld
Jan Willem F. Elte
Manuel Castro Cabezas

Eur J Clin Invest 2012;42:365-70

Abstract

Objectives: Apolipoprotein (apo) B containing lipoproteins are closely linked to atherogenesis. These lipoproteins are transported in plasma and are also associated with blood leukocytes. Our aim was to investigate whether apoB-containing lipoproteins are also present on the surface of erythrocytes and investigate the relationship with the presence of atherosclerosis.

Methods and results: Erythrocyte-bound apo B (ery-apoB) was measured by flowcytometry in subjects with (CAD+) and without coronary artery disease (CAD-), based on coronary angiography or on a history of cardiovascular disease. Intima media thickness (IMT) measurements were carried out using B-mode ultrasound. The relationship between ery-apoB and clinical and subclinical atherosclerosis was evaluated with binary logistic regression. A total of 166 subjects were included (40 CAD+ and 126 CAD-). Apo B was detected on freshly isolated erythrocytes (range: 0.1 to 5.5 a.u.; mean ± SEM 0.86 ± 0.09 a.u.) in all but nine subjects (4 CAD+ and 5 CAD-). Ery-apoB was lower in CAD+ (0.62 ± 0.09 a.u.) compared to CAD- (1.18 ± 0.10 a.u.; $P < 0.001$). Higher ery-apoB was associated with a lower risk of CAD (adjusted OR: 0.003 (95% CI: 0.001 - 0.08; $P < 0.001$), but the protective effect was diminished with increasing age (adjusted OR: 1.10 (95% CI: 1.04 - 1.16; $P < 0.001$). IMT was increased in CAD+ subjects (0.77 ± 0.13 mm) compared to CAD- (0.57 ± 0.14 mm; $P < 0.001$). A significant negative correlation was found between ery-apoB and IMT (Beta=-0.214: 95%CI -0.284 to -0.145; $P<0.001$). There was no association between ery-apoB with plasma apo B (Pearson's r: -0.45; $P = 0.57$).

Conclusions: Human erythrocytes carry apo B-containing lipoproteins. Subjects with atherosclerosis have lower ery-apoB. High ery-apoB may be protective against atherosclerosis, and may reflect an alternative blood cell-mediated lipoprotein transport system in the circulation, in which these lipoproteins less likely interact with the endothelium.

Introduction

All atherogenic lipoproteins carry apolipoprotein (apo) B as structural apolipoprotein [1,2,3]. Increased concentrations of plasma apo B have been identified as strong predictors of cardiovascular disease [4-8].

The classical concept of lipoprotein transport in the circulation suggests that lipoproteins in plasma are modified by a series of enzymatic reactions [1,2]. Earlier studies from our group suggested the presence of a marginated pool of apo B-containing lipoproteins possibly attached to the endothelium or to other cells [9]. More recently, it has been demonstrated that apo B-containing lipoproteins can bind to blood leukocytes and that dietary fatty acids may be taken up by these cells in the circulation [10,11]. Others have provided evidence suggesting that human erythrocytes also bind LDL [12,13]. The clinical and pathophysiological consequences of these observations are have not been established yet.

In this study we measured apo B attached to human erythrocytes (ery-apoB) and evaluated the relationship with clinical and subclinical atherosclerosis.

Materials and methods

Subjects

Participants were recruited from the outpatient clinic of the Diabetes Vascular Center from the Department of Internal Medicine, and from the outpatient clinic of the Department of Cardiology of the Sint Franciscus Gasthuis, Rotterdam, the Netherlands. Subjects were divided into patients with and without atherosclerosis (CAD+ and CAD-, respectively). CAD+ patients were defined as patients with CAD established by coronary angiography or with a history of cardiovascular disease, as described in the International Classification of Disease (ICD-10, codes 100 - 199) [14]. CAD- patients were subjects with angiographically established absence of coronary atherosclerosis or without clinical signs and without a history of clinical atherosclerosis. Exclusion criteria were aberrations in liver and thyroid function and the use of any experimental medication within 6 months before participation. Written informed consent was obtained from each subject. Anthropometric characteristics e.g., weight, length, BMI, waist circumference and blood pressure measurements were recorded. The study was approved by the independent Regional Medical Ethical Committee Rotterdam, Maasstad Hospital, the Netherlands.

Intima media thickness (IMT) of the carotid arteries

Carotid ultrasound scans were carried out using the ART-LAB (Esaote, Italy) by trained and experienced sonographers, who were unaware of the patient's medical history.

Ultrasound scans were performed with the patients 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.

Analytical Methods

All clinical chemistry measurements were performed on freshly drawn blood samples. Baseline renal function, glucose, plasma cholesterol, HDL-C and TG were measured using the LX 20 analyzer (Beckman Coulter, Miami, FL, USA). LDL-C values were calculated using the Friedewald formula. Apo A-I and apo B were determined by nephelometry using an IMMAGE instrument with commercially available kits (Beckman Coulter). Blood cell counts and 5-part leukocyte differentiation were determined automatically using LH 750 analyzers (Beckman Coulter). Thyroid function (TSH) was determined by TSH measurement (Immulite 2500, Siemens, Healthcare Diagnostics, Deerfield, IL, USA).

Apo B on erythrocytes

Blood samples for the determination of binding of apo B on erythrocytes were obtained in tubes containing 5.4 mg K2 EDTA (Becton Dickinson, Plymouth, UK). The staining procedure for apoB was started within one hour after venipuncture. The method has been described in detail previously [10]. To avoid interference of serum lipoproteins the samples were washed three times in PBS supplemented with 0.5% bovine serum albumin (PBS-BSA). The erythrocytes were incubated with a polyclonal goat antibody directed against human apo B (Millipore, Billerica, MA, USA) for 30 minutes in the dark on ice. Subsequently, the erythrocytes were washed with PBS-BSA and incubated with a rabbit anti-goat (RAG) antibody conjugated with FITC (Nordic Immunological Laboratories, Tilburg, the Netherlands) for another 30 minutes in the dark on ice. As a control for background staining each sample was simultaneously stained in parallel without anti-apoB antibodies, but with RAG-FITC. Samples were kept in the dark on ice until measurement. A total of 5000 erythrocytes per sample were analyzed by flowcytometry using an Epics XL-flowcytometer (Beckman Coulter).

Statistical analysis

The binding of apo B on erythrocytes was expressed as the mean difference between fluorescence intensity of apo B-staining and aspecific staining. For statistical analysis IMT was defined as the mean of the six individual measurements. Differences in baseline

characteristics between the CAD+ and CAD- groups were tested by Chi-square test (or Fisher's Exact test, where indicated) for nominal variables, by Student's t-test for continuous variables which were approximately Gaussian distributed, and by Mann-Whitney-U test for skewed variables. Correlation matrix of CAD+ and CAD- was carried out using Pearson Correlation.

The association between ery-apoB and the presence of CAD adjusted for other covariables was evaluated with multiple binary logistic regression (backwards stepwise analysis). The following variables were entered into the model: gender, age, waist, apo A-I, apo B and ery-apoB. For methodological reasons, age was entered into the model as an interaction effect but not as main effect. The effect of age in the multiple binary logistic regression model is presented graphically for fixed values of gender, waist, apo AI and apo B. The association between ery-apoB and IMT as an estimate of subclinical atherosclerosis adjusted for other covariables was evaluated with multiple linear regression, entering the same variables as described earlier.

SPSS v16.0 was used for all analyses. P values < 0.05 (2-tailed) were considered statistically significant.

Results

Baseline characteristics

The study group consisted of 166 subjects, 40 CAD+ and 126 CAD- subjects. The CAD+ subjects were on average older than the CAD- subjects. BMI, diastolic blood pressure and plasma triglycerides were similar between the groups. CAD+ subjects had a significantly higher waist circumference, systolic blood pressure, glucose level and reported more frequently the use of lipid lowering drugs and other medication. IMT was increased in CAD+ subjects compared to CAD- subjects ($P < 0.001$).

Erythrocyte-bound apo B and clinical and subclinical atherosclerosis

In all but nine subjects (4 CAD+ and 5 CAD-; 7.5%) ApoB was detected on freshly isolated erythrocytes (mean ± SEM: 0.86 ± 0.09 a.u.; range: 0.1 to 5.5 au). CAD+ subjects showed a lower mean erythrocyte bound apo B (0.60±0.09 au) compared to CAD- subjects (1.12 ± 0.12 a.u.; $P < 0.001$) (Figure 1).

Table 2 shows the associations between ery-apoB, CAD and IMT. Higher ery-apoB was associated with a lower risk of CAD (adjusted OR: 0.003, 95% CI 0.001 - 0.08), but the protective effect was diminished with increasing age (adjusted OR: 1.10, 95% CI 1.04 – 1.16). Figure 2 illustrates our model of multiple binary logistic regression from Table 2 showing the decrease in protective effect of ery-apoB on CAD with increasing age. Male gender was associated with a higher risk of CAD. We found no significant in-

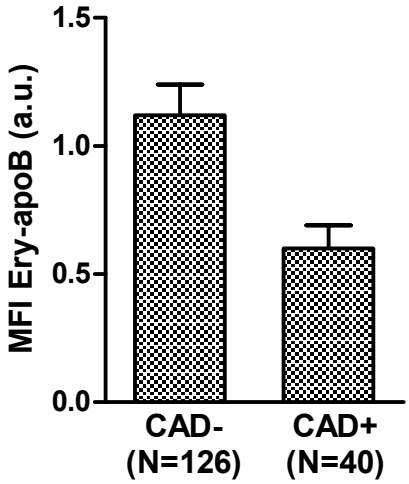

Figure 1: Mean ± SEM expression of apolipoprotein B on erythrocytes in with and without coronary artery disease (CAD+ and CAD-, respectively). Expression of ery-apoB is shown as mean fluorescence intensity (MFI) in arbitrary units (a.u.).

Table 1: Characteristics of study subjects with and without clinical atherosclerosis (CAD).

	CAD- (n = 126)	CAD+ (n = 40)	P
Age (years)	51 (22-81)	73 (35-85)	<0.001
Diabetes mellitus (n, %)	6 (4.8%)	13 (32.5%)	0.002
Male gender (n, %)	37 (44.0%)	28 (71.8%)	<0.001
Waist (cm)	90 ±12	99 ±12	<0.001
Body mass index (kg/m2)	26 ±4.4	27 ±4.2	0.1
Intima media thickness (mm)	0.57±0.13	0.77±0.13	<0.001
Systolic blood pressure (mmHg)	125 ±13	136 ±16	<0.001
Diastolic blood pressure (mmHg)	77 ±9	77 ±11	0.92
Glucose (mmol/l)	5.5 ±1.3	6.6 ±2.0	<0.001
Triglycerides (mmol/l) (median, range)	1.19 (0.34-3.89)	1.36 (0.54-4.66)	0.12
Cholesterol (mmol/l)	5.5 ±1.2	4.4 ±0.7	<0.001
LDL-cholesterol (mmol/l)	3.3 ±1.0	2.5 ±0.7	<0.001
HDL-cholesterol (mmol/l)	1.50 ±0.4	1.20 ±0.3	<0.001
Apolipoprotein B (g/l)	0.99 ±0.3	0.87 ±0.2	0.005
Apolipoprotein AI (g/l)	1.61 ±0.3	1.38 ±0.2	<0.001
No. of medications (median, range)	0 (0-6)	4 (1-7)	<0.001

Data are expressed as mean ± SD unless stated otherwise.

teraction effect between gender and age, nor between gender and ery-apoB. Ery-apoB was negatively associated with IMT (beta: -0.215, adjusted R^2 = 0.249; P < 0.001). The interaction effect between age and ery-apoB for IMT was significant and positive (beta: 0.004), suggesting that the negative association between ery-apoB and IMT was also partially diminished with age. Ery-apoB was not associated with plasma apo B (Pearson's r = -0.45; P = 0.57).

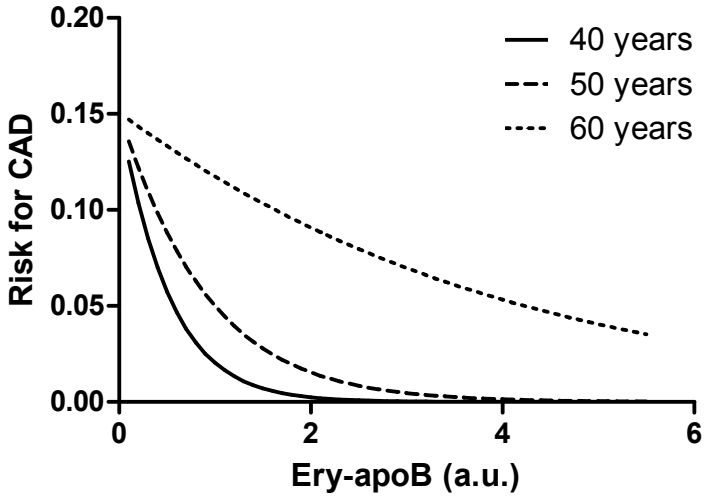

Figure 2: The relationship between ery-apoB and the risk for coronary artery disease (CAD) by age, based on the regression results from Table 2, for females with an apo AI level of 1.55 mmol/l, apo B of 0.96 g/l and waist of 95.7 cm. Expression of ery-apoB is shown as mean fluorescence intensity in arbitrary units (a.u.).

Table 2: Impact of ery-apoB on the presence of clinical atherosclerosis (CAD) and subclinical atherosclerosis (IMT). The impact of ery-apoB on CAD was evaluated by multiple logistic regression analysis (backward stepwise analysis). The impact of ery-apoB on IMT was evaluated with multiple linear regression analysis (backward stepwise analysis).

Parameter	Presence of CAD		IMT *	
	Adjusted OR (95% CI)	P-value	Beta (95% CI)	P-value
Constant	37.37	0.21	0.377 (0.213; 0.541)	0.083
Female gender	0.24 (0.09; 0.67)	0.006	—	—
Waist circumference	1.031 (0.99; 1.072)	0.13	0.003 (0.001; 0.004)	0.001
Apo A-I	0.05 (0.004; 0.57)	0.016	—	—
Apo B	0.10 (0.012; 0.83)	0.032	—	—
Ery-apoB	0.003 (0.001; 0.08)	0.001	-0.214 (-0.284; -0.145)	0.035
Age x ery-apoB	1.10 (1.04; 1.17)	0.001	0.004 (0.003; 0.005)	0.001

* R^2a = 0.2

Discussion

This is the first study showing the binding of apo B on human erythrocytes and its relation to clinical and subclinical atherosclerosis. Flowcytometric measurement of apoB on erythrocytes is technically feasible and can be used as a tool in mechanistic and clinical studies of cellular transport of lipoproteins. In 7.5% of all subjects no signal of ery-apoB was found, but this occurred equally between CAD- and CAD+ subjects. Ery-apoB did not correlate with plasma apo B concentrations and therefore, ery-apoB does not seem to be a mere reflection of plasma apo B concentrations. These results support the concept of different pools of apo B containing lipoproteins in the circulation and different pathways for atherogenic lipoprotein transport in humans [9], as "free" lipoproteins in the plasma compartment and bound to blood cells. So far, we have not been able to quantify the contribution of the cell-bound fraction and this is one of the limitations of the present study. For this purpose, other types of experiments should be designed, most likely using labelled lipoproteins.

As expected, CAD+ subjects in our study were on average older and age was also positively associated with higher IMT. The fact that ery-apoB interacted with age, suggests that binding of apoB-containing lipoproteins to erythrocytes may be a physiological process that becomes less efficient with increasing age (see Table 2, Figure 2), similar to the decreasing hepatic LDL-receptor effect on LDL metabolism with increasing age. No difference in ery-apoB was found when comparing males and females, although apo B metabolism is different between genders resulting in lower apoB plasma concentrations in women [15,16].

Previous studies demonstrated that LDL can be attached to erythrocytes [12], but the clinical relevance of this observation was not investigated. In theory, LDL, VLDL, chylomicrons and their remnants could be bound to erythrocytes. We did not investigate the nature of the apo B-containing lipoproteins attached to the erythrocytes and at this stage we have no data concerning the specific type of lipoproteins detected on the erythrocyte surface, except that erythrocytes carry apo B. We do not know if the decrease in quantity of ery-apoB and the protective effect on CAD with increasing age can be linked to a change in different types of apo B-containing lipoproteins bound to erythrocytes. The general consensus is that all apo B-containing lipoproteins are atherogenic, although we agree that there may be functional differences between lipoproteins.

Earlier studies suggested that the LDL-receptor is not involved in binding of LDL to erythrocytes [13]. Recently we proposed a mechanism involving the complement receptor on human erythrocytes and complement activation on the surface of apo B containing lipoproteins [17]. In our view, the binding of apo B-containing lipoproteins to erythrocytes may have some similarities to the binding of immune complexes ("immune adherence") to erythrocytes and clearance from the circulation by the liver [18,19,20].

Therefore, higher concentrations of apo B-containing lipoproteins on erythrocytes will result in less (free) apo B-containing particles migrating to the subendothelium which can stimulate foam cell formation. This theory remains speculative and ongoing studies in our laboratory will address this issue. The current paper, however, provides further support to explore this phenomenon by showing a close association with clinical and subclinical atherosclerosis.

One of the mechanisms by which binding of apo B-containing lipoproteins to erythrocytes could protect the endothelium is by impeding the interaction between these atherogenic lipoproteins and the endothelial cells. If this mechanism is correct and ery-apoB appears to be a stable parameter and able to predict cardiovascular risk prospectively in relation to classical and recently described risk factors, ery-apoB could become a novel cardiovascular biomarker [21]. The power of this potential risk identifier should be weighed against other atherosclerotic risk factors, like plasma apo A-I, apo B, total cholesterol/HDL and the combination of biomarkers, like N-terminal pro-brain natriuretic peptide, troponin I, cystatin C and C-reactive protein [22,23,24]. For this purpose large prospective cohort studies will be necessary.

In conclusion, high apo B bound to erythrocytes in the circulation may be protective against atherosclerosis, and may reflect an alternative blood cell-mediated lipoprotein transport system in humans, which reduces interaction of these lipoproteins with the endothelium.

