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**LDL apheresis beyond lipid reduction:**

A study of three different apheresis columns in heterozygous familial hypercholesterolemia and *ex vivo*

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Anders Hovland
List of papers

**Paper I**


**Paper II**


**Paper III**


**Paper IV**

**Abbreviations**

FH: Familial Hypercholesterolemia  
LDL: Low-Density Lipoprotein  
PCSK-9: Proprotein convertase subtilisin/kexin type 9  
heFH: Heterozygous Familial Hypercholesterolemia  
hoFH: Homozygous Familial Hypercholesterolemia  
CP: Classical Pathway  
MBL: Mannose Binding Lectin  
PAI-1: Plasminogen Activator Inhibitor-1  
Lp(a): Lipoprotein(a)  
Apo(a): Apolipoprotein(a)  
K 4: Kringle-4  
AV-fistula: arteriovenous fistula  
CRP: C-reactive protein  
RANTES: Regulated upon Activation, Normal T-cell Expressed, and Secreted  
TNF-α: Tumor Necrosis Factor- α  
TAT: Thrombin-antithrombin  
TCC: Terminal Complement Complex  
AP: Alternative Pathway  
C1rs-inh: C1rs-inhibitor  
IL-1ra: Interleukin-1 receptor antagonist  
IP-10: Interferon gamma-induced protein 10
1 Introduction/background

1.1 Atherosclerosis

Atherosclerosis is the hardening/thickening of the arteries (from Greek; arterio-referring to the arteries and sclerosis-calcification/hardening). Arteries are often referred to as the “tubes” of the body, and these tubes can become narrowed due to accumulation of fat and calcification, leading to known diseases such as heart attacks and strokes.

Atherosclerotic diseases are quite common and are a leading cause of morbidity and mortality in the western world. Accordingly, they contribute significantly to health care costs. An American survey has indicated that the prevalence of all cardiovascular disease will increase from 37% in 2010 to 41% in 2030, and the direct cost will increase from 273 billion dollars in 2010 to 818 billion dollars in 2030.\textsuperscript{[1]} The American Heart Association has launched ambitious goals for treating and preventing cardiovascular disease.\textsuperscript{[2]} In Norway, the prevalence of all cardiovascular disease is unknown, however a decline in mortality in cardiovascular diseases in Norway has been observed.\textsuperscript{[3]} We do not have exact data regarding cardiovascular prevalence or mortality in our own region, but the latest registration of myocardial infarctions demonstrated a fairly stable incidence of 300/100 000 per year. Additionally, with the exception of elderly patients, a decline in mortality has also been observed.\textsuperscript{[4]}

Traditional risk factors for atherosclerosis include genetic predisposition, hypercholesterolemia, hypertension, diabetes, smoking, obesity, age and gender. There seems to be a shift in traditional risk factors globally as systolic hypertension is reduced\textsuperscript{[5]}, serum-cholesterol is modestly reduced\textsuperscript{[6]} and there is an increase in body mass index.\textsuperscript{[7]} Western countries have imposed stronger regulations leading to decreased use of tobacco, resulting in reduced coronary artery disease.\textsuperscript{[8]}

The development of an atherosclerotic lesion in the artery wall typically evolves over the course of years as demonstrated by several autopsy studies\textsuperscript{[9,10]} and confirmed in a later ultrasound study.\textsuperscript{[11]} Today there is consistent evidence that atherosclerosis starts with endothelial injury, adhesion of leukocytes to the endothelial layer,
migration of leucocytes to the intima of the arterial wall, formation of foam cells and further build up of an atherosclerotic plaque (\cite{12} and Figure 1 below). Acute coronary events, including acute myocardial infarction, are most often caused by an imposing thrombus on the atherosclerotic plaque resulting from rupture of the thin fibrous cap.\cite{13}

The atherosclerotic spectrum is wide, ranging from acute coronary events such as unstable angina, myocardial infarction and sudden death to peripheral atherosclerosis and stroke.\cite{14}