References

1. Stalenhoef AF, Malloy MJ, Kane JP, Havel RJ. Metabolism of apolipoproteins B-48 and B-100 of triglyceride-rich lipoproteins in normal and lipoprotein lipase-deficient humans. Proc Natl Acad Sci U S A 1984;81:1839-1843
2. Young SG. Recent progress in understanding apolipoprotein B. Circulation 1990;82:1574-1594
3. Elovson J, Chatterton JE, Bell GT, Schumaker VN, Reuben MA, Puppione DL, et al. Plasma very low density lipoproteins contain a single molecule of apolipoprotein B. J Lipid Res 1988;29:1461-1473
4. Walldius G, Jungner I, Holme I, Aastveit AH, Kolar W, Steiner E. High apolipoprotein B, low apolipoprotein A-I, and improvement in the prediction of fatal myocardial infarction (AMORIS Study): A prospective study. Lancet 2001;358:2026-2033
5. Walldius G, Jungner I. The apoB/apoA-I ratio: A strong, new risk factor for cardiovascular disease and a target for lipid-lowering therapy--a review of the evidence. J Intern Med 2006;259:493-519
6. Sniderman AD, Furberg CD, Keech A, Roeters van Lennep JE, Frohlich J, Jungner I, et al. Apolipoproteins versus lipids as indices of coronary risk and as targets for statin treatment. Lancet 2003;361:777-780
7. Lamarche B, Moorjani S, Lupien PJ, Cantin B, Bernard PM, Dagenais GR, et al. Apolipoprotein A-I and B levels and the risk of ischemic heart disease during a five-year follow-up of men in the Quebec Cardiovascular Study. Circulation 1996;94:273-278
8. Barter PJ, Ballantyne CM, Carmena R, Castro Cabezas M, Chapman MJ, Couture P, et al. Apo B versus cholesterol in estimating cardiovascular risk and in guiding therapy: Report of the Thirty-Person/Ten-Country Panel. J Intern Med 2006;259:247-258
9. Verseyden C, Meijssen S, Castro Cabezas M. Effects of atorvastatin on fasting plasma and marginated apolipoproteins B48 and B100 in large, triglyceride-rich lipoproteins in familial combined hyperlipidemia. J Clin Endocrinol Metab 2004;89:5021-5029
10. Alipour A, van Oostrom AJ, Izraeljan A, Verseyden C, Collins JM, Frayn KN, et al. Leukocyte activation by triglyceride-rich lipoproteins. Arterioscler Thromb Vasc Biol 2008;28:792-797
11. Gower RM, Wu H, Foster GA, Devaraj S, Jialal I, Ballantyne CM, et al. CD11c/CD18 expression is upregulated on blood monocytes during hypertriglyceridemia and enhances adhesion to vascular cell adhesion molecule-1. Arterioscler Thromb Vasc Biol 2011;31:160-166
12. Hui DY, Noel JG, Harmony JA. Binding of plasma low density lipoproteins to erythrocytes. Biochim Biophys Acta 1981;664:513-526
13. Hui DY, Harmony JA. Interaction of plasma lipoproteins with erythrocytes. I. Alteration of erythrocyte morphology. Biochim Biophys Acta 1979;550:407-424
14. WHO. ICD10 2nd edition, international statistical classification of disease and health related problems. Geneva: World Health Organization 2005
15. Matthan NR, Jalbert SM, Barrett PH, Dolnikowski GG, Schaefer EJ, Lichtenstein AH. Gender-specific differences in the kinetics of nonfasting TRL, IDL, and LDL apolipoprotein B-100 in men and premenopausal women. Arterioscler Thromb Vasc Biol 2008;28:1838-1843
16. Gardner CD, Tribble DL, Young DR, Ahn D, Fortmann SP. Population frequency distributions of HDL, HDL(2), and HDL(3) cholesterol and apolipoproteins A-I and B in healthy men and women and associations with age, gender, hormonal status, and sex hormone use: The Stanford Five City Project. Prev Med 2000;31:335-345
17. Bovenberg SA, Alipour A, Elte JW, Rietveld AP, Janssen JW, van de Geijn GJ, et al. Cell-mediated lipoprotein transport: A novel anti-atherogenic concept. Atheroscler Suppl 2010;11:25-29

18. Pascual M, Schifferli JA. Another function of erythrocytes: Transport of circulating immune complexes. Infusionstherapie und Transfusionsmedizin 1995;22:310-315
19. Cornacoff JB, Hebert LA, Smead WL, VanAman ME, Birmingham DJ, Waxman FJ. Primate erythrocyte-immune complex-clearing mechanism. J Clin Invest 1983;71:236-247
20. Nardin A, Lindorfer MA, Taylor RP. How are immune complexes bound to the primate erythrocyte complement receptor transferred to acceptor phagocytic cells? Mol Immunol 1999;36:827-835
21. Eagle KA, Ginsburg GS, Musunuru K, Aird WC, Balaban RS, Bennett SK, et al. Identifying patients at high risk of a cardiovascular event in the near future: Current status and future directions: Report of a national heart, lung, and blood institute working group. Circulation 2010;121:1447-1454
22. Ray KK, Cannon CP, Cairns R, Morrow DA, Ridker PM, Braunwald E. Prognostic utility of apoB/AI, total cholesterol/HDL, non-HDL cholesterol, or hs-CRP as predictors of clinical risk in patients receiving statin therapy after acute coronary syndromes: Results from PROVE-IT-TIMI 22. Arterioscler Thromb Vasc Biol 2009;29:424-430
23. Schmidt C, Wikstrand J. High apoB/apoA-I ratio is associated with increased progression rate of carotid artery intima-media thickness in clinically healthy 58-year-old men: Experiences from very long-term follow-up in the Air Study. Atherosclerosis 2009;205:284-289
24. Zethelius B, Berglund L, Sundstrom J, Ingelsson E, Basu S, Larsson A, et al. Use of multiple biomarkers to improve the prediction of death from cardiovascular causes. N Engl J Med 2008;358:2107-2116

Chapter 7

Erythrocyte-bound apolipoprotein B in relation to atherosclerosis, serum lipids and ABO blood group

Boudewijn Klop
Gert-Jan M. van de Geijn
Sarah A. Bovenberg
Noëlle van der Meulen
Jan Willem F. Elte
Erwin Birnie
Tjin L. Njo
Hans J.W. Janssen
Addy van Miltenburg
J. Wouter Jukema
Manuel Castro Cabezas

PLoS One 2013;8:e75573

Abstract

Introduction: Erythrocytes carry apolipoprotein B on their membrane, but the determining factors of erythrocyte-bound apolipoprotein B (ery-apoB) are unknown. We aimed to explore the determinants of ery-apoB to gain more insight into potential mechanisms.

Methods: Subjects with and without CVD were included (n = 398). Ery-apoB was measured on fresh whole blood samples using flow cytometry. Subjects with ery-apoB levels ≤ 0.20 a.u. were considered deficient. Carotid intima media thickness (CIMT) was determined as a measure of (subclinical) atherosclerosis.

Results: Mean ery-apoB value was 23.2% lower in subjects with increased CIMT (0.80 ± 0.09 mm, n = 140) compared to subjects with a normal CIMT (0.57 ± 0.08 mm, n = 258) (P = 0.007, adjusted P < 0.001). CIMT and ery-apoB were inversely correlated (Spearman's r: -0.116, P = 0.021). A total of 55 subjects (13.6%) were considered ery-apoB deficient, which was associated with a medical history of CVD (OR: 1.86, 95% CI 1.04 – 3.33; adjusted OR: 1.55; 95% CI 0.85 – 2.82). Discontinuation of statins in 54 subjects did not influence ery-apoB values despite a 58.4% increase in serum apolipoprotein B. Subjects with blood group O had significantly higher ery-apoB values (1.56 ± 0.94 a.u.) when compared to subjects with blood group A (0.89 ± 1.15 a.u), blood group B (0.73 ± 1.12 a.u.) or blood group AB (0.69 ± 0.69 a.u.) (P-ANOVA = 0.002).

Conclusion: Absence or very low values of ery-apoB are associated with clinical and subclinical atherosclerosis. While serum apolipoprotein B is not associated with ery-apoB, the ABO blood group seems to be a significant determinant.

Introduction

The cardiovascular complications of atherosclerosis remain a major health problem in the general population. Atherosclerosis is a slowly progressive disease, induced by numerous risk factors contributing to lipid deposition, inflammation and atherothrombosis [1]. Apolipoprotein (apo) B is the structural protein of the atherogenic lipoproteins including chylomicrons and their remnants, VLDL, IDL and LDL. Large studies have shown that the concentration of serum apo B is a strong predictor of CVD [2-4]. Lipoproteins are found in the fluid phase where they are metabolized and transported to specific organs. However, there is also evidence of a marginated pool of apo B containing lipoproteins attached to the endothelium and to circulating blood cells [5,6]. Close interaction exists between circulating leukocytes and apo B containing lipoproteins as has been demonstrated in human studies [7-9].

Erythrocytes represent the largest blood cell population and make up more than 99% of the total cellular space in blood [10]. It has been suggested that the exchange of cholesterolesters between LDL and erythrocyte membranes may be substantial, which can only be explained by binding of LDL to erythrocytes and not by accidental collision [11,12]. Recently, it was shown that erythrocytes may contribute to reverse cholesterol transport with impairment of reverse cholesterol transport in anemic mice [13]. In a relatively small pilot study, binding of apolipoprotein (apo) B containing lipoproteins to erythrocytes (ery-apoB) was proposed as a protective factor for cardiovascular disease (CVD) [14]. In this study no clear correlation was observed between serum apo B concentrations and ery-apoB and no significant determinants of ery-apoB were detected.

We investigated the association of ery-apoB with carotid intima media thickness (CIMT) and CVD in a larger study population. In addition, we tested whether statins and serum apo B concentrations influence ery-apoB values in a separate group of subjects. Finally, we explored the association between the ABO blood group system and ery-apoB values.

Materials and methods

Subjects

Participants were recruited from the outpatient clinics of the Diabetes Vascular Center and the department of Cardiology, Sint Franciscus Gasthuis in Rotterdam, for the measurement of ery-apoB. The inclusion was carried out between July 2009 and February 2013. Both subjects with and without a history of CVD were included since we expected an atheroprotective effect from ery-apoB. A history of CVD was defined as the presence of at least one of the following conditions before inclusion: a myocardial infarction, angina

pectoris based on clinical characteristics, documented coronary artery disease based on a coronary angiogram, a cerebral infarction or the presence of peripheral artery disease. Exclusion criteria were age <18 years or the use of any experimental medication within 6 months before participation. Anthropometric characteristics, e.g. weight, length, BMI, waist circumference and blood pressure measurements were recorded. Carotid ultrasound scans were carried out to measure carotid intima media thickness (CIMT) using the ART-LAB (Esaote, Italy), which has been described in detail previously [14].

ABO blood groups were obtained from the clinical registry system and if not available, participants were asked to have their ABO blood group determined in our hospital on a separate occasion.

A separate group of subjects who used statins for primary cardiovascular prevention discontinued their statin therapy to investigate the effect of statins and subsequent changes in serum apo B concentrations on ery-apoB values. Participants using statins, but not other lipid lowering drugs, visited the outpatient clinic for baseline measurements including ery-apoB and they were asked to discontinue statin therapy for 6 weeks followed by a second visit including a second blood draw with the measurement of ery-apoB. The subjects were fasting during both visits. This substudy was registered at Clinicaltrials.gov (NCT01634906).

All subjects provided written informed consent. The studies were approved by the independent Regional Medical Ethical Committee Rotterdam, Maasstad Hospital, the Netherlands.

In vivo measurement of ery-apoB

Blood samples for the determination apo B bound to erythrocytes were obtained in tubes containing 5.4 mg K2 EDTA (Becton Dickinson, Plymouth, UK). The staining procedure was started within one hour after venipuncture. The method has been described in detail previously [7,14]. To avoid interference of serum lipoproteins, the samples were washed three times in PBS supplemented with 0.5% BSA (PBS-BSA). The erythrocytes were incubated with a polyclonal goat antibody directed against human apo B (catalogue no. AB742; Millipore, Billerica, MA, USA) for 30 minutes in the dark on ice. Subsequently, the erythrocytes were washed with PBS-BSA and incubated with a rabbit anti-goat (RAG) antibody conjugated with FITC (Nordic Immunological Laboratories, Tilburg, the Netherlands) for another 30 minutes in the dark on ice. As a control for background staining, each sample was simultaneously stained in parallel without anti-apo B antibodies, but with RAG-FITC. Samples were kept in the dark on ice until measurement. A total of 5000 erythrocytes per sample were analysed by flow cytometry using an Epics XL-flow cytometer (Beckman Coulter, Miami, Florida, USA). An FC500 flow cytometer (Beckman Coulter) was used in case of the statin withdrawal sub-study. Before each use, the optics and settings of the flow cytometer were checked with Flow-Check

and Flow-Set beads (Beckman Coulter). Identical flow cytometric settings were used for the complete study.

Laboratory measurements

All clinical and haematological chemistry measurements were carried out on freshly drawn blood samples and analysed in the Department of Clinical Chemistry, Sint Franciscus Gasthuis. Baseline glucose, plasma cholesterol, HDL-C, triglycerides, C-reactive protein, creatine kinase, ALAT and ASAT were measured using the LX20 and DxC analyzers (Beckman Coulter). LDL-C values were calculated using the Friedewald formula. Apo A-I and apo B were determined by nephelometry using an IMMAGE instrument with commercially available kits (Beckman Coulter). Blood cell counts were determined automatically using LH750 and DxH800 analyzers (Beckman Coulter). Thyroid function (TSH) was determined by TSH measurement (Immulite 2500; Siemens, Healthcare Diagnostics, Deerfield, Illinois, USA). The ABO blood group was determined by standard procedures using agglutination techniques (Galileo Echo; Immucor Gamma, Heppignies, Belgium).

Statistics

The binding of apo B on erythrocytes was expressed as the mean difference between fluorescence intensity of apo B-staining and control background staining in arbitrary units (a.u.). The mean fluorescence intensity reflects the amount of apo B per erythrocyte. For statistical analysis, CIMT was defined as the mean of the six individual measurements. Subjects were divided into two groups: a normal CIMT (< 0.70 mm) or increased CIMT (≥ 0.70 mm). Linear regression analysis (backward stepwise) was used to test the differential impact of ery-apoB on CIMT adjusted for multiple variables. The variables included were age, gender, a medical history of CVD, BMI, HDL-C, T2DM and the following interaction effects: CIMT x gender, CIMT x CVD, CIMT x T2DM, CIMT x age, CIMT x BMI, CIMT x HDL-C, BMI x HDL-C, BMI x T2DM, CVD x age, T2DM x age, T2DM x HDL-C, HDL-C x gender, CVD x HDL-C, CVD x BMI. Subjects with ery-apoB levels ranging from undetectable to 0.20 a.u. were considered ery-apoB deficient. Since leukocyte count and HDL-C were significantly different between ery-apoB deficient and sufficient subjects, the impact of ery-apoB deficiency on CVD risk was adjusted for leukocyte count and HDL-C using multiple binary logistic regression analysis.

A power calculation demonstrated that at least 53 subjects were necessary to demonstrate a significant difference of 0.25 ± 0.64 a.u. in ery-apoB after statin withdrawal (power 80%, alpha 0.05 two-sided). The subjects with ABO blood group measurements were divided into tertiles based upon their ery-apoB values to test the distribution of the ABO blood group between the respective tertiles with the Chi-square test. Differences in continuous variables between two groups were tested with the independent Student's *t*-test. One-way ANOVA with LSD as post-hoc analysis was used for comparing

multiple groups. The LSD test was not corrected for multiple comparisons. However, we performed the LSD test only when the overall ANOVA resulted in a P value less than 0.05.

Skewed variables, which included ery-apoB, triglycerides and C-reactive protein, were logarithmically transformed before analysis, but non-transformed data are shown in the text and tables. Correlations were obtained using the bivariate Spearman's correlation. All statistical analyses were performed using PASW statistics version 18.0 (IBM SPSS Statistics, New York, United States). A P-value of < 0.05 (two sided) was regarded as statistical significant.

Results

Carotid intima media thickness and ery-apoB

A total of 409 subjects were included in the study. Levels of ery-apoB were missing in 11 subjects due to technical failures and they were left out of the analysis. A total of 258 subjects (64.8%) showed a normal CIMT and 140 (35.2%) had an increased CIMT. Baseline characteristics of these subjects are shown in Table 1. Subjects with an increased CIMT were older and were more frequently male and obese. They had more frequently a medical history of CVD and used more often statins. The serum apo B concentration was similar between the two groups. Ery-apoB was significantly lower in subjects with increased CIMT (0.89 ± 0.83 a.u.) compared to subjects with a normal CIMT (1.16 ± 0.92; P = 0.007). The difference between the two groups remained significant after adjustment for multiple variables (0.71 ± 0.08 a.u. versus 0.60 ± 0.14 a.u.; P < 0.001). A reverse correlation existed between CIMT and ery-apoB (Spearman's r: -0.116, P = 0.021).

Ery-apoB deficiency and CVD risk

A total of 55 subjects (13.8%) were deficient for ery-apoB with undetectable to very low (≤ 0.20 a.u.) ery-apoB values. The remaining subjects were classified as ery-apoB sufficient (n = 343). Characteristics of the two groups are shown in Table 1. The two groups were comparable, except for a higher leukocyte count and lower HDL-C in the ery-apoB deficient group. The risk of CVD was significantly higher in the ery-apoB deficient group compared to the ery-apoB sufficient group (60.0% vs 44.6%, P = 0.034).

Ery-apoB deficiency was associated with an almost two-fold increased prevalence of a medical history of CVD (OR 1.86; 95% CI 1.04 – 3.33). However, after adjustment for HDL-C and leukocytes the association did not reach statistical significance (adjusted OR 1.55; 95% CI 0.85 – 2.82).

Table 1: Characteristics of subjects based on carotid intima media thickness and on deficiency of ery-apoB (ery-apoB ≤0.20 a.u.) and ery-apoB sufficiency (ery-apoB >0.20 a.u.).

	CIMT < 0.70 (n = 258)	CIMT ≥ 0.70 (n = 140)	P-value	Ery-apoB deficiency (n = 55)	Ery-apoB sufficiency (n = 343)	P-value
Age (years)	54.2 ± 11.7	65.8 ± 8.7	<0.001	60.8 ± 13.3	58.0 ± 11.9	0.11
Male gender (n, %)	125 (48.4)	94 (67.1)	<0.001	31 (56.4)	187 (54.4)	0.78
History of T2DM (n, %)	31 (12.0)	35 (25.0)	0.001	13 (23.6)	58 (16.9)	0.22
History of CVD (n, %)	99 (38.4)	91 (65.0)	<0.001	33 (60.0)	153 (44.6)	0.03
Use of statins (n, %)	100 (43.4)	97 (69.3)	<0.001	35 (63.6)	173 (50.7)	0.08
BMI (kg/m^2)	26.4 ± 4.5	27.9 ± 4.6	0.001	27.5 ± 4.8	27.0 ± 4.6	0.41
CIMT (mm)	0.57 ± 0.08	0.80 ± 0.09	<0.001	0.673 ± 0.132	0.646 ± 143	0.19
Hemoglobin (mmol/l)	8.8 ± 0.7	8.8 ± 0.9	0.72	8.8 ± 0.86	8.8 ± 0.7	0.89
Erythrocytes (*10^{12}/l)	4.6 ± 0.4	4.6 ± 0.4	0.33	4.6 ± 0.4	4.6 ± 0.4	0.80
Leukocytes (*10^9/l)	6.5 ± 1.9	6.9 ± 1.7	0.04	7.2 ± 1.6	6.5 ± 1.8	0.02
Platelets (*10^9/l)	232 ± 54	237 ± 59	0.47	244 ± 58	232 ± 56	0.13
C-reactive protein (mg/l)	2.7 ± 3.0	3.3 ± 3.0	0.10	3.6 ± 3.9	2.7 ± 2.8	0.06
Total cholesterol (mmol/l)	4.9 ± 1.2	4.7 ± 1.1	0.12	4.7 ± 1.1	4.9 ± 1.2	0.35
LDL-C (mmol/l)	2.9 ± 1.1	2.7 ± 1.0	0.19	2.7 ± 1.0	2.8 ± 1.1	0.51
HDL-C (mmol/l)	1.44 ± 0.42	1.31 ± 0.38	0.01	1.26 ± 0.36	1.41 ± 0.42	0.01
Triglycerides (mmol/l)	1.44 ± 1.01	1.68 ± 0.93	0.03	1.70 ± 0.97	1.50 ± 0.99	0.16
Apo B (g/l)	0.93 ± 0.29	0.93 ± 0.25	0.96	0.92 ± 0.26	0.93 ± 0.27	0.78
Apo AI (g/l)	1.56 ± 0.32	1.50 ± 0.24	0.10	1.48 ± 0.25	1.55 ± 0.31	0.14

Abbreviations: BMI = body mass index; CIMT = carotid intima media thickness; apo = apolipoprotein.

The effect of statin use on ery-apoB

The impact of statin withdrawal on serum apo B and ery-apoB values were tested in a group of 54 subjects who discontinued their statin therapy for 6 weeks. As expected, total cholesterol, LDL-C, triglycerides and serum apo B increased, whereas creatine kinase and aspartate aminotransferase decreased 6 weeks after statin withdrawal (Table 2). Ery-apoB remained unchanged before and after discontinuation with a mean change in ery-apoB of -0.04 ± 0.39 (P = 0.49) after statin withdrawal. Ery-apoB at baseline was strongly correlated to ery-apoB after discontinuing statin therapy for 6 weeks (Spearman r: 0.828; P < 0.001) (Figure 1). Ery-apoB did not correlate with serum apo B before (Spearman r: -0.025; P = 0.857) nor after 6 weeks of statin withdrawal (Spearman r: 0.038; P = 0.786).

Table 2: Changes in ery-apoB and other parameters of subjects who discontinued statin therapy for 6 weeks (n = 54).

	Baseline	After 6 weeks of statin withdrawal	P-value
Age (years)	58.8 ± 8.7	-	-
Male gender (n, %)	29 (53.7%)	-	-
BMI (kg/m^2)	28.5 ± 5.1	28.4 ± 5.1	0.45
Waist (cm)	103.1 ± 13.4	104.3 ± 13.9	0.09
Total cholesterol (mmol/l)	4.6 ± 1.0	6.9 ± 1.4	<0.001
LDL-C (mmol/l)	2.5 ± 0.8	4.6 ± 1.1	<0.001
HDL-C (mmol/l)	1.40 ± 0.41	1.35 ± 0.39	0.08
Triglycerides (mmol/l)	1.72 ± 1.12	2.46 ± 2.49	0.007
Apolipoprotein B (g/l)	0.89 ± 0.26	1.41 ± 0.32	<0.001
Apolipoprotein AI (g/l)	1.41 ± 0.22	1.41 ± 0.23	0.84
Erythrocytes (*10^{12}/l)	4.85 ± 0.46	4.86 ± 0.44	0.83
Leukocytes (*10^9/l)	6.5 ± 1.7	6.3 ± 1.5	0.27
C-reactive protein (mg/l)	2.60 ± 2.74	3.26 ± 5.18	0.32
ALAT (U/l)	30.6 ± 11.4	28.6 ± 12.6	0.10
ASAT (U/l)	21.8 ± 14.8	19.1 ± 12.3	0.01
Creatine kinase (U/l)	122 ± 76	106 ± 63	0.02
Ery-apoB (a.u.)	1.08 ± 0.68	1.05 ± 0.57	0.49

Abbreviations: ALAT = alanine aminotransferase; ASAT = aspartate aminotransferase; ery-apoB = erythrocyte bound apolipoprotein B.

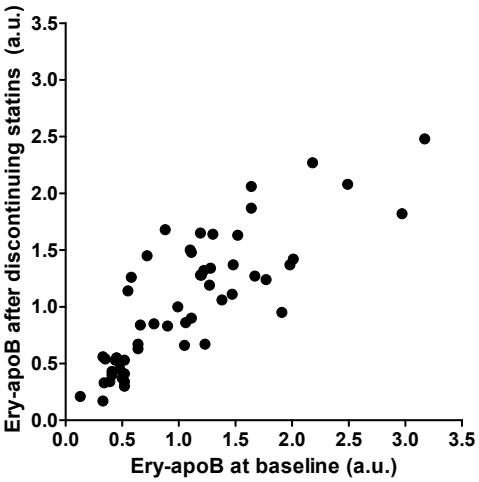

Figure 1: Erythrocyte-bound apolipoprotein B (ery-apoB) was measured in subjects using statins (n = 54) at baseline and after discontinuing statin therapy for 6 weeks. Individual ery-apoB levels remained fairly stable during the 6 weeks of follow-up since ery-apoB at baseline was strongly correlated to ery-apoB after discontinuing statin therapy for 6 weeks (Spearman r: 0.828; P < 0.001).

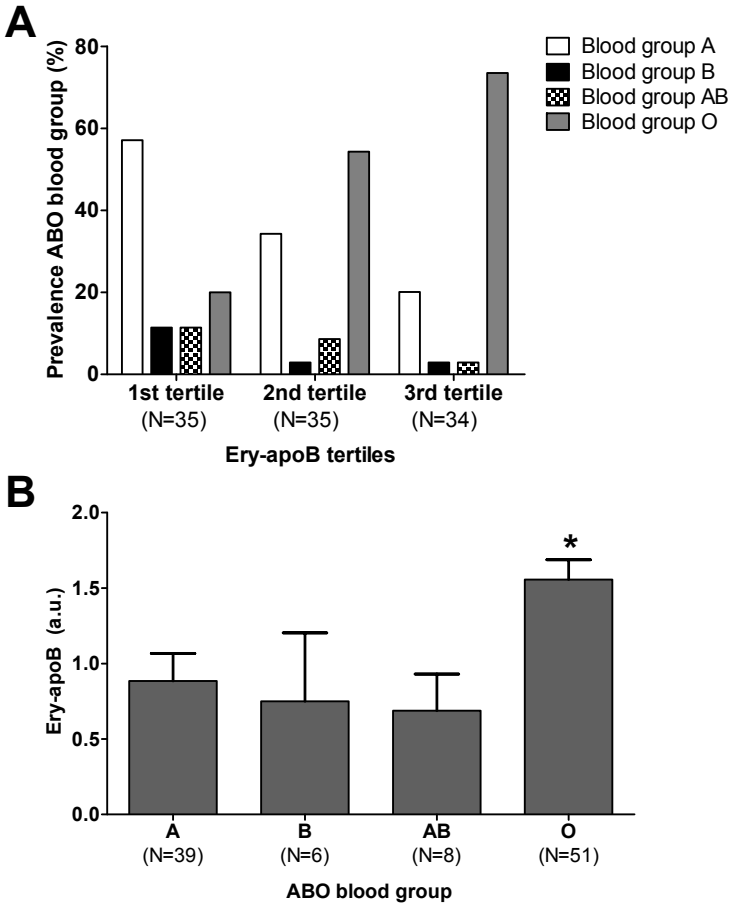

Figure 2: The prevalence of the ABO blood group phenotypes per tertile are shown (A). Tertiles are based upon erythrocyte-bound apolipoprotein B (ery-apoB). The first tertile represents the group with the lowest ery-apoB, whereas the third tertile represents the subjects with the highest ery-apoB. The prevalence of ABO blood group phenotypes was significantly different between the three groups (P = 0.002). Ery-apoB levels were almost two-fold increased in subjects with blood group O when compared to subjects with blood group A, B or AB (P-ANOVA < 0.001) (B). *P < 0.05 when compared to subjects with blood group A, B or AB.

Relationship between ABO blood group and ery-apoB

The ABO blood group was included in the analyses to evaluate factors related to ery-apoB. The ABO blood group was available in only 104 subjects of the original cohort. Subjects were divided into tertiles based on their ery-apoB value. The mean ery-apoB value for each of the tertiles were 0.17 ± 0.11 a.u., 0.98 ± 0.36 a.u. and 2.46 ± 0.76 a.u., respectively. The prevalence of CVD was lowest in the third tertile (55.9%) when compared to the first (82.3%) and second tertile (82.3%) with a similar trend for the use of statins (Table 3). The prevalence of blood group A, B and AB were markedly higher in the first tertile when compared to the third tertile, whereas blood group O was much more

Table 3: Characteristics of a select group of subjects with measurements of erythrocyte-bound apolipoprotein B (ery-apoB) and ABO blood group phenotype. Subjects were divided into tertiles based on their ery-apoB levels.

	1st tertile (n = 35)	2nd tertile (n = 35)	3rd tertile (n = 34)	P-value
Age (years)	62.8 ± 12.2	61.8 ± 8.9	60.9 ± 8.3	0.75
Ery-apoB (a.u.)	0.17 ± 0.11[2,3]	0.98 ± 0.36[1,3]	2.46 ± 0.76[1,2]	<0.001
Blood group A (n, %)	20 (57.1)	12 (34.3)	7 (20.1)	0.002
Blood group B (n, %)	4 (11.4)	1 (2.9)	1 (2.9)	
Blood group AB (n, %)	4 (11.4)	3 (8.6)	1 (2.9)	
Blood group O (n, %)	7 (20.0)	19 (54.3)	25 (73.5)	
Male gender (n, %)	21 (60.0)	26 (74.3)	16 (47.1)	0.07
History of T2DM (n, %)	9 (25.7)	6 (17.1)	7 (20.1)	0.68
History of CVD (n, %)	29 (82.3)	29 (82.3)	19 (55.9)	0.01
Use of statins (n, %)	29 (82.3)	31 (88.6)	21 (61.8)	0.03
BMI (kg/m^2)	27.9 ± 4.4	27.7 ± 4.9	26.0 ± 5.2	0.22
Hemoglobin (mmol/l)	8.9 ± 1.0	8.8 ± 0.7	8.8 ± 0.7	0.98
Erythrocytes (*10^{12}/l)	4.7 ± 0.5	4.6 ± 0.3	4.5 ± 0.4	0.19
Leukocytes (*10^9/l)	7.3 ± 1.6[2,3]	6.6 ± 1.5[1]	6.2 ± 1.2[1]	0.01
Platelets (*10^9/l)	236 ± 56	224 ± 55	237 ± 40	0.50
C-reactive protein (mg/l)	3.3 ± 3.3[3]	3.0 ± 3.4	1.7 ± 1.2[1]	0.02
Total cholesterol (mmol/l)	4.5 ± 0.8	4.4 ± 0.9	4.6 ± 1.1	0.63
LDL-C (mmol/l)	2.4 ± 0.7	2.4 ± 0.9	2.5 ± 0.9	0.90
HDL-C (mmol/l)	1.31 ± 0.35	1.37 ± 0.42	1.52 ± 0.52	0.12
Triglycerides (mmol/l)	1.85 ± 1.18	1.69 ± 1.11	1.47 ± 0.77	0.40
Apolipoprotein B (g/l)	0.87 ± 0.20	0.83 ± 0.21	0.84 ± 0.24	0.75
Apolipoprotein AI (g/l)	1.52 ± 0.28	1.59 ± 0.31	1.64 ± 0.35	0.26

[1] Significantly different when compared to the corresponding 1st tertile ($P < 0.05$)
[2] Significantly different when compared to the corresponding 2nd tertile ($P < 0.05$)
[3] Significantly different when compared to the corresponding 3rd tertile ($P < 0.05$)

prevalent in the second and third tertile when compared to the first tertile ($P = 0.002$) (Figure 2A).

Ery-apoB levels were also analyzed when subjects were divided according to the ABO blood group phenotype. Subjects with blood group A showed higher LDL-C concentrations when compared to subjects with blood group O (Table 4). Mean ery-apoB levels were 1.56 ± 0.94 a.u. in subjects with blood group O (n = 51), which was almost two-fold higher when compared to subjects with blood group A (n = 39) (0.89 ± 1.15 a.u.; $P < 0.001$), blood group B (n = 6) (0.73 ± 1.12 a.u.; $P = 0.02$) or blood group AB (n = 8) (0.69 ± 0.69 a.u.; $P = 0.015$) (Figure 2B).

Table 4: Characteristics of a select group of subjects with measurements of erythrocyte-bound apolipoprotein B (ery-apoB) and ABO blood group phenotype. Subjects were divided according to ABO blood group phenotype.

	A (n = 39)	B (n = 6)	AB (n = 8)	O (n = 51)	P-value
Age (years)	61.7 ± 9.5	56.7 ± 16.7	67.6 ± 8.6	61.7 ± 9.3	0.23
Male gender (n, %)	24 (61.5)	3 (50.0)	5 (62.5)	31 (60.8)	0.96
History of T2DM (n, %)	9 (23.1)	0 (0.0)	3 (37.5)	10 (19.6)	0.38
History of CVD (n, %)	27 (69.2)	4 (66.7)	7 (87.5)	39 (76.5)	0.67
Use of statins (n, %)	27 (69.2)	5 (83.3)	8 (100.0)	41 (80.3)	0.30
BMI (kg/m^2)	27.0 ± 5.2	28.3 ± 4.2	27.9 ± 3.8	27.1 ± 5.0	0.92
Hemoglobin (mmol/l)	8.8 ± 0.8	8.9 ± 0.6	8.5 ± 1.1	8.9 ± 0.8	0.72
Erythrocytes (*10^{12}/l)	4.6 ± 0.4	4.7 ± 0.4	4.4 ± 0.6	4.6 ± 0.4	0.53
Leukocytes (*10^9/l)	7.1 ± 1.6	6.1 ± 1.2	6.8 ± 1.0	6.5 ± 1.6	0.22
Platelets (*10^9/l)	235 ± 56	237 ± 48	252 ± 63	226 ± 45	0.55
C-reactive protein (mg/l)	3.4 ± 3.9	1.8 ± 1.0	2.7 ± 2.5	2.2 ± 1.8	0.34
Ery-apoB (a.u.)	0.89 ± 1.15*	0.73 ± 1.12*	0.69 ± 0.69*	1.56 ± 0.94***	<0.001
Total cholesterol (mmol/l)	4.7 ± 1.0	4.4 ± 1.3	4.1 ± 0.8	4.4 ± 0.9	0.18
LDL-C (mmol/l)	2.7 ± 0.9*	1.9 ± 1.0	2.3 ± 0.6	2.2 ± 0.8**	0.04
HDL-C (mmol/l)	1.34 ± 0.37	1.63 ± 0.65	1.30 ± 0.41	1.42 ± 0.46	0.41
Triglycerides (mmol/l)	1.65 ± 0.85	1.88 ± 1.83	1.09 ± 0.60	1.76 ± 1.11	0.36
Apolipoprotein B (g/l)	0.91 ± 0.24	0.75 ± 0.24	0.80 ± 0.18	0.82 ± 0.19	0.12
Apolipoprotein AI (g/l)	1.57 ± 0.29	1.57 ± 0.27	1.52 ± 0.30	1.60 ± 0.35	0.90

*Significantly different when compared to blood group O ($P < 0.05$)
**Significantly different when compared to blood group A ($P < 0.05$)
***Significantly different when compared to blood group A, B or AB (all $P < 0.05$)

Discussion

Our study confirmed the association of ery-apoB with clinical and subclinical atherosclerosis in an extended study population [14]. Ery-apoB was lower in subjects with increased CIMT and very low to undetectable values of ery-apoB were associated with an increased CVD risk. Recently, it was demonstrated in a mouse model that erythrocytes contribute to reverse cholesterol transport, particularly when the number of HDL particles is low [13]. Erythrocyte mediated reverse cholesterol transport by binding of atherogenic apo B containing lipoproteins could potentially explain the atheroprotective effect of ery-apoB suggested by the present data. In addition, we hypothesize a protective mechanism in which apo B containing lipoproteins bound to erythrocytes are less likely to interact with the endothelium [6,14]. The observation that anemia is associated with the development of CVD is also in line with our hypothesis [15].

We did not observe any changes in ery-apoB in the group of subjects who discontinued their statin therapy, despite a marked increase in serum apo B. In our comparison between subjects with normal and increased CIMT we did not correct for the use of statins, although subjects with increased CIMT used statins more frequently. In the present study we clearly demonstrated that statins do not influence ery-apoB since there was no change after discontinuation. In addition, there was no association between serum apo B and ery-apoB [14]. We have chosen to study the effects of statin withdrawal for a period of 6 weeks on ery-apoB, because serum concentrations of apo B and LDL-C return to baseline within 4 weeks. In addition, the lifespan of erythrocytes is approximately 12 weeks and half of all erythrocytes would have been renewed within 6 weeks, which potentially may have shown changes in the renewed erythrocyte population. We hypothesize that the adherence of apo B containing lipoproteins to erythrocytes is dynamic with a continuous process of adherence and release of lipoproteins. Therefore, we presume that a period of 6 weeks of statin withdrawal would have been sufficient to study the effects of statins and changes in serum apo B on ery-apoB. Furthermore, we do not think that it would be ethical for patients to refrain from a necessary treatment for a longer period of time.

The exact binding mechanism of apo B containing lipoproteins to erythrocytes has not yet been elucidated. The LDL receptor seems to be a logical candidate to bind apo B containing lipoproteins to erythrocytes but it is not expressed on erythrocytes [11,12]. Therefore, other binding mechanisms must be operational. The exchange of cholesterolesters between LDL and erythrocyte membranes may be substantial and could not be explained by accidental collision [11,12]. Here we show for the first time that the ABO blood group system may influence the binding of apo B containing lipoproteins to erythrocytes. Interestingly, we found that ery-apoB levels were almost two-fold increased in subjects with blood group O when compared to subjects with blood group A, B or AB. We have to admit that the ABO blood group was only available in 104 of the total 409 subjects and that the number of subjects with blood group B and AB was low. This could have resulted in a selection bias. However, the results were so striking, that we decided to include them in the current paper. Studies are underway in our laboratory to gain more insight in the relationship between blood groups and ery-apoB. It is tempting to speculate that different carbohydrate groups on the apo B molecule interact with fucose, galactose, N acetyl-galactosamine or N acetyle-glucosamine, which make up the A, B and H antigens [16].