Figure 1. 1a shows the normal artery wall with the endothelial lining and the adventitia. 1b depicts the altered endothelium with adhesion of leukocytes, entering the intima with formation of cholesterol filled foam cells. 1c shows further development of the atherosclerotic plaque with influx of smooth muscle cells from the adventitia of the vessel wall. In 1d the final complication due to thrombosis resulting from rupture of the fibrous cap is shown. Reprinted by permission from Macmillan Publishers Ltd: Nature. Libby P, Ridker PM and Hansson G. Progress and challenges in translating the biology of atherosclerosis. Nature 2011;473:317-25. Copyright 2011.
1.2 Familial Hypercholesterolemia

Familial hypercholesterolemia (FH) is an autosomal dominant inherited disease leading to high levels of low-density lipoprotein (LDL-) cholesterol and increased risk of premature atherosclerotic disease.

1.2.1 Historical aspects/disease mechanism

Although there are indications that the disease has been present for a long time\[15\], the link between lipid stigmata including xantomae and premature coronary artery disease was initially acknowledged in the first half of the twentieth century.\[16-18\] Several studies during the 1950s characterized the genetic features of the disease.\[19,20\] However, the landmark studies by Brown and Goldstein firmly documented the link between familial hypercholesterolemia and the structure of the LDL-receptor.\[21-26\] They demonstrated that mutations in the gene (chromosome 19) coding for the LDL-receptor led to reduced LDL-receptor activity and increased levels of LDL-cholesterol. The prevailing form of hypercholesterolemia is heterozygous in which approximately 50% of the LDL-receptors are missing. Other forms of autosomal dominant hypercholesterolemia have since been discovered, including those associated with apolipoprotein B mutations\[27\] or mutations in the gene PCSK9 (encoding proprotein convertase subtilisin/kexin type 9).\[28\] In addition, a rare autosomal recessive form has been described.\[29,30\]

The heterozygous form of FH (heFH) is quite common; most studies indicate a prevalence of 1/500 in white Caucasians\[26\], while subpopulations in certain parts of the world indicate a prevalence of 1/100 \[31,32\]. A Norwegian study found a prevalence of 1/300 in Østfold \[33\] estimating approximately 16500 persons with heFH in Norway today. Recent genetic testing has revealed 5273 positive FH mutation tests in Norway (TP Leren, personal communication, 27 October 2011).
1.2.2 Clinical features/diagnosis

Persons with heFH have an increased risk of premature atherosclerosis compared with matched individuals without the disease. Before the statin-era (a statin is cholesterol lowering medication; the correct term is 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors), approximately half of the men with FH had overt ischemic heart disease before the age of 50. Later registry data have confirmed the increased risk of atherosclerotic disease associated with FH, the risk seems reduced when treated adequately. A recent non-invasive study has demonstrated substantial sub-clinical coronary artery disease in persons with FH, despite adequate treatment.

heFH leads to increased levels of LDL-cholesterol in the serum; this is a highly penetrating trait and in an adult population the LDL-cholesterol level is about 9 mmol/L if no treatment is given. A Norwegian study has shown that children/adolescents (2-18 years of age) have an average LDL-cholesterol of 5.6 mmol/L. This feature of elevated LDL-cholesterol may be the only manifestation of the disease during the first decade. Later on more typical stigmata such as arcus cornea and tendon xantomas (figure 2) often occur.

Figure 2.
Xantoma in the achilles tendon, pathognomonic for FH.
(Picture from the Lipid Clinic, Nordland Hospital)
The most frequent form of premature atherosclerosis in heFH is coronary heart disease ranging from stable angina to acute coronary syndromes including sudden cardiac death.\cite{26} Furthermore, in heFH patients without symptoms of coronary artery disease, an invasive study has demonstrated more coronary atherosclerosis than in matched controls.\cite{42} This finding was recently reproduced non-invasively.\cite{40} Some data indicate that the risk of aortic root and valve disease is increased in heFH \cite{43}, a finding disputed by others.\cite{44}