The ABO blood group system has already been associated with plasma cholesterol concentrations more than 40 years ago [17,18]. Blood group O has been associated with slightly lower cholesterol levels [19], whereas others have found associations between blood group A and elevated plasma concentrations for total cholesterol [20,21], and

LDL-C [21]. We found similar results in our study with elevated plasma LDL-C levels in subjects with blood group A.

Besides with lipids, the ABO blood group system has been associated with CVD. Factors involved in this association are potentially Factor VIII, Von Willebrand Factor, endothelial molecules and platelet proteins [22,23]. Recently, two large prospective cohort studies plus a meta-analysis demonstrated that the non-O blood group has a higher risk of CVD (RR 1.11; 95%CI 1.05 – 1.18) and that 6.27% of the CVD cases were attributable to inheriting the non-O blood group [24]. Our finding that ery-apoB is associated with both an atheroprotective effect similar to blood group O is in line with these observations. Prospective data regarding ery-apoB and incident CVD are still lacking and the clinical value of ery-apoB as a biomarker in cardiovascular risk prediction needs to be determined.

In conclusion, absence or very low ery-apoB is associated with the presence of clinical and subclinical atherosclerosis. The evaluation of ery-apoB as a clinical useful risk factor for CVD needs to be determined in a large prospective study. Blood group O was strongly associated with high ery-apoB and may provide a new explanation for the association between the ABO blood group system and CVD and plasma lipids. Statins and serum apo B do not affect ery-apoB.

References

1. Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med 1999;340:115-126
2. Lamarche B, Moorjani S, Lupien PJ, Cantin B, Bernard PM, Dagenais GR, et al. Apolipoprotein A-I and B levels and the risk of ischemic heart disease during a five-year follow-up of men in the Quebec Cardiovascular Study. Circulation 1996;94:273-278
3. Boekholdt SM, Arsenault BJ, Mora S, Pedersen TR, LaRosa JC, Nestel PJ, et al. Association of LDL cholesterol, non-HDL cholesterol, and apolipoprotein B levels with risk of cardiovascular events among patients treated with statins: A meta-analysis. JAMA 2012;307:1302-1309
4. Mora S, Rifai N, Buring JE, Ridker PM. Fasting compared with nonfasting lipids and apolipoproteins for predicting incident cardiovascular events. Circulation 2008;118:993-1001
5. Verseyden C, Meijssen S, Castro Cabezas M. Effects of atorvastatin on fasting plasma and marginated apolipoproteins B48 and B100 in large, triglyceride-rich lipoproteins in familial combined hyperlipidemia. J Clin Endocrinol Metab 2004;89:5021-5029
6. Bovenberg SA, Alipour A, Elte JW, Rietveld AP, Janssen JW, van de Geijn GJ, et al. Cell-mediated lipoprotein transport: A novel anti-atherogenic concept. Atheroscler Suppl 2010;11:25-29
7. Alipour A, van Oostrom AJ, Izraeljan A, Verseyden C, Collins JM, Frayn KN, et al. Leukocyte activation by triglyceride-rich lipoproteins. Arterioscler Thromb Vasc Biol 2008;28:792-797
8. Gower RM, Wu H, Foster GA, Devaraj S, Jialal I, Ballantyne CM, et al. CD11c/CD18 expression is upregulated on blood monocytes during hypertriglyceridemia and enhances adhesion to vascular cell adhesion molecule-1. Arterioscler Thromb Vasc Biol 2011;31:160-166
9. van Oostrom AJ, van Wijk J, Castro Cabezas M. Lipaemia, inflammation and atherosclerosis: Novel opportunities in the understanding and treatment of atherosclerosis. Drugs 2004;64 Suppl 2:19-41
10. Hinderling PH. Red blood cells: A neglected compartment in pharmacokinetics and pharmacodynamics. Pharmacol Rev 1997;49:279-295
11. Hui DY, Noel JG, Harmony JA. Binding of plasma low density lipoproteins to erythrocytes. Biochim Biophys Acta 1981;664:513-526
12. Hui DY, Harmony JA. Interaction of plasma lipoproteins with erythrocytes. I. Alteration of erythrocyte morphology. Biochim Biophys Acta 1979;550:407-424
13. Hung KT, Berisha SZ, Ritchey BM, Santore J, Smith JD. Red blood cells play a role in reverse cholesterol transport. Arterioscler Thromb Vasc Biol 2012;32:1460-1465
14. Bovenberg SA, Klop B, Alipour A, Martinez-Hervas S, Westzaan A, van de Geijn GJ, et al. Erythrocyte-associated apolipoprotein B and its relationship with clinical and subclinical atherosclerosis. Eur J Clin Invest 2012;42:365-70
15. Sarnak MJ, Tighiouart H, Manjunath G, MacLeod B, Griffith J, Salem D, et al. Anemia as a risk factor for cardiovascular disease in the atherosclerosis risk in communities (ARIC) study. J Am Coll Cardiol 2002;40:27-33
16. Sasak WV, Lown JS, Colburn KA. Human small-intestinal apolipoprotein B-48 oligosaccharide chains. Biochem J 1991;274:159-165
17. Oliver MF, Geizerova H, Cumming RA, Heady JA. Serum-cholesterol and ABO and rhesus blood-groups. Lancet 1969;2:605-606
18. Langman MJ, Elwood PC, Foote J, Ryrie DR. ABO and Lewis blood-groups and serum-cholesterol. Lancet 1969;2:607-609
19. Garrison RJ, Havlik RJ, Harris RB, Feinleib M, Kannel WB, Padgett SJ. ABO blood group and cardiovacular disease: The Framingham Study. Atherosclerosis 1976;25:311-318

20. Wong FL, Kodama K, Sasaki H, Yamada M, Hamilton HB. Longitudinal study of the association between ABO phenotype and total serum cholesterol level in a Japanese cohort. Genet Epidemiol 1992;9:405-418
21. George VT, Elston RC, Amos CI, Ward LJ, Berenson GS. Association between polymorphic blood markers and risk factors for cardiovascular disease in a large pedigree. Genet Epidemiol 1987;4:267-275
22. Zhang H, Mooney CJ, Reilly MP. ABO blood groups and cardiovascular diseases. Int J Vasc Med 2012;2012:641917
23. Carpeggiani C, Coceani M, Landi P, Michelassi C, L'Abbate A. ABO blood group alleles: A risk factor for coronary artery disease. An angiographic study. Atherosclerosis 2010;211:461-466
24. He M, Wolpin B, Rexrode K, Manson JE, Rimm E, Hu FB, et al. ABO blood group and risk of coronary heart disease in two prospective cohort studies. Arterioscler Thromb Vasc Biol 2012;32:2314-2320

Chapter 8

Differential regulation of complement activation by native and acetylated LDL affects binding to complement receptor 1

Boudewijn Klop
Pieter van der Pol
Robin van Bruggen
Yanan Wang
Joseph O'Flynn
Tjin L. Njo
Hans W. Janssen
J. Wouter Jukema
Ton J. Rabelink
Patrick C. N. Rensen
Cees van Kooten
Manuel Castro Cabezas

Submitted

Abstract

Introduction: Lipoproteins can induce complement activation resulting in opsonization and binding of these complexes to complement receptors. We investigated the binding of opsonized native LDL and acetylated LDL (acLDL) to the complement receptor 1 (CR1).
Methods: Binding of complement factors C3b, IgM, C1q, mannose-binding lectin (MBL) and properdin to LDL and acLDL were investigated by ELISA. Subsequent binding of opsonized LDL and acLDL to CR1 on CR1-transfected Chinese Hamster Ovarian cells (CHO-CR1) was tested by flow cytometry.
Results: Both native LDL and acLDL induced complement activation with subsequent C3b opsonization upon incubation with normal human serum. Opsonized LDL and acLDL bound to CR1. Binding to CHO-CR1 was reduced by EDTA, whereas MgEGTA only reduced the binding of opsonized LDL, but not of acLDL suggesting involvement of the alternative pathway in the binding of acLDL to CR1. *In vitro* incubations showed that LDL bound C1q, whereas acLDL bound to C1q, IgM and properdin. MBL did neither bind to LDL nor to acLDL.
Conclusion: CR1 is able to bind opsonized native LDL and acLDL. Binding of LDL to CR1 is mediated via the classical pathway, whereas binding of acLDL is mediated via both the classical and alternative pathways. Binding of lipoproteins to CR1 may be of clinical relevance due to the ubiquitous cellular distribution of CR1.

Introduction

The complement system is part of the innate immune system, which provides protection against micro-organisms. Complement activation occurs via three different pathways: the *classical pathway* that depends on IgG and/or IgM antibodies and C-reactive protein (CRP), the *alternative pathway* initiated by factor B, factor D and properdin and the *lectin pathway* initiated by the recognition of neutral sugars like mannose and fructose via mannose-binding lectin (MBL) and ficolins [1-3]. All three pathways lead to the conversion of complement component (C) 3, the most abundant complement component in serum, into C3a and C3b via surface-bound C3-convertases. The final step of the complement cascade is the formation of the membrane attack complex, resulting in removal of pathogens and immune complexes from the circulation [2,4,5].

The complement system and its activation are not only important in microbial defence but also in the development of atherosclerosis, both by anti- and pro-atherogenic properties [6,7]. In addition, the complement system has been linked with lipoprotein metabolism, which is closely linked to atherosclerosis, e.g. by binding of complement factors to lipoproteins. C3 and C4 have been found on high density lipoproteins (HDL) [8] and MBL may be involved in postprandial lipid metabolism with effects on chylomicron and very low density lipoprotein (VLDL) handling [9]. Properdin has recently been linked to postprandial lipid metabolism as well [10]. Modified forms of low density lipoproteins (LDL), like acetylated LDL (acLDL), oxidized LDL (oxLDL) or enzymatically degraded LDL have been shown to induce complement activation [6,11], which was enhanced by the addition of CRP [6]. Finally, modified forms of LDL bind C3b and C1q [12].

Although binding of lipoproteins to specific lipoprotein receptors and heparan sulphate proteoglycans is well established [13,14], binding to complement receptors may also occur as suggested previously by our group [15]. In theory, C3b-opsonized lipoproteins may bind to the complement receptor 1 (CR1, also known as CD35 or C3b receptor), which is present on many types of cells such as erythrocytes, endothelial cells and inflammatory cells [16,17]. CR1 on erythrocytes is important for "immune adherence", which functions as a clearing mechanism for immune complexes [18,19] and microorganisms [20]. CR1 also facilitates phagocytosis of immune complexes by monocytes, macrophages and neutrophils [17]. Therefore, CR1-mediated cellular binding of opsonized lipoproteins may also contribute to removal of lipoproteins from plasma [15].

The aim of this study was to investigate whether CR1 can bind native LDL and acLDL and which complement activation pathways may be involved. The clinical relevance of this question is based on the recent finding that circulating erythrocytes carry apolipoprotein B-containing lipoproteins on their surface [21]. Since erythrocytes express CR1 and not the classical lipoprotein receptors, CR1-mediated lipoprotein binding by erythrocytes may contribute to the clearance of these particles similarly to the earlier

mentioned process of immune adherence. For this purpose, a cell model with CR1 transfected Chinese ovarian hamster cells was used for complement activation experiments *in vitro*.

Materials and methods

CR1 cell model

Transfected Chinese Hamster Ovary cells expressing the complement receptor 1 (CHO-CR1) and negative controls (CHO) with CD46 cloned in reverse orientation were kindly provided by J.P. Atkinson and M.K. Liszewski (Washington University School of Medicine, St Louis, Needham, MA, USA) and by S. Rooijakkers (University Medical Center Utrecht, Utrecht, the Netherlands) [22,23]. The cells were cultured at 37°C in DMEM/F12 + GLUTAMAX (HAM) (1:1) medium (GIBCO, Invitrogen, Carlsbad, California, USA) with 1% penicillin-streptomycin, 0.5 µg/ml G-418 (Sigma-Aldrich, St. Louis, Missouri, USA) and 10% heat-inactivated FCS. Trypsin was used to harvest the cells for experiments. A total of 200.000 cells per sample were used for each experiment. Expression and functionality of CR1 on CHO-CR1 was confirmed.

Binding of LDL and acLDL to Chinese Hamster Ovary cells

BODIPY® fluorochrome labeled LDL and acLDL were purchased (Invitrogen Molecular Probes, Eugene, Oregon, United States) and all experiments were conducted within two weeks after arrival of the labeled lipoproteins. All experiments regarding the binding of LDL and acLDL to CHO-CR1 and CHO were conducted with these labeled lipoproteins. CHO-CR1 and CHO were incubated with 10 µg/ml LDL or acLDL or with medium as control for 30 minutes on ice in the dark followed by flow cytometric analysis (FACSCalibur, Becton Dickinson, Franklin Lakes, New Jersey, USA). To investigate whether LDL binding to CHO could be reduced by blocking the LDL receptor, CHO cells were pre-incubated with an anti-human LDL receptor blocking antibody (catalog number AF2148, R&D Systems, Minneapolis, United States) for 30 minutes prior to LDL incubation. In addition, fluorescent LDL (10 µg/ml) was incubated with either normal human serum (NHS) or non-labeled LDL in different concentrations to test whether competition for binding to CHO cells occurred between fluorescent LDL, LDL present in NHS or with native LDL. Fluorescent LDL was incubated with NHS or with non-labeled LDL in culture medium for 60 minutes on 37°C in the dark prior to incubation with CHO-CR1 and CHO.

Binding of complement-opsonized LDL and acLDL to CR1

In experiments where it was necessary to impede complement activation heat inactivated serum (HIS) (at 56°C for 30 minutes) was used. Labeled LDL and acLDL (10 µg/

ml) were incubated with either 10% NHS or 10% HIS in culture medium for 60 minutes on 37°C in the dark. Next CHO and CHO-CR1 were incubated with either LDL in NHS, acLDL in NHS, LDL in HIS, acLDL in HIS or in culture medium as control for 30 minutes in the dark on ice. Binding of LDL or acLDL to CHO and CHO-CR1 was detected by flow cytometry (FACSCalibur, Becton Dickinson).

Inactivation of different complement pathways

In order to inactivate the different complement pathways, the following experiments were carried out. Anti-C1q (20 µg/ml) was added to 10% NHS to inactivate the classical pathway. The lectin pathway was blocked by addition of D-mannose (200 mM) to 10% NHS. MgEGTA (20 mM) was used to block both the classical and lectin pathway. EDTA (20 mM) was used to block all three complement pathways. NHS with either anti-C1q, D-mannose, MgEGTA or EDTA was left on room temperature for ten minutes before incubation with labeled LDL (10 µg/ml) or acLDL (10 µg/ml). After the samples were incubated for 60 minutes on 37°C in the dark, CHO-CR1 were incubated with the different samples for 30 minutes in the dark on ice and LDL and acLDL binding was assessed by flow cytometry.

Binding of complement factors to LDL and acLDL by ELISA

Human lipoproteins were isolated according to the technique described by Redgrave et al [24]. In short, PBS with 0.3 mM EDTA with densities of 1.0063 g/L, 1.019 g/L and 1.063 g/L were prepared by using KBr. Serum was collected from non-fasting healthy volunteers, which was adjusted to 1.21 g/L by the addition of KBr. A gradient was prepared and centrifuged with 40,000 rpm with slow acceleration and deceleration for 16 hours at a temperature of 4°C. The LDL fraction was aspirated with a glass pipette and subsequently dialyzed overnight against PBS pH 7.4 using a dialysis cassette (Thermo Scientific, Pierce Protein Research Products, Rockford, Illinois, USA). The protein concentration was determined using the BCA protein assay kit (Thermo Scientific, Pierce Protein Research Products). In addition, a portion of LDL was acetylated according to the technique described by Van Berkel et al [25]. AcLDL was prepared by mixing LDL with saturated sodium acetate and acetic anhydride. After acetylation, the acLDL was dialyzed overnight against PBS buffer with pH 7.4.

Human properdin [26], human IgM [27], human C1q [28] and human MBL [27] were purified as described previously. Ninety-six well, flat bottom plates (NUNC, Thermo Fisher Scientific, Rochester, New York, USA) were coated with either LDL (50 µg/ml), acLDL (50 µg/ml) or human serum albumin (HSA) (1 mg/ml) overnight at room temperature. The remaining sites were blocked with 1% HSA in PBS and incubated for 1 hour at 37°C. Wells were washed with PBS tween 0.05% including 2.5 mM $CaCl_2$ and incubated with either NHS, human IgM, C1q, MBL or properdin diluted in Veronal buffer supplemented

with 1% HSA, 2.5 mM $CaCl_2$, 2.5 mM $MgCl_2$ and 0.05% tween for 1 hour at 37°C. The plate was washed again and incubated with either monoclonal antibodies against human C3 (RFK22, in-house, Leiden University Medical Center, Leiden, the Netherlands), monoclonal anti-human IgM (HB57, hybridoma obtained from the American Type Culture Collection, Manassas, VA, United States), monoclonal antibodies against human C1q (mAb 2214, kindly provided by Prof. C. E. Hack, Sanquin Blood Supply Foundation, Amsterdam, The Netherlands) monoclonal antibodies against human MBL (3E7, Hycult Biotech, Uden, the Netherlands) or rabbit anti-human properdin (in house, Leiden University Medical Center) followed by HRP-conjugated F(ab')2 from goat IgG anti-dig (Boehringer Mannheim, Mannheim, Germany) or streptavidin-HRP conjugate (Zymed Laboratories, South San Francisco, California, USA) with ABTS/H_2O_2 (Sigma) to measure the deposition of C3b, IgM, C1q, MBL and properdin, respectively on LDL and acLDL. The optical density (OD) was measured at 415nm using a microtiter plate reader (Model 680 Microtiter Reader, Bio-Rad Laboratories, California, United States). BSA instead of HSA was used as negative control for the determination of properdin binding. Mannan (Sigma-Aldrich) was used as a positive control for MBL deposition and IgM as a positive control for C1q binding.

The contribution of the classical and lectin pathways to C3b opsonisation of LDL and acLDL were investigated. A similar kind of ELISA for the assessment of C3b deposition was done with some adjustments. Different concentrations of anti-C1q or D-mannose were added to a fixed concentration of 10% NHS and left on room temperature for ten minutes, which was used to incubate the LDL and acLDL coated plates. Human IgM (2.6 µg/ml) and mannan (10 µg/ml) were used as controls for either inhibition of the classical or lectin pathway.

Statistics

Differences between groups were tested using the unpaired *t*-test when comparing two unrelated groups. One-way ANOVA with LSD as post-hoc analysis was used when multiple groups were compared. Graphpad 5.0 (Prism) and PASW 18.0 (SPSS, IBM) were used for the statistical analyses. A P-value of less than 0.05 (two-sided) was regarded as statistical significant.

Results

C3b-opsonization of LDL and acLDL

To determine whether both native LDL and acLDL were able to induce complement activation with subsequent C3b opsonization LDL and acLDL were incubated with NHS. This resulted in C3b opsonization of LDL and acLDL as established by ELISA (Figure

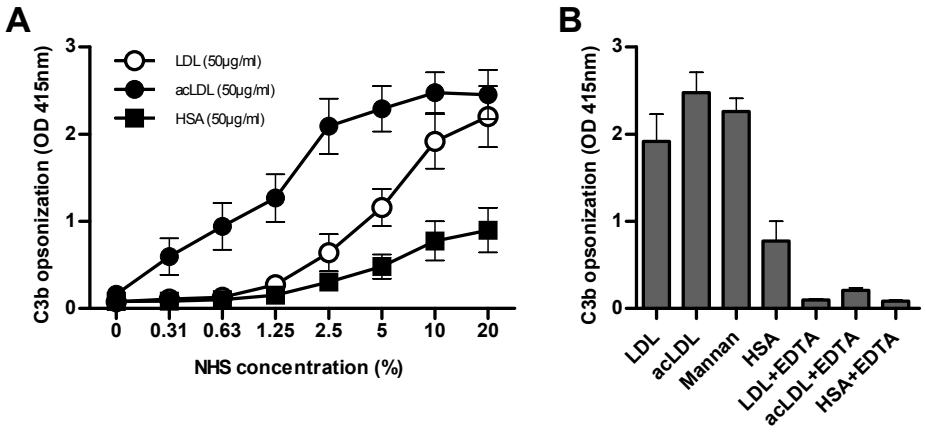

Figure 1: C3b opsonization of acLDL occurred already at very low concentrations of normal human serum (NHS), but concentrations of at least 2.5% were necessary to establish C3b opsonization of LDL (A). C3b opsonization occurred on both LDL and acLDL upon incubation with 10% NHS. Mannan served as a positive control, whereas human serum albumin (HSA) served as a negative control. The addition of EDTA to NHS prevented complement activation and C3b opsonization of LDL and acLDL completely (B). Every figure represents the mean ± SEM of at least three experiments.

1A). C3b opsonization of acLDL occurred already at NHS concentrations as low as 0.3%, whereas concentrations of at least 2.5% were necessary to induce C3b-opsonization of LDL. Addition of EDTA to NHS prevented C3b opsonization of LDL and acLDL (Figure 1B).

Binding of opsonized LDL and acLDL to CR1

The capability of CR1 to bind opsonized LDL and acLDL was tested. For this purpose a cell model with CHO-CR1 and control CHO cells was used. Binding of opsonized LDL to CHO-CR1 was significantly higher compared to CHO (11.6 ± 1.1 a.u. vs 5.5 ± 0.3 a.u., $P<0.05$). Similar results were found for opsonized acLDL (8.3 ± 1.4 a.u. vs 4.8 ± 0.8 a.u., $P<0.05$) (Figure 2A). This binding was dose-dependently reduced when LDL and acLDL were incubated with different ratios of HIS and NHS (Figures 2B and C).

Binding of non-opsonized LDL and acLDL to CHO and CHO-CR1 was similar between CHO and CHO-CR1 (Figure 3A). Binding of LDL to CHO via the LDL receptor was demonstrated by a reduction in LDL binding to CHO by pre-incubation of CHO with an LDL receptor blocking antibody (Figure 3B). Similar reductions in LDL binding to CHO were achieved by inducing competition between fluorescent labeled LDL with increasing concentrations of NHS or native LDL (Figures 3C and D).

Binding of human IgM, C1q, MBL and properdin to LDL and acLDL

The binding of several complement factors to LDL and acLDL was tested in order to explore which complement factors may be involved in CR1-bound LDL and acLDL.

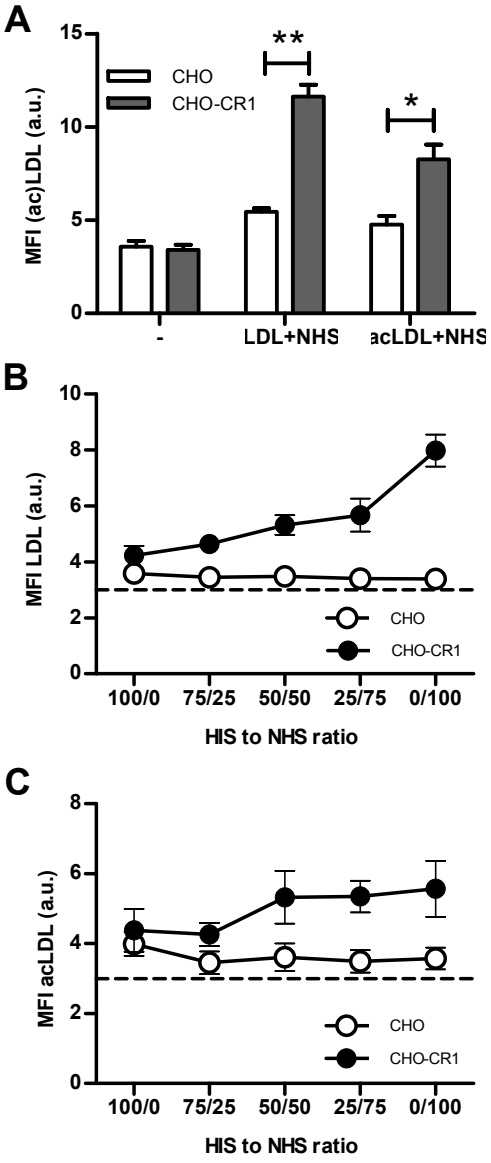

Figure 2: CHO-CR1 bound significantly more LDL (10 µg/ml) and acLDL (10 µg/ml) than CHO when LDL and acLDL were first incubated with normal human serum (NHS) (A). LDL and acLDL binding to CHO-CR1 was diminished when heat inactivated serum (HIS) was used to pre-incubate LDL and acLDL instead of NHS (B and C). The dotted horizontal lines in the figures represent the autofluorescent background signal. *P < 0.05, **P < 0.01

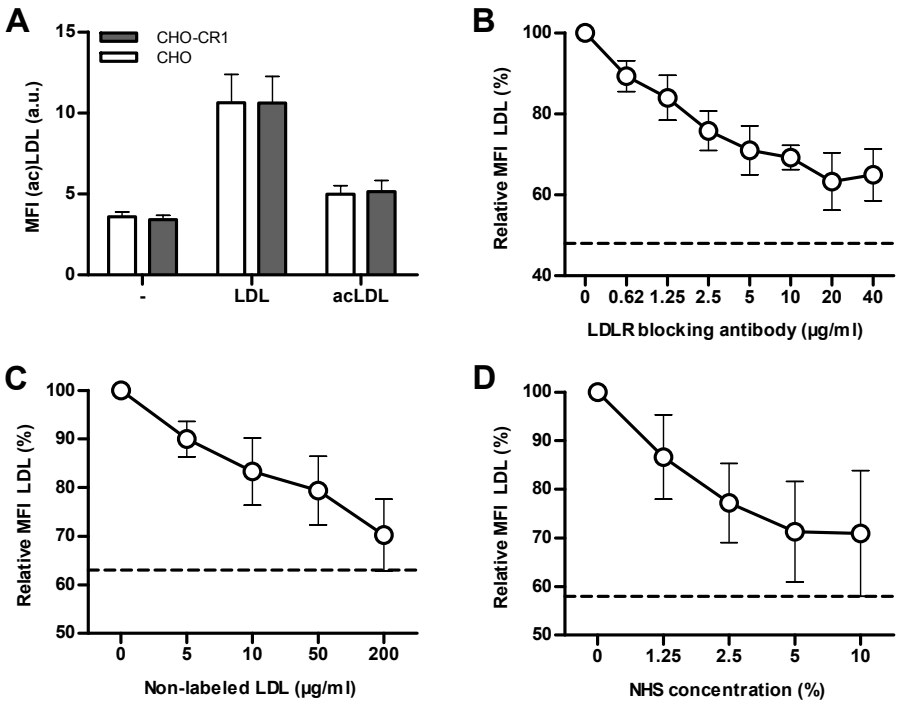

Figure 3: Non-opsonized LDL and acLDL binding to CHO cells was investigated by flow cytometry. CHO and CHO-CR1 bound LDL (10 µg/ml) in a similar proportion, but binding of acLDL (10 µg/ml) was less clear (A). Binding of LDL was reduced when CHO were pre-incubated with increasing concentrations anti-human LDL receptor antibodies (B). Comparable reductions in LDL binding to CHO were observed when fluorescent LDL (10 µg/ml) was combined with increasing concentrations of native (non-labeled) LDL (C) or normal human serum (NHS) (D). The dotted horizontal lines represent the autofluorescent background signal.

Therefore, binding of purified forms of IgM, C1q, MBL and properdin to LDL and acLDL coated plates was established by ELISA. IgM bound to acLDL dose dependently, but not to LDL when the lipoproteins were incubated with IgM (Figure 4A and B). C1q bound to both LDL and acLDL, but binding to acLDL was more than two-fold higher when compared to LDL or to the positive control IgM (Figure 4C). Binding of C1q to acLDL was already observed at concentrations of 0.08 µg/ml, whereas C1q binding to LDL or natural IgM were observed at concentrations above 1 µg/ml (Figure 4D). MBL did not bind to either LDL or acLDL, whereas MBL effectively bound to mannan, which served as a positive control for MBL binding (Figure 4E and F). Properdin bound to acLDL, but not to LDL (Figure 4G and H). Altogether these results imply that opsonization of either LDL or acLDL may involve different pathways of complement activation.

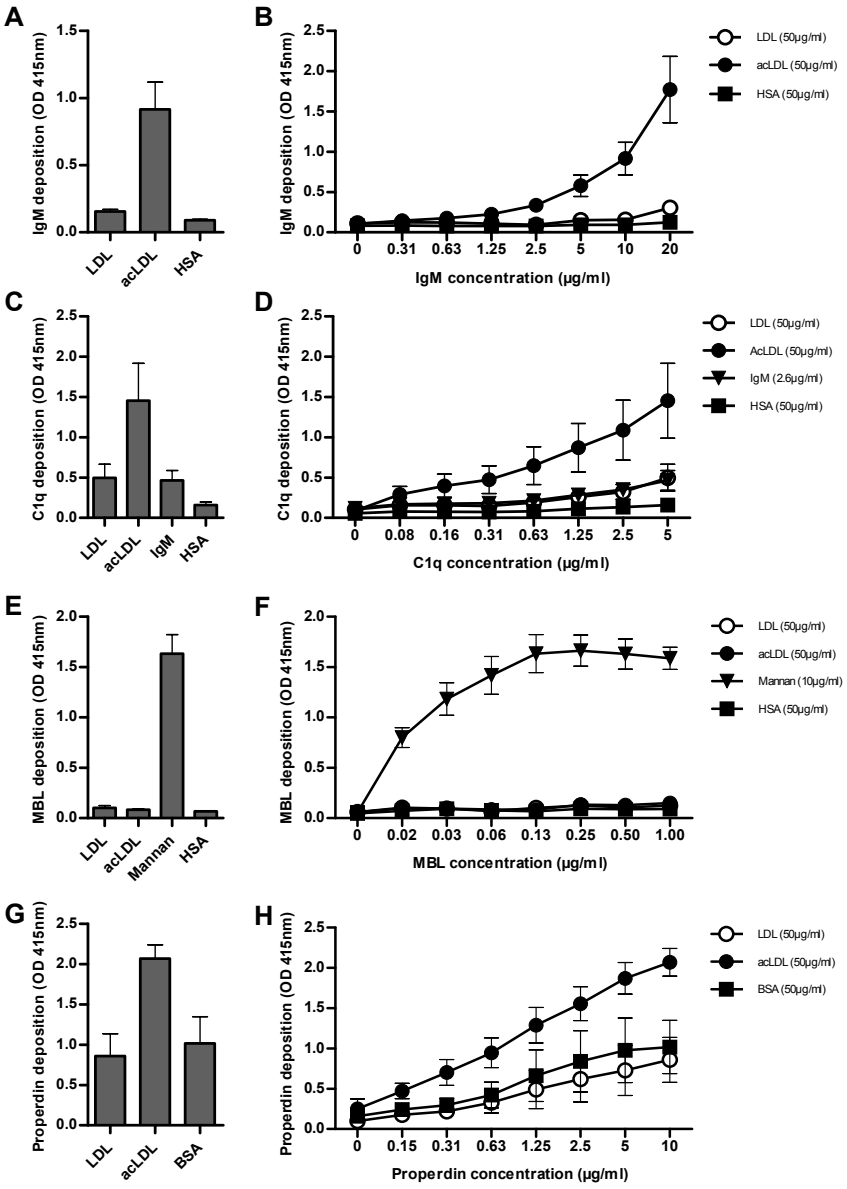

Figure 4: Binding of different complement factors to LDL and acLDL was investigated by ELISA. Isolated natural IgM (10 µg/ml) bound to acLDL, but not to LDL (A) and a clear dose response curve observed with natural IgM binding to acLDL (B). Isolated C1q (5 µg/ml) bound to LDL to a similar extent as the positive control natural IgM, whereas C1q was highly bound by acLDL (C) with a clear dose response curve (D). Isolated mannose binding lectin (MBL) (0.13 µg/ml) did not bind to either LDL or acLDL, in contrast to mannan, which served as a positive control (E), which was also observed with higher concentrations of MBL (F). Properdin (10 µg/ml) highly bound acLDL, but not LDL (G). The binding of properdin to acLDL was already observed at very low concentrations (H). Every figure represents the mean ± SEM of at least three experiments.

Involvement of different complement pathways in the binding of LDL and acLDL to CR1

The involvement of the classical and lectin pathways in C3b opsonization of LDL and acLDL were investigated. For this purpose C3b opsonization of LDL and acLDL was detected using a C3b-ELISA on LDL or acLDL coated plates incubated with NHS substituted with different concentrations of either anti-C1q or D-mannose. C3b opsonization of LDL was almost completely diminished by anti-C1q at an anti-C1q concentration of 5 µg/ml or higher. C3b opsonization of acLDL was only gradually reduced by anti-C1q with a maximum decrease in C3b opsonization of acLDL of 36% at an anti-C1q concentration of 10 µg/ml (Figure 5A). D-mannose was able to block C3b opsonization of LDL at a maxi-

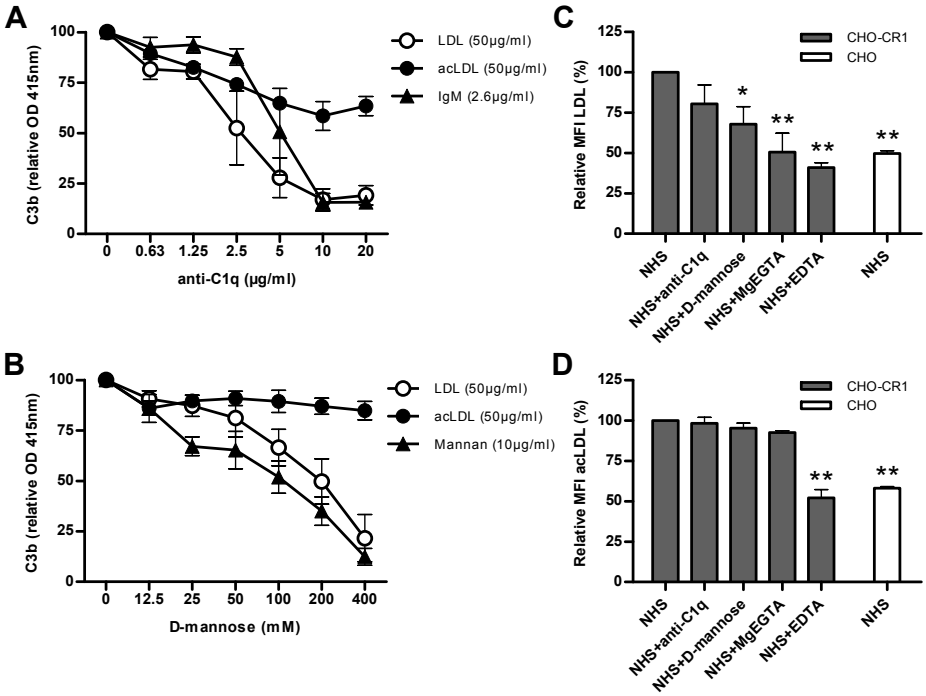

Figure 5: C3b opsonization of LDL was highly reduced by the addition of anti-C1q to 10% NHS, whereas C3b opsonization of acLDL was only modestly reduced by antiC1q (20 µg/ml). The dose response pattern of anti-C1q and its reduction on C3b deposition on LDL is almost comparable to its control IgM, whereas increasing concentrations of anti-C1q showed a more linear reduction in C3b opsonization of acLDL (A). The addition of D-mannose to 10% NHS reduced C3b deposition on LDL, but not on acLDL. The reduction in C3b opsonization of LDL by D-mannose was almost similar to its control mannan (B). Binding of LDL to CHO-CR1 was significantly reduced by the addition of D-mannose (100 mM), MgEGTA (20 mM) or EDTA (20mM) to normal human serum (NHS). Addition of anti-C1q (20 µg/ml) to NHS resulted in a non-significant reduction in LDL binding to CHO-CR1 (C). AcLDL binding to CHO-CR1 was only reduced by the addition of EDTA to NHS, but not by anti-C1q, D-mannose or MgEGTA (D). Every figure represents the relative mean ± SEM of at least three experiments. *P < 0.05, **P < 0.01

mum concentration of 400 mM in a similar way as for mannan. In contrast, D-mannose did not affect C3b opsonization of acLDL (Figure 5B).

In addition, we conducted experiments to test, which complement pathways were involved in LDL and acLDL binding to CHO-CR1. For this purpose we incubated CHO-CR1 with fluorescent labeled LDL and acLDL, which were incubated with NHS substituted with either anti-C1q, D-mannose, MgEGTA or EDTA. By blocking the classical pathway with anti-C1q LDL binding to CHO-CR1 was slightly decreased. When the lectin pathway was blocked with D-mannose LDL binding to CHO-CR1 was significantly decreased. LDL binding to CHO-CR1 could also be inhibited with MgEGTA, which blocks both the classical and lectin pathway but not the alternative pathway, and EDTA, which blocks all three complement pathways (Figure 5C). AcLDL binding to CHO-CR1 was significantly decreased by the addition of EDTA and slightly affected by anti-C1q, but not by D-mannose or MgEGTA (Figure 5D).

Discussion

The current experiments show for the first time that opsonized LDL and acLDL can bind to CR1. LDL and acLDL can activate the complement system through different pathways with subsequent C3b opsonization of these lipoproteins followed by binding to CR1.