Homozygotes in FH (hoFH) are rare (1/1000 000), and at this time there are 7 patients with this diagnosis in Norway (Leiv Ose, personal communication 31 October 2011). These patients have LDL-cholesterol levels ranging from 15 to 20 mmol/L, and often have severe atherosclerotic complications in the first two decades of life.\cite{26} However, new register data show a survival benefit of modern lipid lowering therapy.\cite{45}

1.2.3 Treatment

There is general consensus that LDL-cholesterol reduction is mandatory in patient with FH.\cite{46-48} This should be achieved with lifestyle modification and in most instances by means of statin therapy. A new European consensus paper also suggests that statin therapy should be started in children above the age of 10 with heFH and/or if the LDL- cholesterol is above 5 mmol/L.\cite{49} The European treatment goal is LDL-cholesterol below 2.5 mmol/L\cite{47}, while the treatment goal in the US is an LDL-cholesterol reduction of more than 50% \cite{48}. If statin therapy cannot be tolerated, bile acid sequestrants, ezetimibe or niacin are treatment options. If LDL-cholesterol levels cannot be controlled by means of medication, LDL apheresis is the next treatment step (see 1.4). New treatment options for elevated LDL-cholesterol including apolipoprotein B synthesis inhibitors and Cholesterol Ester Transfer Protein Inhibitors are currently being tested.\cite{50}
1.3 Inflammation in atherosclerosis and FH

Inflammation is closely linked to atherosclerotic diseases \(^{[51-53]}\), and new data support that in patients with FH, the degree of inflammation is increased.\(^{[54,55]}\)

Accordingly, statins, the most widely used drug for FH treatment have been shown to reduce inflammation.\(^{[56,57]}\) Our group has accordingly shown that statin-treated FH patients have the same inflammatory profile and endothelial function as controls.\(^{[58]}\)

Previously there have been few cross-over studies on how different LDL apheresis columns affect inflammation.

1.3.1 The complement system and atherosclerosis

The complement system is part of our innate defence against infections and consists of about 30 proteins mainly secreted by hepatic cells and monocytes. While complement is a “resting defence”, it can quickly be activated by events such as infections or detection of foreign surfaces. There are three different mechanisms by which the complement system can be activated via the classical pathway (CP), the mannose binding lectin pathway (MBL) and the alternative pathway (figure 3). These different activation paths lead to a common pathway involving formation of C3a and C5a and other split products. From this point, the cascade continues to the terminal pathway with resulting lysis of the target cell (figure 3). The complement system is central in the pathophysiology of several diseases including atherosclerosis \(^{[59]}\) which it can both promote and protect against.\(^{[60-62]}\) More specifically, it has recently been demonstrated that a polymorphism in complement factor H (Y402H) decreases cardiovascular risk in patients with FH.\(^{[63]}\)
Figure 3 shows the three different paths leading to activation of the complement system, the common pathway, and finally to formation of the terminal complement complex. “Reprinted from Trends Immunol, 23:61-63, Mollnes TE, Song WC, Lambris JD. “Complement in inflammatory tissue damage and disease” with permission from Elsevier.

1.3.2 Cytokines and atherosclerosis

Cytokines are small proteins functioning as signalling molecules in the nervous- and the immune system. Cytokines can be categorized as pro-inflammatory, and hence pro-atherosclerotic, or anti-inflammatory.\textsuperscript{[52,64,65]} There are data supporting that untreated FH patients have a pro-inflammatory cytokine profile.\textsuperscript{[54,66,67]} Few studies have examined cytokines prospectively in LDL apheresis in FH.
1.4 Hemostasis and atherosclerosis
Previous studies have shown that factors important in hemostasis, including fibrinogen and plasminogen activator inhibitor-1 (PAI-1), are associated with increased risk of coronary artery disease.\[68-71\] However, these factors have not been associated with increased risk of coronary artery disease in a cross section of patients with treated familial hypercholesterolemia.\[72\] There are few prospective data on the effect of LDL apheresis regarding these parameters.