Recently, it was demonstrated *in vitro* that complement activation occurred when epithelial cells were incubated with oxLDL in NHS supplemented cell cultures. This resulted in increased C3b levels and the formation of membrane attack complexes and could be prevented by using HIS instead of NHS [11]. This study is in line with our results since we observed C3b opsonization and subsequent binding of LDL and acLDL to CR1 only after incubation with NHS. Our experiments also demonstrated that besides acLDL, native LDL was able to induce complement activation.

Enzymatically modified LDL (E-LDL) activates the C1 complex via the classical pathway [29]. Our data suggest that LDL binds to CR1 primarily via C3b formed by activation of the classical pathway, whereas acLDL binds to CR1 via both the classical and alternative pathways. We were able to demonstrate C1q binding to native LDL in a similar proportion as to IgM, but only with high concentrations of purified C1q. In contrast, a previous study showed that reconstitution of purified C1q to 10% C1q-depleted serum resulted only in C3b deposition on modified forms of LDL, but not on native LDL [11]. It is known that complement activity of serum decreases during C1q depletion and our results showed that acLDL was three times more potent in binding C1q when compared to native LDL or natural IgM [30]. This may explain the contrast with earlier results [11,30], since we observed that only sufficiently high enough NHS concentrations lead to C3b-opsonization of native LDL.

We also observed a reduction in CR1-bound LDL with decreased C3b opsonization with high concentrations of D-mannose. These results were unexpected since binding of MBL to LDL or acLDL could not be established in our hands, which is in line with published data [31]. A different study demonstrated binding of MBL to acLDL, but reconstitution of MBL deficient serum with purified MBL did not result in complement activation on acLDL [12]. Therefore, the exact contribution of MBL and the lectin pathway to the binding of LDL to CR1 needs to be explored further.

We studied the importance of the alternative pathway in these processes in addition to the classical and lectin pathways. Properdin was able to bind acLDL, but not native LDL. Moreover, MgEGTA, which blocks selectively the classical and lectin pathway but not the alternative pathway, did not affect acLDL binding to CHO-CR1, whereas it significantly reduced LDL binding to CHO-CR1. These results illustrate the importance of the alternative pathway in the binding of acLDL to CR1. It was already described that oxLDL is able to increase factor B in vitro, which is a stimulator of the alternative pathway [11]. Properdin has been linked to lipid metabolism before. Properdin deficient mice

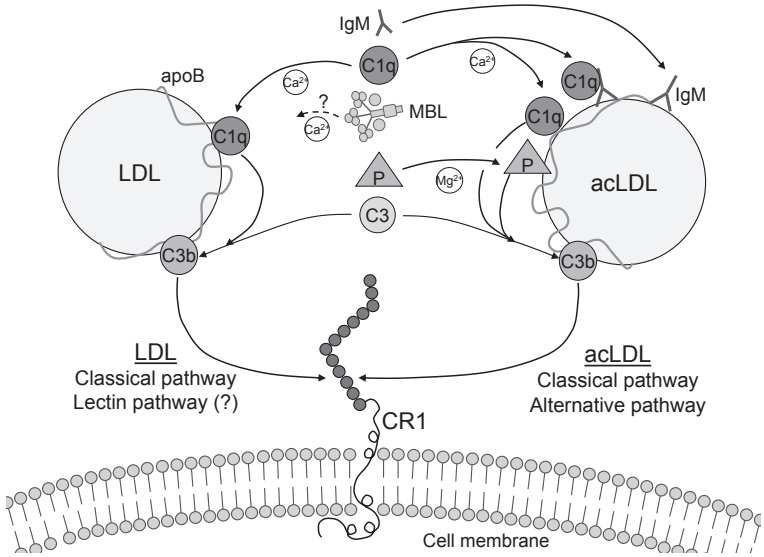

Figure 6: Schematic overview of the mechanism of CR1-bound LDL and acLDL. Binding of LDL to CR1 is mediated via the classical pathway. C1q binds first to LDL by C3b opsonization, which serves as the ligand for CR1. The role of Mannose-binding lectin (MBL) and the lectin pathway in CR1-bound LDL remains unclear since we were unable to show MBL binding to LDL despite an effect of alienating the lectin pathway. Binding of acLDL to CR1 is mediated via the classical and alternative pathway. AcLDL can be bound by both C1q and properdin (P), which are able to initiate the classical and alternative pathway, respectively. In addition, IgM, which is able to bind C1q, can also bind to acLDL. The LDL and modified LDL binding capacity of CR1 may provide an additional mechanism for cellular bound apolipoprotein B containing lipoproteins.

have an increased postprandial triglyceride response [10]. It is tempting to speculate that properdin deficient mice have increased postprandial lipemia due to a lack of properdin-mediated alternative complement activation with reduced CR1 binding of lipoproteins, but definitive evidence is yet lacking. We have only investigated properdin and alternative complement activation with native LDL and acLDL, but not with triglyceride-rich lipoproteins like VLDL and chylomicrons. Therefore, we cannot extrapolate our results to the metabolism of postprandial lipoproteins.

In Figure 6 a schematic model is provided, summarizing the complement pathways and its complement factors involved in LDL and acLDL binding to CR1. This model proposes that the classical pathway is involved in both LDL and acLDL binding to CR1, whereas the alternative pathway via properdin also regulates acLDL binding to CR1. The exact contribution of MBL and the lectin pathway in LDL binding to CR1 remains unclear.

Our data suggest an additional cellular binding mechanism for LDL and modified LDL via CR1, which is ubiquitously distributed throughout the human body. CR1 was first isolated from human erythrocytes where CR1 facilitates immune adherence of immune complexes and microorganisms to erythrocytes [18-20,32]. CR1 is also present on various inflammatory cells. Monocytes, macrophages and neutrophils are able to internalize CR1 as a defense mechanism for phagocytosis of CR1-bound immune complexes in contrast to erythrocytes [17].

The expression of CR1 by inflammatory cells is highly variable within subjects, as reported previously showing that an increase in CR1 expression by monocytes and neutrophils occurs upon the ingestion of a fat meal [33]. On average, monocytes and neutrophils express 20000 to 140000 CR1 molecules per cell [34,35]. In theory, monocytes, macrophages and neutrophils may internalize opsonized forms of LDL via CR1 with subsequent clearance of LDL from the circulation, but with the risk of increased activation and initiation of an inflammatory reaction. Internalization of lipoproteins by neutrophils has been associated with increased neutrophil activation demonstrated by an increased expression of degranulation markers, which contributes to endothelial dysfunction [36]. In addition, uptake of modified LDL by macrophages stimulates intracellular transcription of the C3 gene with subsequent increased secretion of C3 by macrophages [37]. In theory, this could lead to additional opsonization of circulating lipoproteins.

In contrast, erythrocyte-mediated lipoprotein clearance may have anti-atherogenic properties. Erythrocytes express only approximately 300-800 CR1 molecules per erythrocyte, but most of the total CR1 in the circulation is present on erythrocytes since they form the majority of the cellular blood compartment [38]. The liver clears immune complexes and microorganisms bound to erythrocytes at high rates [18]. Something similar may occur with CR1-mediated erythrocyte-bound LDL, which may be an atheroprotective mechanism [15,39]. Recently it was shown that erythrocytes contribute to reverse

cholesterol transport by an unknown mechanism [40]. We propose that this could be a CR1-mediated process in which opsonized LDL bind to CR1 present on erythrocytes. This may explain the presence of apolipoprotein B, the structural protein of LDL and of the triglyceride-rich lipoproteins, on circulating erythrocytes, as described by our group recently [21,39]. We have recently shown that the presence of apolipoprotein B on erythrocytes is associated to lower intima media thickness values of the carotids in humanst [21,39]. Therefore, the capacity of erythrocytes to bind atherogenic lipoproteins via CR1 may be atheroprotective.

It should be noted that all studies presented here have been done *in vitro* and it is still not clear whether LDL and modified forms of LDL are able to induce complement activation *in vivo*. In our experiments C3b was not present on freshly isolated LDL or acLDL and addition of NHS was necessary to initiate C3b opsonization of LDL and acLDL. 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 due to avid binding to CR1 expressing cells like endothelial cells, leukocytes and erythrocytes [15,21,36,39]. Animal and clinical studies are necessary to further elucidate the importance of lipoprotein mediated complement activation and the presence of CR1-mediated cellular lipoprotein transport *in vivo*.

In conclusion, CR1 is able to bind opsonized native LDL and acLDL. Binding of LDL to CR1 is mediated via the classical pathway, whereas binding of acLDL is mediated via both the classical and alternative pathways.

References

1. Oksjoki R, Kovanen PT, Pentikainen MO. Role of complement activation in atherosclerosis. Curr Opin Lipidol 2003;14:477-482
2. Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: A key system for immune surveillance and homeostasis. Nat Immunol 2010;11:785-797
3. Kojima M, Presanis JS, Sim RB. The mannose-binding lectin (MBL) route for activation of complement. Adv Exp Med Biol 2003;535:229-250
4. Walport MJ. Complement. First of two parts. N Engl J Med 2001;344:1058-1066
5. Walport MJ. Complement. Second of two parts. N Engl J Med 2001;344:1140-1144
6. Bhakdi S, Torzewski M, Klouche M, Hemmes M. Complement and atherogenesis: Binding of CRP to degraded, nonoxidized LDL enhances complement activation. Arterioscler Thromb Vasc Biol 1999;19:2348-2354
7. van Oostrom AJ, Alipour A, Plokker TW, Sniderman AD, Castro Cabezas M. The metabolic syndrome in relation to complement component 3 and postprandial lipemia in patients from an outpatient lipid clinic and healthy volunteers. Atherosclerosis 2007;190:167-173
8. Vaisar T, Pennathur S, Green PS, Gharib SA, Hoofnagle AN, Cheung MC, et al. Shotgun proteomics implicates protease inhibition and complement activation in the antiinflammatory properties of HDL. J Clin Invest 2007;117:746-756
9. Alipour A, van Oostrom AJ, Van Wijk JP, Verseyden C, Plokker HW, Jukema JW, et al. Mannose binding lectin deficiency and triglyceride-rich lipoprotein metabolism in normolipidemic subjects. Atherosclerosis 2009;206:444-450
10. Gauvreau D, Roy C, Tom FQ, Lu H, Miegueu P, Richard D, et al. A new effector of lipid metabolism: Complement factor properdin. Mol Immunol 2012;51:73-81
11. Ebrahimi KB, Fijalkowski N, Cano M, Handa JT. Decreased membrane complement regulators in the retinal pigmented epithelium contributes to age-related macular degeneration. J Pathol 2013;229:729-742
12. Fraser DA, Tenner AJ. Innate immune proteins C1q and mannan-binding lectin enhance clearance of atherogenic lipoproteins by human monocytes and macrophages. J Immunol 2010;185:3932-3939
13. Goldstein JL, Brown MS. The LDL receptor. Arterioscler Thromb Vasc Biol 2009;29:431-438
14. Mitra S, Goyal T, Mehta JL. Oxidized LDL, LOX-1 and atherosclerosis. Cardiovasc Drugs Ther 2011;25:419-429
15. Bovenberg SA, Alipour A, Elte JW, Rietveld AP, Janssen JW, van de Geijn GJ, et al. Cell-mediated lipoprotein transport: A novel anti-atherogenic concept. Atheroscler Suppl 2010;11:25-29
16. Langeggen H, Berge KE, Johnson E, Hetland G. Human umbilical vein endothelial cells express complement receptor 1 (CD35) and complement receptor 4 (CD11c/CD18) in vitro. Inflammation 2002;26:103-110
17. Fearon DT, Wong WW. Complement ligand-receptor interactions that mediate biological responses. Annu Rev Immunol 1983;1:243-271
18. Cornacoff JB, Hebert LA, Smead WL, VanAman ME, Birmingham DJ, Waxman FJ. Primate erythrocyte-immune complex-clearing mechanism. J Clin Invest 1983;71:236-247
19. Waxman FJ, Hebert LA, Cornacoff JB, VanAman ME, Smead WL, Kraut EH, et al. Complement depletion accelerates the clearance of immune complexes from the circulation of primates. J Clin Invest 1984;74:1329-1340
20. Nelson Jr RA. The immune-adherence phenomenon. Science 1953;118:733-737

21. Klop B, van de Geijn GJ, Bovenberg SA, van der Meulen N, Elte JW, Birnie E, et al. Erythrocyte-bound apolipoprotein B in relation to atherosclerosis, serum lipids and ABO blood group. PLoS One 2013;8:e75573
22. Makrides SC, Scesney SM, Ford PJ, Evans KS, Carson GR, Marsh HC, Jr. Cell surface expression of the C3b/C4b receptor (CR1) protects Chinese hamster ovary cells from lysis by human complement. J Biol Chem 1992;267:24754-24761
23. Nickells M, Hauhart R, Krych M, Subramanian VB, Geoghegan-Barek K, Marsh HC, Jr., et al. Mapping epitopes for 20 monoclonal antibodies to CR1. Clin Exp Immunol 1998;112:27-33
24. Redgrave TG, Roberts DC, West CE. Separation of plasma lipoproteins by density-gradient ultracentrifugation. Anal Biochem 1975;65:42-49
25. Van Berkel TJ, Nagelkerke JF, Harkes L, Kruijt JK. Processing of acetylated human low-density lipoprotein by parenchymal and non-parenchymal liver cells. Involvement of calmodulin? Biochem J 1982;208:493-503
26. Gaarkeuken H, Siezenga MA, Zuidwijk K, van Kooten C, Rabelink TJ, Daha MR, et al. Complement activation by tubular cells is mediated by properdin binding. Am J Physiol Renal Physiol 2008;295:F1397-1403
27. Roos A, Bouwman LH, Munoz J, Zuiverloon T, Faber-Krol MC, Fallaux-van den Houten FC, et al. Functional characterization of the lectin pathway of complement in human serum. Mol Immunol 2003;39:655-668
28. Nauta AJ, Trouw LA, Daha MR, Tijsma O, Nieuwland R, Schwaeble WJ, et al. Direct binding of C1q to apoptotic cells and cell blebs induces complement activation. Eur J Immunol 2002;32:1726-1736
29. Biro A, Thielens NM, Cervenak L, Prohaszka Z, Fust G, Arlaud GJ. Modified low density lipoproteins differentially bind and activate the C1 complex of complement. Mol Immunol 2007;44:1169-1177
30. van der Pol P, Roos A, Berger SP, Daha MR, van Kooten C. Natural IgM antibodies are involved in the activation of complement by hypoxic human tubular cells. Am J Physiol Renal Physiol 2011;300:F932-940
31. Faro J, Chen Y, Jhaveri P, Oza P, Spear GT, Lint TF, et al. L-ficolin binding and lectin pathway activation by acetylated low-density lipoprotein. Clin Exp Immunol 2008;151:275-283
32. Fearon DT. Regulation of the amplification C3 convertase of human complement by an inhibitory protein isolated from human erythrocyte membrane. Proc Natl Acad Sci U S A 1979;76:5867-5871
33. Klop B, van de Geijn GJ, Njo TL, Janssen HW, Rietveld AP, van Miltenburg A, et al. Leukocyte cell population data (Volume Conductivity Scatter) in postprandial leukocyte activation. Int J Lab Hematol 2013;6:644-651
34. Quadri RA, Schifferli JA. Over-estimation of the number of complement receptor type 1 (CR1) on erythrocytes. Scand J Immunol 1992;36:125-130
35. Wilson JG, Wong WW, Schur PH, Fearon DT. Mode of inheritance of decreased C3b receptors on erythrocytes of patients with systemic lupus erythematosus. N Engl J Med 1982;307:981-986
36. Alipour A, van Oostrom AJ, Izraeljan A, Verseyden C, Collins JM, Frayn KN, et al. Leukocyte activation by triglyceride-rich lipoproteins. Arterioscler Thromb Vasc Biol 2008;28:792-797
37. Mogilenko DA, Kudriavtsev IV, Trulioff AS, Shavva VS, Dizhe EB, Missyul BV, et al. Modified low density lipoprotein stimulates complement C3 expression and secretion via liver X receptor and toll-like receptor 4 activation in human macrophages. J Biol Chem 2012;287:5954-5968
38. Hinderling PH. Red blood cells: A neglected compartment in pharmacokinetics and pharmacodynamics. Pharmacol Rev 1997;49:279-295

39. Bovenberg SA, Klop B, Alipour A, Martinez-Hervas S, Westzaan A, van de Geijn GJ, et al. Erythrocyte-associated apolipoprotein B and its relationship with clinical and subclinical atherosclerosis. Eur J Clin Invest 2012;42:365-370
40. Hung KT, Berisha SZ, Ritchey BM, Santore J, Smith JD. Red blood cells play a role in reverse cholesterol transport. Arterioscler Thromb Vasc Biol 2012;32:1460-1465

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 adherance 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 erythematodes [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 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

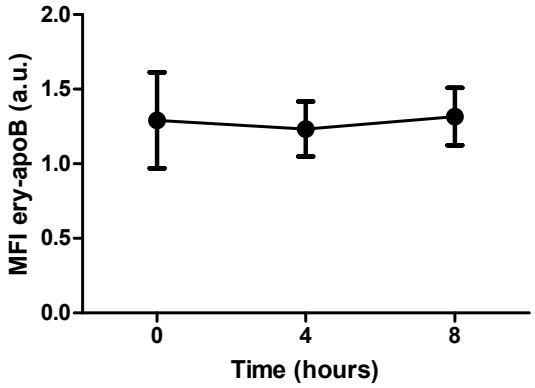

Figure 1: A postprandial change in erythrocyte-bound apolipoprotein B (ery-apoB) could not be detected. Ery-apoB was measured up to eight hours after the ingestion of a standardized oral fat loading test (n = 12). Data represent mean ± SEM. MFI = mean fluorescent intensity.

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).

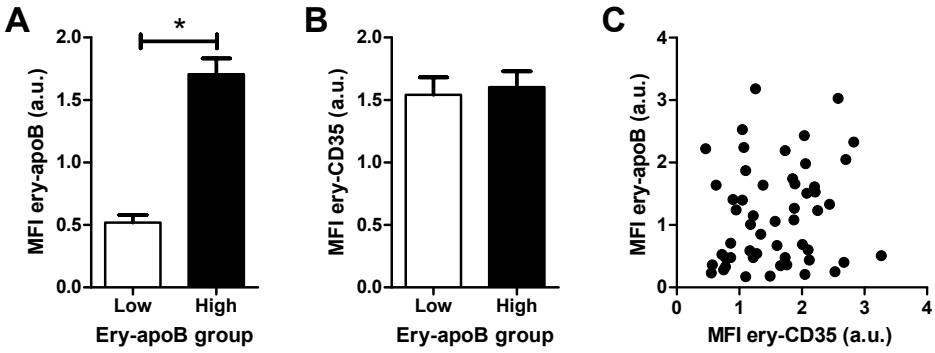

Figure 2: Erythrocyte-bound apolipoprotein B (ery-apoB) was unrelated to the expression of complement receptor 1 (CR1 or CD35) by erythrocytes. Subjects with low (n = 25) and high (n = 28) ery-apoB (≤ 0.20 a.u. and ≥ 2.00 a.u., respectively) were asked to have their ery-apoB measured again on a separate 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

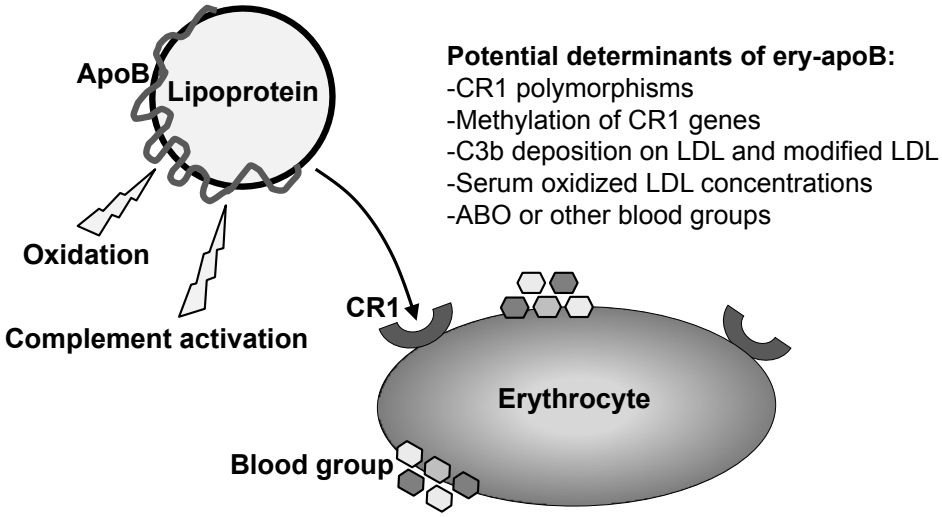

Figure 3: Potential determinants of erythrocyte-bound apolipoprotein B (ery-apoB), which should be further explored in future studies.

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

1. Jorgensen AB, Frikke-Schmidt R, West AS, Grande P, Nordestgaard BG, Tybjaerg-Hansen A. Genetically elevated non-fasting triglycerides and calculated remnant cholesterol as causal risk factors for myocardial infarction. Eur Heart J 2013;34:1826-1833
2. Klop B, Proctor SD, Mamo JC, Botham KM, Castro Cabezas M. Understanding postprandial inflammation and its relationship to lifestyle behaviour and metabolic diseases. Int J Vasc Med 2012;2012:947417
3. Mamo JC, Wheeler JR. Chylomicrons or their remnants penetrate rabbit thoracic aorta as efficiently as do smaller macromolecules, including low-density lipoprotein, high-density lipoprotein, and albumin. Coron Artery Dis 1994;5:695-705
4. Varbo A, Benn M, Tybjaerg-Hansen A, Jorgensen AB, Frikke-Schmidt R, Nordestgaard BG. Remnant cholesterol as a causal risk factor for ischemic heart disease. J Am Coll Cardiol 2013;61:427-436
5. Mora S, Glynn RJ, Boekholdt SM, Nordestgaard BG, Kastelein JJ, Ridker PM. On-treatment non-high-density lipoprotein cholesterol, apolipoprotein B, triglycerides, and lipid ratios in relation to residual vascular risk after treatment with potent statin therapy: Jupiter (Justification for the Use of statins in Prevention: an Intervention Trial Evaluating Rosuvastatin). J Am Coll Cardiol 2012;59:1521-1528
6. Boekholdt SM, Arsenault BJ, Mora S, Pedersen TR, LaRosa JC, Nestel PJ, et al. Association of LDL cholesterol, non-HDL cholesterol, and apolipoprotein B levels with risk of cardiovascular events among patients treated with statins: A meta-analysis. JAMA 2012;307:1302-1309
7. Alipour A, Valdivielso P, Elte JW, Janssen HW, Rioja J, van der Meulen N, et al. Exploring the value of apoB48 as a marker for atherosclerosis in clinical practice. Eur J Clin Invest 2012;42:702-708
8. Nordestgaard BG, Benn M, Schnohr P, Tybjaerg-Hansen A. Nonfasting triglycerides and risk of myocardial infarction, ischemic heart disease, and death in men and women. JAMA 2007;298:299-308
9. Bansal S, Buring JE, Rifai N, Mora S, Sacks FM, Ridker PM. Fasting compared with nonfasting triglycerides and risk of cardiovascular events in women. JAMA 2007;298:309-316
10. Klop B, Elte JW, Cabezas MC. Dyslipidemia in obesity: Mechanisms and potential targets. Nutrients 2013;5:1218-1240
11. Do R, Willer CJ, Schmidt EM, Sengupta S, Gao C, Peloso GM, et al. Common variants associated with plasma triglycerides and risk for coronary artery disease. Nat Genet 2013;45:1345-1352
12. Klop B, Rego AT, Castro Cabezas M. Alcohol and plasma triglycerides. Curr Opin Lipidol 2013;24:321-326
13. Klop B, Jukema JW, Rabelink TJ, Castro Cabezas M. A physician's guide for the management of hypertriglyceridemia: The etiology of hypertriglyceridemia determines treatment strategy. Panminerva Med 2012;54:91-103
14. Catapano AL, Reiner Z, De Backer G, Graham I, Taskinen MR, Wiklund O, et al. ESC/EAS guidelines for the management of dyslipidaemias the task force for the management of dyslipidaemias of the European Society of Cardiology (ESC) and the European Atherosclerosis Society (EAS). Atherosclerosis 2011;217 Suppl 1:1-44
15. Sniderman AD, Williams K, Contois JH, Monroe HM, McQueen MJ, de Graaf J, et al. A meta-analysis of low-density lipoprotein cholesterol, non-high-density lipoprotein cholesterol, and apolipoprotein B as markers of cardiovascular risk. Circ Cardiovasc Qual Outcomes 2011;4:337-345

16. Berglund L, Brunzell JD, Goldberg AC, Goldberg IJ, Sacks F, Murad MH, et al. Evaluation and treatment of hypertriglyceridemia: An Endocrine Society clinical practice guideline. J Clin Endocrinol Metab 2012;97:2969-2989
17. Sidhu D, Naugler C. Fasting time and lipid levels in a community-based population: A cross-sectional study. Arch Intern Med 2012;172:1707-1710
18. Langsted A, Freiberg JJ, Nordestgaard BG. Fasting and nonfasting lipid levels: Influence of normal food intake on lipids, lipoproteins, apolipoproteins, and cardiovascular risk prediction. Circulation 2008;118:2047-2056
19. Klop B, Cohn JS, van Oostrom AJ, van Wijk JP, Birnie E, Castro Cabezas M. Daytime triglyceride variability in men and women with different levels of triglyceridemia. Clin Chim Acta 2011;412:2183-2189
20. Khera AV, Mora S. Fasting for lipid testing: Is it worth the trouble?: Comment on "fasting time and lipid levels in a community-based population". Arch Intern Med 2012;172:1710-1711
21. Touma Z, Gladman DD, Ibanez D, Urowitz MB. Ability of non-fasting and fasting triglycerides to predict coronary artery disease in lupus patients. Rheumatology (Oxford) 2012;51:528-534
22. Varbo A, Benn M, Tybjaerg-Hansen A, Nordestgaard BG. Elevated remnant cholesterol causes both low-grade inflammation and ischemic heart disease, while elevated low-density lipoprotein cholesterol causes ischemic heart disease without inflammation. Circulation 2013;128:1298-1309
23. van Oostrom AJ, Sijmonsma TP, Verseyden C, Jansen EH, de Koning EJ, Rabelink TJ, et al. Postprandial recruitment of neutrophils may contribute to endothelial dysfunction. J Lipid Res 2003;44:576-583
24. Alipour A, van Oostrom AJ, Izraeljan A, Verseyden C, Collins JM, Frayn KN, et al. Leukocyte activation by triglyceride-rich lipoproteins. Arterioscler Thromb Vasc Biol 2008;28:792-797
25. Jackson KG, Poppitt SD, Minihane AM. Postprandial lipemia and cardiovascular disease risk: Interrelationships between dietary, physiological and genetic determinants. Atherosclerosis 2012;220:22-33
26. Klop B, van de Geijn GJ, Njo TL, Janssen HW, Rietveld AP, van Miltenburg A, et al. Leukocyte cell population data (Volume Conductivity Scatter) in postprandial leukocyte activation. Int J Lab Hematol 2013;6:644-651
27. Strohacker K, Breslin WL, Carpenter KC, Davidson TR, Agha NH, McFarlin BK. Moderate-intensity, premeal cycling blunts postprandial increases in monocyte cell surface CD18 and CD11a and endothelial microparticles following a high-fat meal in young adults. Appl Physiol Nutr Metab 2012;37:530-539
28. Tarcin O, Yavuz DG, Ozben B, Telli A, Ogunc AV, Yuksel M, et al. Effect of vitamin D deficiency and replacement on endothelial function in asymptomatic subjects. J Clin Endocrinol Metab 2009;94:4023-4030
29. Dong Y, Stallmann-Jorgensen IS, Pollock NK, Harris RA, Keeton D, Huang Y, et al. A 16-week randomized clinical trial of 2000 international units daily vitamin D3 supplementation in black youth: 25-hydroxyvitamin D, adiposity, and arterial stiffness. J Clin Endocrinol Metab 2010;95:4584-4591
30. Spittler A, Willheim M, Leutmezer F, Ohler R, Krugluger W, Reissner C, et al. Effects of 1 alpha,25-dihydroxyvitamin D3 and cytokines on the expression of MHC antigens, complement receptors and other antigens on human blood monocytes and U937 cells: Role in cell differentiation, activation and phagocytosis. Immunology 1997;90:286-293
31. Oh J, Weng S, Felton SK, Bhandare S, Riek A, Butler B, et al. 1,25(OH)2 vitamin D inhibits foam cell formation and suppresses macrophage cholesterol uptake in patients with type 2 diabetes mellitus. Circulation 2009;120:687-698

32. Cutini PH, Campelo AE, Agriello E, Sandoval MJ, Rauschemberger MB, Massheimer VL. The role of sex steroids on cellular events involved in vascular disease. J Steroid Biochem Mol Biol 2012;132:322-330
33. Deitch EA, Ananthakrishnan P, Cohen DB, Xu da Z, Feketeova E, Hauser CJ. Neutrophil activation is modulated by sex hormones after trauma-hemorrhagic shock and burn injuries. Am J Physiol Heart Circ Physiol 2006;291:H1456-1465
34. Correale J, Ysrraelit MC, Gaitan MI. Gender differences in 1,25 dihydroxyvitamin D3 immunomodulatory effects in multiple sclerosis patients and healthy subjects. J Immunol 2010;185:4948-4958
35. Deleskog A, Piksasova O, Silveira A, Gertow K, Baldassarre D, Veglia F, et al. Serum 25-hydroxyvitamin D concentration in subclinical carotid atherosclerosis. Arterioscler Thromb Vasc Biol 2013;33:2633-2638
36. van Oostrom AJ, van Wijk J, Castro Cabezas M. Lipaemia, inflammation and atherosclerosis: Novel opportunities in the understanding and treatment of atherosclerosis. Drugs 2004;64 Suppl 2:19-41
37. Bermudez B, Lopez S, Varela LM, Ortega A, Pacheco YM, Moreda W, et al. Triglyceride-rich lipoprotein regulates apoB48 receptor gene expression in human THP-1 monocytes and macrophages. J Nutr 2012;142:227-232
38. Bovenberg SA, Klop B, Alipour A, Martinez-Hervas S, Westzaan A, van de Geijn GJ, et al. Erythrocyte-associated apolipoprotein B and its relationship with clinical and subclinical atherosclerosis. Eur J Clin Invest 2012;42:365-370
39. Klop B, van de Geijn GJ, Bovenberg SA, van der Meulen N, Elte JW, Birnie E, et al. Erythrocyte-bound apolipoprotein B in relation to atherosclerosis, serum lipids and abo blood group. PLoS One 2013;8:e75573
40. Hung KT, Berisha SZ, Ritchey BM, Santore J, Smith JD. Red blood cells play a role in reverse cholesterol transport. Arterioscler Thromb Vasc Biol 2012;32:1460-1465
41. Bovenberg SA, Alipour A, Elte JW, Rietveld AP, Janssen JW, van de Geijn GJ, et al. Cell-mediated lipoprotein transport: A novel anti-atherogenic concept. Atheroscler Suppl 2010;11:25-29
42. Goldstein JL, Brown MS. The LDL receptor. Arterioscler Thromb Vasc Biol 2009;29:431-438
43. Mitra S, Goyal T, Mehta JL. Oxidized LDL, LOX-1 and atherosclerosis. Cardiovasc Drugs Ther 2011;25:419-429
44. Fearon DT, Wong WW. Complement ligand-receptor interactions that mediate biological responses. Annu Rev Immunol 1983;1:243-271
45. Cornacoff JB, Hebert LA, Smead WL, VanAman ME, Birmingham DJ, Waxman FJ. Primate erythrocyte-immune complex-clearing mechanism. J Clin Invest 1983;71:236-247
46. Waxman FJ, Hebert LA, Cornacoff JB, VanAman ME, Smead WL, Kraut EH, et al. Complement depletion accelerates the clearance of immune complexes from the circulation of primates. J Clin Invest 1984;74:1329-1340
47. Ng YC, Schifferli JA, Walport MJ. Immune complexes and erythrocyte CR1 (complement receptor type 1): Effect of CR1 numbers on binding and release reactions. Clin Exp Immunol 1988;71:481-485
48. Buraczynska M, Ksiazek P, Wacinski P, Zukowski P, Dragan M, Bednarek-Skublewska A. Complement receptor 1 gene polymorphism and cardiovascular disease in dialyzed end-stage renal disease patients. Hum Immunol 2010;71:878-882

Chapter 10

Nederlandse samenvatting

“De Interactie tussen Lipoproteïnen, het Complement Systeem en Bloedcellen in Relatie tot Atherosclerose”

Inleiding

Cholesterol en triglyceriden zijn vetten en kunnen daarom niet oplossen in water of bloed. Daarom worden cholesterol en triglyceriden "verpakt" samen met specifieke eiwitten tot een lipoproteïne ("cholesterolpartikel"), zodat het cholesterol en de triglyceriden vervoerd kunnen worden door het bloed. De stofwisseling van cholesterol en triglyceriden (vetzuren) is complex en gaat verder dan "goed" en "slecht" cholesterol. In beide gevallen gaat het om dezelfde stof (cholesterol), maar de verpakking waarin het door het bloed vervoerd wordt, bepaalt of het cholesterol in de vaatwand afgezet wordt (het slechte cholesterol, het zogenaamde LDL-cholesterol) of juist uit de vaatwand kan worden teruggehaald (het goede cholesterol, het HDL-cholesterol).

De beschreven studies in dit proefschrift zijn voornamelijk gericht op de stofwisseling en transport van de "slechte" lipoproteïnen met het apolipoproteïne (apo) B als kenmerkende eiwit. Hierbij is altijd precies één apo B per "slechte" lipoproteïne aanwezig. Na een maaltijd worden cholesterol en triglyceriden opgenomen in de dunne darm en verpakt met apo B48 om vervolgens in de bloedbaan terecht te komen. De lipoproteïnen na een maaltijd (chylomicronen en VLDL) bevatten vooral triglyceriden uit de maaltijd en een relatief kleine hoeveelheid cholesterol. In de bloedbaan worden vervolgens de triglyceriden uit deze lipoproteïnen vrijgemaakt voor energiegebruik of voor opslag van energie in vetcellen. Uiteindelijk blijven kleine, compacte lipoproteïnen over waar voornamelijk nog cholesterol in aanwezig is, de zogenaamde low-density lipoproteïnen (LDL). Alle "slechte" lipoproteïnen met het apo B: de chylomicronen, VLDL en LDL kunnen zorgen voor cholesterolafzetting in de vaatwand (atherosclerose, oftewel aderverkalking).

De behandeling van verhoogde triglyceriden

Het cholesterol in zowel LDL als in VLDL en chylomicronen draagt bij aan de ontwikkeling van atherosclerose wat uiteindelijk leidt tot hart- en vaatziekten (bijvoorbeeld een hartinfarct of beroerte). Daarnaast zijn verhoogde triglyceriden ook gerelateerd aan hart- en vaatziekten, onder andere omdat zij een goede maat vormen voor een toename in VLDL en chylomicronen die niet gemeten worden wanneer het LDL-cholesterol bepaald wordt in het bloed. Daarom is naast het LDL-cholesterol, ook de behandeling van verhoogde triglyceriden belangrijk, waarbij de behandeling wordt bepaald door de onderliggende aandoening. Zo kunnen overgewicht, diabetes mellitus (suikerziekte), alcoholgebruik en specifieke erfelijke aandoeningen leiden tot verhoogde triglyceriden met elk hun eigen behandeling. In **Hoofdstuk 2** worden de diagnostiek en behandeling van patiënten met verhoogde triglyceriden besproken onderbouwd door de huidige

wetenschappelijke literatuur. Gewichtsverlies en een vetarm dieet zijn het belangrijkste in de behandeling van verhoogde triglyceriden, wat meestal afdoende is bij patiënten met alleen overgewicht. Echter medicamenteuze behandeling is meestal noodzakelijk bij patiënten met een erfelijke stoornis waarbij de triglyceriden niet uit de chylomicronen en VLDL gehaald kunnen. Fibraten hebben dan de voorkeur, omdat zij het vrijmaken van triglyceriden uit de lipoproteïnen stimuleren, evenals het metabolisme van de vetzuren afkomstig uit die triglyceriden. Bij patiënten met hart- en vaatziekten en/of diabetes mellitus worden in eerste instantie statines, de klassieke cholesterolverlagers, geadviseerd, eventueel aangevuld met een fibraat. De doseringen van de statines dienen opgehoogd te worden bij deze patienten die ook een hypertriglyceridemie hebben totdat de streefwaarden van apo B (< 0.8 g/l) danwel non-HDL-C (< 3.3 mmol/l) behaald is.