1.5 Lipoprotein (a) and familial hypercholesterolemia
The Lipoprotein(a) [Lp(a)] particle is a complex of a hydrophobic LDL-cholesterol-particle and a hydrophilic apolipoprotein(a) [Apo(a)] –particle. The isoforms and thus the size of apo(a) depend on the number of so-called kringle 4 (K 4) repeats.\[73\] Risk of atherosclerosis is associated with levels of Lp(a), and smaller isoforms are thought to be more atherogenic.\[74-76\] Lp(a) level is thought to be an important risk factor for atherosclerosis for individuals with FH.\[77,78\] Standard lipid lowering therapy, however, has failed to lower Lp(a) levels.\[79\] There is ample evidence that LDL apheresis lowers Lp(a) effectively \[80,81\], however there are few prospective studies where evaluation of the effect on the size of the Lp(a) particles have been performed.

1.6 LDL apheresis in FH
LDL apheresis is an extracorporeal treatment in which the patient’s blood is passed through an apheresis machine with filters/columns that selectively remove the LDL-cholesterol, resembling how a patient with renal failure is treated with hemodialysis to clear “waste products”. The procedure is performed in specialist centers throughout the world, when the LDL levels cannot be controlled with medications alone, or when patients cannot tolerate the medication.
1.6.1 Apheresis in clinical medicine/historical aspects

Apheresis was first performed in Paris in 1967 by means of plasma exchange with removal of serum cholesterol[82], and was first described in the English literature in 1975.[83] The technique has since evolved from non-specific plasma exchange to more selective LDL-cholesterol removal. Some advocate use of the term lipid apheresis, as several lipoproteins are removed including chylomicrons, very low-density lipoprotein (VLDL) and LDL-cholesterol.[80] Most systems used today utilize a column that “selectively” removes LDL-cholesterol from blood or from plasma. Venous access is needed, either through a venous catheter or through an arteriovenous (A-V) fistula. Anticoagulation is mandatory during treatment. LDL apheresis has also been used in order to remove Lp(a) as mentioned above (1.3), and there are some case reports on using LDL apheresis to lower elevated triglycerides, including from our center.[84]

Figure 4. Schematic drawing of LDL apheresis. Blood is passed from the patient through a central line or an A-V fistula, through the apheresis system. In the example shown, blood is passed through a plasma separation column, and the plasma is then passed through the LDL apheresis column removing the lipoproteins. The "rinsed" plasma is then mixed with the cellular components before being returned to the patient. Plasma-sep: Plasma separation column. LDL-apher: LDL apheresis column.
1.6.2 LDL apheresis in FH

There is general agreement that LDL apheresis should be used in patients with FH when LDL-cholesterol levels cannot be otherwise controlled. In the US, the Food and Drug Administration has approved LDL apheresis for the following indications:

- FH homozygotes with LDL-cholesterol > 12.8 mmol/L
- FH heterozygotes with LDL-cholesterol > 7.7 mmol/L
• FH heterozygotes with LDL-cholesterol >5.1 mmol/L if they have concomitant coronary artery disease

The British, German and international criteria are quite similar.\textsuperscript{[87]}

1.6.3 Possible unwanted effects of the treatment

Side effects occur with all types of treatment and in a small clinical study, our group found a high proportion of reported side effects in LDL apheresis.\textsuperscript{[88]} As we pointed out in a current review, even if the treatment seems well tolerated, further prospective studies need to be carried out in this field.\textsuperscript{[89]} This is mainly due to the fact that many of the studies report different kinds of apheresis treatment (other than LDL apheresis) together with LDL apheresis. The proportion of side effects varies widely between the centers, suggesting that side effects are not registered uniformly. This area of research concerning side effects should be further explored as apheresis is administered weekly, and possibly for the life of the patient.

1.6.4 Biocompatibility regarding LDL apheresis

“Biocompatibility refers to the ability of a biomaterial to perform its desired function with respect to a medical therapy, without eliciting any undesirable local or systemic effects in the recipient or beneficiary of that therapy, but generating the most appropriate beneficial cellular or tissue response in that specific situation, and optimizing the clinically relevant performance of that therapy.”\textsuperscript{[90]} Thus when performing extracorporeal treatment such as LDL apheresis, it becomes paramount that the interaction between blood and biomaterials does not elicit any unwanted responses. Along these lines, in addition to LDL-cholesterol lowering, apheresis should be beneficial to the patient with regard to total lipid profile (beyond LDL-), complement activation, cytokine response, and hemostatic and fibrinolytic factors. Biocompatibility as such, for the patient receiving LDL apheresis, concerns the achievement of a beneficial lipid-, complement-, cytokine-, hemostatic and fibrinolytic response, all of which are clinically relevant for patients prone to atherosclerotic diseases. Several studies have shown that complement activation precedes later inflammatory responses\textsuperscript{[91-94]}, and the complement system is therefore of particular interest in biocompatibility studies.
1.6.5 Concluding remarks on previous studies of LDL apheresis

There are numerous studies on LDL apheresis (using this term in a Medline search retrieves 1083 results (1st November 2011). Most of the studies have focused on efficacy regarding LDL-cholesterol lowering. However, many of these studies are retrospective, small and address different kinds of patients (both hoFH and heFH patients and sometimes also familial combined hyperlipidemia). There are very few studies that prospectively compare different types of LDL apheresis columns/systems with regard to complement, cytokines, hemostasis, fibrinolysis and Lp(a) in a controlled population with heFH and thus exploring biocompatibility issues.
2 Aims of study

2.1 General aims
The general aim of the study was to explore, in a prospective manner, how three different LDL apheresis columns/systems affected complement, cytokines, hemostasis, fibrinolysis and Lp(a) in a group of patients with genetically proven heFH. Secondly, we wanted to study biocompatibility issues raised by the clinical study in an ex vivo setting.

2.2 Specific aims

Paper I
The aim of the first paper was to explore how three different LDL apheresis columns (DL-75, LA-15 and EC-50W) affected lipoprotein proportions, sensitive CRP, complement activation products and cytokine profile in a clinical in vivo setting including a group of patients with FH.

Paper II
The aim of the second paper was to explore how DL-75, LA-15 and EC-50W columns affected hemostatic and fibrinolytic parameters in vivo in the same group of patients with FH.

Paper III
The aim of the third clinical paper was to explore how the three LDL apheresis columns affected the levels of Lp(a) during treatment, and if the isoform of the apolipoprotein was important with regard to Lp(a) lowering in vivo, in the same patients described above.

Paper IV
The aim of the fourth paper was to address biocompatibility regarding the DL-75, LA-15 and EC-50W columns with complement activation as the read-out, and hence to address the “complement compatibility” of the three columns in a closed circuit ex vivo model with freshly donated blood from healthy donors. Complement was chosen as read-out, in the first of several ex-vivo papers as complement activation in
previous studies have been shown to precede other inflammatory responses in a number of settings
3 Materials and Methods

3.1 Paper I-III
The first three papers were based on a clinical trial including three patients with known heFH (from mutation testing), all three established in LDL apheresis in our Department of Nephrology/Department of Cardiology/Lipid Clinic due to intolerance to statins (muscle pain) before the study. Therefore the number of patients was low, but at the time of the study our hospital was the largest center for LDL apheresis in Norway, illustrating the challenge in performing systematic studies on this topic. Furthermore, we took a number of methodological measures to counteract the limited number of participants. All apheresis treatments were performed in one site, by an experienced/consistent group of apheresis nurses and doctors. All the lab samples were handled systematically, and in the same way, with short distance (three minutes walking) to the research lab for further handling by experienced research staff. The patients had six treatments with each different LDL apheresis columns (DL-75, LA-15 and EC-50W), thus there were 18 samples taken before and after apheresis for each of the three patients. A prospective cross-over design was chosen, with random order for each patient in order to minimize carry-over effects.

Before and after treatment comparisons for two groups were carried out by means of T-test, while comparisons of more than two groups were done by means of ANOVA. If groups were not equally distributed, non-parametric statistics was chosen.