De variabiliteit van triglyceriden gedurende de dag

Een probleem bij het meten van triglyceriden in het bloed is dat de waarde afhankelijk is van het feit of de patiënt wel of niet gegeten heeft. In nuchtere toestand zijn de triglyceriden het laagst en stijgen door het eten gedurende de dag. Daarom wordt in de regel geadviseerd om triglyceriden en cholesterol (waarbij het LDL-cholesterol geschat wordt met behulp van de triglyceridenwaarde) te meten in nuchtere toestand. Ondanks een nuchtere toestand zijn triglyceriden reeds zeer variabel. Wij hebben de variabiliteit van triglyceriden in nuchtere toestand en niet-nuchtere toestand onderzocht en met elkaar vergeleken in **Hoofdstuk 3**. Het bleek dat de variabiliteit van triglyceriden binnen een individu hoog is, namelijk ongeveer 25%, maar dat deze redelijk hetzelfde blijft gedurende de dag. De absolute variabiliteit van triglyceriden in nuchtere toestand bedroeg 0.28 – 0.35 mmol/l en steeg tot maximaal 0.28 – 0.69 mmol/l voor het slapen gaan. Dit was ondanks de geleidelijke triglyceriden stijging in het bloed van 0.45 mmol/l bij vrouwen en 1.05 mmol/l bij mannen gedurende de dag. Derhalve zal het gebruik van niet-nuchtere triglyceridewaarden in plaats van nuchtere de nauwkeurigheid van het voorspellen van het risico op hart- en vaatziekten weinig beïnvloeden. Deze kennis kan bijdragen aan de implementatie van het meten van triglyceriden en cholesterol in niet-nuchtere toestand wat in de klinische praktijk wat prettiger is voor patiënten.

Activatie van witte bloedcellen na de maaltijd

De ontwikkeling van atherosclerose komt niet alleen door afzetting van cholesterol in de vaatwand, maar het afweersysteem levert ook een belangrijke bijdrage. Daarnaast beïnvloeden lipoproteïnen die rijk zijn aan triglyceriden, die met name na de maaltijd

vrijkomen, de circulerende witte bloedcellen. De witte bloedcellen zijn bedoeld voor de afweer tegen bacteriën en virussen, maar zij reageren ook op chylomicronen en VLDL alsof zij indringers van buitenaf zijn. Na een maaltijd is dan ook een activatie van witte bloedcellen in het bloed te meten. De witte bloedcellen scheiden cytokinen (activerende stoffen) uit, nemen toe in grootte en krijgen een verhoogd aantal ankers om te kunnen plakken aan de vaatwand met schadelijke gevolgen. De mate van activatie van witte bloedcellen is te meten, maar vereist veel werk in het laboratorium. Recent is ontdekt dat geautomatiseerde meetapparatuur in het klinische laboratorium op een eenvoudige manier patiënten met een infectie kan onderscheiden van patiënten zonder infectie door middel van een drietal parameters: de mate waarin de witte bloedcel licht verstrooit, de grootte van de witte bloedcel en de elektrische geleiding door een witte bloedcel. Wij waren geïnteresseerd of dezelfde geautomatiseerde meetapparatuur ook dergelijke veranderingen in witte bloedcellen na vetinname kon meten. Uit ons onderzoek beschreven in **Hoofdstuk 4** blijkt dat dit inderdaad het geval is, waarbij de lichtverstrooiing van witte bloedcellen 1.4 – 1.7% afneemt na de maaltijd, terwijl de grootte van de witte bloedcellen licht toeneemt wat inderdaad duidt op activatie van de witte bloedcellen. Deze resultaten kunnen nieuw onderzoek naar activatie van witte bloedcellen na de maaltijd vereenvoudigen in de toekomst.

Vitamine D en activatie van witte bloedcellen na de maaltijd

De activatie van witte bloedcellen na een maaltijd is schadelijk voor de vaatwand, doordat de cellen die de vaatwand bekleden zich anders gaan gedragen door de schadelijke stoffen die witte bloedcellen uitscheiden na een maaltijd. Daarnaast kunnen geactiveerde witte bloedcellen die cholesterol hebben opgenomen ook de vaatwand binnendringen wat kan leiden tot atherosclerose. Tot nu toe zijn er weinig behandelingen bekend om de maaltijd geïnduceerde activatie van witte bloedcellen te verminderen. Wij hebben onderzocht in **Hoofdstuk 5** of de toediening van vitamine D mogelijk de schadelijke activatie van witte bloedcellen kon tegengaan. Vitamine D heeft namelijk niet alleen effect op de botstofwisseling, maar ook op witte bloedcellen en hart- en vaatziekten. Gezonde proefpersonen ondergingen tweemaal een slagroomproef, een controle proef zonder vitamine D en een tweede slagroomproef na vitamine D suppletie. Gedurende de slagroomproef werd zowel bij de mannelijke als de vrouwelijke proefpersonen na vitamine D suppletie een verbetering van de elasticiteit van de slagaders waargenomen. Daarnaast was er na vitamine D de activatie van witte bloedcellen na de maaltijd verminderd, maar alleen bij vrouwen. De expressie van CD11b en CD35, celstructuren die toenemen op het celoppervlak van witte bloedcellen tijdens activatie, daalden 10.5 – 17.0% bij vrouwen na vitamine D suppletie vergeleken met de controle slagroomproef. Een

dergelijke vermindering in activatie in witte bloedcellen werd echter niet waargenomen bij mannen. Waarom er een verschil tussen de mannelijke en vrouwelijke proefpersonen in de activatie van witte bloedcellen werd waargenomen is nog niet duidelijk, maar we vermoeden een samenwerking tussen vitamine D en oestrogenen die een gunstig effect op de witte bloedcellen bewerkstelligt. De resultaten van dit onderzoek tonen wederom aan dat vitamine D een positief effect heeft op de slagaders, maar onduidelijk is of dit direct gerelateerd is aan een verminderde activatie van witte bloedcellen na een maaltijd.

Transport van lipoproteïnen door rode bloedcellen

Aangezien de interactie tussen lipoproteïnen met witte bloedcellen gerelateerd is aan de ontwikkeling van atherosclerose, waren we geïnteresseerd of binding van lipoproteïnen aan rode bloedcellen ook een relatie heeft met atherosclerose. Ongeveer 99% van alle cellen in het bloed zijn namelijk rode bloedcellen die voornamelijk belangrijk zijn voor het transport van zuurstof. Theoretisch zou het beschermend zijn als de rode bloedcellen de slechte lipoproteïnen (met het eiwit apo B) door binding zouden wegvangen en zo het contact tussen de lipoproteïnen en de vaatwand kunnen voorkomen. In **Hoofdstuk 6** wordt beschreven dat patiënten met hart- en vaatziekten minder apo B op hun rode bloedcellen hebben dan gezonde controlepersonen (0.62 ± 0.09 a.u. versus 1.18 ± 0.10 a.u.). Tevens bleek dat hoe lager de hoeveelheid apo B gebonden aan de rode bloedcellen was, hoe dikker de vaatwand van de halsslagader (correlatiecoëfficiënt van -0.214). Deze resultaten zijn suggestief voor een beschermend effect op de ontwikkeling van atherosclerose, wanneer rode bloedcellen veel slechte lipoproteïnen met apo B aan zich binden.

De resultaten werden nog eens bevestigd in een uitgebreidere studie beschreven in **Hoofdstuk 7**, waarbij proefpersonen met een verdikte halsslagader 23.2% minder apo B aan hun rode bloedcellen gebonden hadden in vergelijking tot proefpersonen met een normale dikte van hun halsslagader. Tevens werd aangetoond dat de hoeveelheid apo B op rode bloedcellen niet werd beïnvloed door het tijdelijk staken van cholesterolverlagende statines (-0.04 ± 0.39 a.u.) of door de hoeveelheid vrije lipoproteïnen met apo B in het bloed. De correlatiecoëfficiënt tussen het vrije apo B en het apo B gebonden aan rode bloedcellen bedroeg namelijk slechts -0.025 tot 0.038.

Met deze twee studies hebben we aangetoond dat apo B gebonden aan rode bloedcellen geassocieerd is met hart- en vaatziekten onafhankelijk van het gebruikelijke lipidenspectrum dat dagelijks gemeten wordt in de klinische praktijk. Opvallend was dat mensen met bloedgroep O gemiddeld twee keer zoveel apo B op hun rode bloedcellen hadden als mensen met bloedgroep A, B of AB. Een duidelijke verklaring hiervoor is nog

niet gevonden. Echter dit effect van de ABO bloedgroep kan een aanwijzing zijn voor het potentiële bindingsmechanisme van lipoproteïnen aan rode bloedcellen.

De complement receptor 1 als mechanisme voor celgebonden lipoproteïnen

Het is bijzonder dat lipoproteïnen aan rode bloedcellen kunnen binden, omdat deze cellen geen bekende receptoren of ankers hebben om lipoproteïnen te kunnen binden. Naast zuurstoftransport kunnen rode bloedcellen ook bacteriën en antilichaamcomplexen binden en vervoeren om hen vervolgens af te geven aan witte bloedcellen of aan de lever of milt voor een effectieve afweerreactie. Dit mechanisme op rode bloedcellen functioneert via de specifieke complement receptor 1 die op de rode en witte bloedcellen aanwezig is. De complement receptor 1 maakt deel uit van het complement systeem, wat deel uitmaakt van het niet-specifieke afweersysteem. De activatie van het complement systeem kan via drie verschillende routes lopen: de klassieke route, de lectine route en de alternatieve route. Uiteindelijk leidt activatie van van één van deze routes tot complement activatie, opsoniseren van celoppervlakten met complementfactoren en eventueel verval van cellen/bacterieën. Wij hebben onderzocht of de binding van LDL en speciaal gemodificeerd LDL (die beiden ook het eiwit apo B bevatten) kan binden aan de complement receptor 1.

Hoofdstuk 8 beschrijft de resultaten van dit onderzoek. Met behulp van laboratoriumproeven en gemuteerde kweekcellen bleek het normale en gemodificeerde LDL inderdaad aan de complement receptor 1 te kunnen binden. Het LDL en gemodificeerd LDL konden echter niet op een directe manier aan de complement receptor 1 binden, maar alleen na vorming van complement component C3b door activatie van het complementsysteem. Tevens vonden we dat de activatie van het complement systeem door LDL en gemodificeerd LDL licht verschilde, waarbij de klassieke route voor beide belangrijk was, maar dat ook de alternatieve route geactiveerd werd door gemodificeerd LDL.

Deze resultaten kunnen een verklaring geven hoe apo B-bevattende lipoproteïnen aan rode, maar ook witte bloedcellen kunnen binden aangezien de complement receptor 1 ook in grote aantallen op witte bloedcellen aanwezig is. Onderzocht zal moeten worden of dit daadwerkelijk ook het geval is in het menselijk lichaam aangezien de experimenten beschreven in **Hoofdstuk 8** allemaal in een kunstmatige proefopstelling ("*in vitro*") waren verricht.

Concluderend

Er is toenemend bewijs dat het niet noodzakelijk is om cholesterol en triglyceriden in nuchtere toestand te meten, maar dat een niet-nuchtere meting in de meeste gevallen ook kan volstaan. De ontwikkeling van atherosclerose is complex en gaat veel verder dan alleen het cholesterolgehalte in het bloed. Atherosclerose, vet- en cholesterolstofwisseling en het immuunsysteem inclusief rode bloedcellen zijn nauw met elkaar verbonden. Hierbij lijkt het immuunsysteem zowel schadelijk als beschermend tegen de ontwikkeling van atherosclerose te kunnen werken. Vitamine D kan deels de schadelijke activatie van witte bloedcellen na vetinname voorkomen en heeft een gunstig effect op de elasticiteit van van de slagaders. Rode bloedcellen kunnen juist een beschermend effect uitoefenen op de vaatwand door middel van transport van apo B-bevattende lipoproteïnen. Dit transportmechanisme kan mogelijk plaatsvinden door middel van het complement systeem met de complement receptor 1 danwel de ABO bloedgroep of een combinatie van beide.

Chapter 11

Additional information

List of publications

M.A. de Vries, **B. Klop**, S.A. Eskes, T.L.J.M. van der Loos, F.J.M. Klessens-Godfroy, J. Wiebolt, H.W. Janssen, E.M. Westerman, M. Castro Cabezas. The postprandial situation as a pro-infammatory condition. Clin Investig Arterioscler 2014, *in press*

M.A. de Vries, **B. Klop**, M. Castro Cabezas. The use of the non-fasting lipid profile in lipid-lowering therapy in clinical practice: Point of view. Atherosclerosis 2014;234:473-475

B. Klop, G.J.M. van de Geijn, E. Birnie, T.L. Njo, H.W. Janssen, H.G. Jansen, J.W. Jukema, J.W.F. Elte, M. Castro Cabezas. Gender differences in vitamin D3 mediated effects on postprandial leukocyte activation and arterial stiffness. Eur J Clin Nutr 2014;68:635-637

P.W. Raess, G.J.M. van de Geijn, T.L. Njo, **B. Klop**, D. Sukhachev, G. Wertheim, T. McAleer, S.R. Master, A. Bagg. Automated screening for myelodysplastic syndromes through analysis of complete blood count and cell population data parameters. Am J Hematol 2014;89:369-374

B. Klop, C. Verseyden, J. Ribalta, J. Salazar, L. Masana, M. Castro Cabezas. MTP gene polymorhisms and postprandial lipemia in familial combined hyperlipidemia: effects of treatment with atorvastatin. Clin Investig Arterioscler 2014;26:49-56

A. Torres do Rego, **B. Klop**, E. Birnie, J.W.F. Elte, V. Cachofeiro Ramos, L.A. Alvarez-Sala Walther, M. Castro Cabezas. Diurnal triglyceridemia in relation to alcohol intake in men. Nutrients 2013;5:5114-5126

D.F. van Breukelen-van der Stoep, **B. Klop**, D. van Zeben, J.M.W. Hazes, M. Castro Cabezas. Cardiovascular risk in rheumatoid arthritis: how to lower the risk? Atherosclerosis 2013;231:163-172

Van Ramshorst GH, **Klop B**, Hop WC, Israelsson LA, Lange JF. Closure of midline laparotomies by means of small stitches: practical aspects of a new technique. Surg Technol Int 2013;23:23-30

B. Klop, G.J.M. van de Geijn, S.A. Bovenberg, N. van der Meulen, J.W.F. Elte, E. Birnie, T.L. Njo, J.W. Janssen, A. van Miltenburg, J.W. Jukema, M. Castro Cabezas. Erythrocyte-bound apolipoprotein B in relation to atherosclerosis, serum lipids and ABO blood group. PLoS One 2013;8:e75573

B. Klop, G.J.M. van de Geijn, T.L. Njo, J.W. Janssen, A.P. Rietveld, A. van Miltenburg, L. Fernández-Sender, J.W.F. Elte, M. Castro Cabezas. Leukocyte cell population data (VCS) in postprandial leukocyte activation. Int J Lab Hematol 2013;35:644-651

B. Klop, J.W.F. Elte, M. Castro Cabezas. Dyslipidemia in obesity: mechanisms and potential targets. Nutrients 2013;5:1218-1240

B. Klop, A. Torres do Rego, M. Castro Cabezas. Alcohol and plasma triglycerides. Curr Opin Lipidol 2013;24:321-326

M. Castro Cabezas, J.P.H. van Wijk, J.W.F. Elte, **B. Klop**. Effects of metformin on the regulation of free fatty acids in insulin resistance: a double blind, placebo controlled study. J Nutr Metab 2012;2012:394623

B. Klop, J.W. Jukema, T.J. Rabelink, M. Castro Cabezas. A physician's guide for the management of hypertriglyceridemia: the etiology of hypertriglyceridemia determines treatment strategy. Panminerva Med 2012;54:91-103

B. Klop, M. Castro Cabezas. Chylomicrons: a key biomarker and risk factor of cardiovascular disease and for the understanding of obesity. Curr Cardiovasc Risk Rep, 2012;6:27-34

B. Klop, S.D. Proctor, J.C. Mamo, K.M. Botham, M. Castro Cabezas. Understanding postprandial inflammation and its relationship to lifestyle behaviour and metabolic diseases. Int J Vasc Med 2012;2012:947147

S.A. Bovenberg, **B. Klop**, A. Alipour, S. Martinez-Hervas, A. Westzaan, G.J.M. van de Geijn J.W. Janssen, T. Njo, E. Birnie, R. van Mechelen, A.P. Rietveld, J.W.F. Elte, M. Castro Cabezas. Erythrocyte-associated apolipoprotein B and its relationship with clinical and subclinical atherosclerosis. Eur J Clin Invest 2012;42:365-370

B. Klop, T.M. van de Berg, A.P. Rietveld, J. Chaves, J.T. Real, J.F. Ascaso, R. Carmena, J.W.F. Elte, M. Castro Cabezas. AT1 receptor gene polymorphisms in relation to postprandial lipemia. Int J Vasc Med 2012;2012:271030

B. Klop, J.S. Cohn, A.J.H.H.M. van Oostrom, J.P.H. van Wijk, E. Birnie, M. Castro Cabezas. Daytime triglyceride variability in men and women with different levels of triglyceridemia. Clin Chim Acta 2011;412:2183-2189

B. Klop, M.G. Scheffer, E. McFadden, F. Bracke, B. van Gelder. Treatment of pacemaker-induced superior vena cava syndrome by balloon angioplasty and stenting. Neth Heart J 2011;19:41-46

B. Klop, J.W.F. Elte, M. Castro Cabezas. Cholesterolhomeostase en enterohepatische connectie: nieuwe inzichten in cholesterolabsorptie. Ned Tijdschr Geneeskd 2010;154:A2503

Curriculum vitae

The author of this thesis was born in Woerdense Verlaat on February the 26th 1984. In 2002 he graduated from the Coornhert Gymnasium and started medical school at the Erasmus University in Rotterdam the same year. During his last year of medical training he investigated the implementation of a new surgical technique to close abdominal wounds at the surgical department of the Erasmus MC and Sundsvall Hospital (Sweden) under supervision of prof. dr. L.A. Israelsson, prof. dr. J.F. Lange and dr. G.H. van Ramshorst. This experience provoked his interest for medical research and showed him the available scientific possibilities of non-academic hospitals. After receiving his medical degree in October 2008 he started as a cardiology resident at the Maasstad Ziekenhuis in Rotterdam until April 2010.

In May 2010, Boudewijn started his work on this thesis at the department of Internal medicine of the Sint Franciscus Gasthuis in Rotterdam. He was supervised by dr. M. Castro Cabezas en dr. J.W.F. Elte in collaboration with prof. dr. J.W. Jukema from the department of Cardiology from the Leiden University Medical Center. The majority of the research described in this thesis was performed in close collaboration with the department of Clinical Chemistry at the Sint Franciscus Gasthuis itself (dr. J.W. Janssen, drs. T.L. Njo and dr. G.J.M. van de Geijn). Part of the research was also performed at the department of Nephrology at the Leiden University Medical Center (prof. dr. C. van Kooten and prof. dr. T.J. Rabelink) and at Sanquin in Amsterdam (dr. R. van Bruggen).

After finishing his research in May 2013, he started as a cardiology resident at the Erasmus MC and started his specialty training in Cardiology in April 2014, which takes part in the Albert Schweitzer Hospital (dr. E.F.H. van Bommel and dr. M.J.M. Kofflard) and Erasmus MC (dr. J.W. Deckers and dr. T.W. Galema).

Besides being a physician, Boudewijn likes to make sculptures as a hobby. The sculpture shown throughout this thesis was specially crafted by the author to illustrate the described research.