3.2 Paper IV
In the clinical study we noted activation of the complement system including differences between the columns. However the site-, the degree of- and the time course of complement activation were not known. This prompted us to carry out an ex vivo experiment in which healthy donor blood was passed through the same columns we tested in the clinical study. Blood from six donors was passed through the columns (DL-75, LA-15 and EC-50W) three times, thus there were a total of 18 experiments; six for each column. During each ex vivo procedure, samples were taken at different sites (two to four sites, depending on the column type (plasma
separation versus whole blood) at different times (0, 15, 30, 60, 120, 180 and 240 minutes).

Figure 6. Schematic ex vivo apheresis. 6A demonstrates the whole blood system with sampling before and after the LDL apheresis column. 6B shows the two plasma separation systems (LA-15 and EC-50W), in which samples were drawn before and after both the plasma separation columns and the apheresis columns.
4 Summary of main results

4.1. Paper I

In this paper we found that while all columns reduced LDL-cholesterol significantly, the three columns affected the complement system and cytokine activity differently. LDL-cholesterol was reduced by 67%, 75% and 68% by columns DL-75, LA-15 and EC-50W (all p<0.001), and sensitive C-reactive protein (CRP) was reduced with 77%, 72% and 43% for the same columns respectively (all p<0.001). TCC increased equally for all columns indicating that the complement cascade was activated to completion for all systems. There was a substantial increase in fragment Bb, reflecting alternative complement activation, while C4d was only marginally affected. The anaphylatoxins C3a and C5a were lowered more with the adsorption columns DL-75 and LA-15 than with the filtration column EC-50W. Accordingly, the adsorption columns DL-75 and LA-15 behaved similarly regarding cytokines, while the filtration column EC-50W tended to differ from the two other systems by statistical analyses. Some of the pro-inflammatory cytokines (RANTES and TNF-α) were reduced more by the adsorption- than filtration columns.

4.2 Paper II

This paper focused on the hemostatic changes in LDL apheresis in the clinical study, and there were differences between the three columns. Fibrinogen was reduced significantly for all columns; 28%, 32% and 48% for columns DL-75, LA-15 and EC-50W respectively. EC-50W was most effective when comparing all columns. Thrombin-antithrombin (TAT) increased with all columns, and TAT increased the most with DL-75, and the least with LA-15. Plasminogen activator inhibitor-1 (PAI-1) was reduced with all columns, especially for DL-75 which was significantly different from the EC-50W, which lowered PAI-1 least.
4.3 Paper III

Paper III demonstrated that all the columns lowered Lp(a) significantly, (p<0.001); 70%, 74% and 75% for columns DL-75, LA-15 and EC-50W respectively without significant differences between the columns. The three patients had dominating small isoforms of Apo(a); 14 and 32 K 4 domains, and the apheresis treatment did not affect the relative proportion of the two isoforms. The reduction in Lp(a) correlated with the reduction in LDL-cholesterol for all the columns together (r=0.51, p<0.01).

4.4 Paper IV

In the experimental ex vivo study we found that the apheresis columns used in the clinical studies affected complement activation and complement factors differently. Regarding the final common terminal complement complex (TCC), DL-75 was inert while the two plasma separation based systems (LA-15 and EC-50W) generated substantial amounts of TCC. Classical pathway activation was revealed by C4d and C1rs-C1inhibitor complexes for the plasma separation systems, while activation through the alternative pathway, measured by Bb, was most pronounced for LA-15. The anaphylatoxins C3a and C5a were equally induced by the plasma columns of LA-15 and EC-50W, however they were more efficiently cleared by the LA-15 column, leaving higher levels of the anaphylatoxin to enter the patient from the EC-50W column.
5 Discussion

5.1 Complement activation (paper I and IV)

Several papers have emphasized the importance of the complement system in the evolvement of atherosclerosis.\textsuperscript{[60-62,95]} Formation of TCC via the final common pathway is associated with atherosclerosis.\textsuperscript{[62]} Activation of the complement system via the alternative pathway appears to be pro-inflammatory, whereas activation via the classical/lectin pathways appears to be protective.\textsuperscript{[62]} The anaphylatoxins C3a and C5a are pro-inflammatory, and indeed C5a has been associated with plaque ruptures\textsuperscript{[95]} known to lead to clinical events such as acute myocardial infarctions. Furthermore, it has been shown that the degree of complement activation measured by TCC is inversely correlated with outcome in the acute clinical setting.\textsuperscript{[96]}

TCC

Fadul et al. have previously shown that in clinical LDL apheresis in hoFH patients (plasma separation before apheresis), TCC increases after the plasma separation column and decreases after the LDL apheresis column, suggesting adsorption.\textsuperscript{[97]} In our clinical study (paper I) we found an increase in TCC for all columns in patients (AV-fistula) immediately after completion of LDL apheresis. The treatment time differed between the two studies, as did the form of FH, with our study involving only heFH. However, the findings suggest that extracorporeal treatment in the form of LDL apheresis induces complement activation to completion. In the \textit{ex vivo} study, we were able to examine the changes in complement in greater detail due to the use of several sampling sites and several sample times. Clearly, TCC increased substantially after the plasma separation columns in LA-15 and DL-75, but all the LDL apheresis columns (DL-75, LA-15 and EC-50W) seemed to clear TCC from the circulation over time. Accordingly, after 120 minutes the pre-return values of TCC were quite similar between the three systems, but during the first 120 minutes the TCC returned to the patient was systematically higher with the two plasma separation based systems than with the whole blood system DL-75, suggesting lesser complement activation with the latter. Therefore, over time, both LA-15 and EC-50W seem to clear the TCC produced as a result of activation in the plasma separation columns. However, this could also be a result of eventual coating of the apheresis columns over time, resulting in attenuation of the complement activation analogue. This is consistent with studies describing
the “first pass syndrome” in which allergic reactions to hemodialysis membranes were reduced with repetitive use.\textsuperscript{[98]}

\textit{Complement Activation Pathway}

Several studies indicate that the alternative pathway (AP) of complement activation is important when foreign surfaces interact with blood\textsuperscript{[99-101]}, even if other pathways are also important when considering biomaterials used in medicine.\textsuperscript{[102]} In our clinical study (paper I) we found evidence for activation through the alternative pathway for all columns, especially for the plasma separation based systems. However, only marginal changes in C4d were observed, suggesting that the classical/lectin pathways were of lesser importance. Our \textit{ex vivo} study, however, demonstrated that in addition to activation through the alternative pathway, the classical pathway was also important. There was an increase in C4d for both plasma separation columns, but not so for the whole blood column. The increase in C4d correlated positively with C1rs-inh, suggesting activation via the classical pathway. The EC-50W column cleared C4d to the least degree, and thus had higher pre-return values of C4d than the other columns. Activation through the classical pathway is traditionally thought to be dependent on antibodies, possibly suggesting binding of antibodies to the plasma separation columns. In addition, other mediators such as CRP, beta-amyloid and polyanions may also be able to induce this system.\textsuperscript{[59]} The clinical meaning of this finding is not obvious as activation through the classical/lectin pathways are associated with protection against atherosclerosis.\textsuperscript{[62]}
Anaphylatoxins

Fadul et al. showed that the anaphylatoxin C3a increased significantly after the plasma separation column, but decreased after the LDL apheresis column.\(^{97}\) In our clinical study we found that C3a increased for all the columns after treatment, while C5a decreased after treatment. The highest increase in C3a and lowest reduction in C5a was shown for the filtration column EC-50W, and thus the EC-50W column was least beneficiary with regards to the anaphylatoxins which are regarded as pro-atherogenic.\(^{62,95}\) In the ex vivo study (paper IV) the same tendency observed for TCC was seen here. That is, low levels of C3a and C5a for the whole blood system were observed while the two plasma separation systems increased C3a and C5a substantially.

The adsorption column LA-15 removed these anaphylatoxins over time, reducing pre-return values. However, the filtration column EC-50W was not able to lower the concentration of C3a and C5a, leading to higher levels of pre-return C3a and C5a. This could be important to individual patients with high risk of atherosclerotic disease as indeed is true of patients with heFH.\(^{38,39}\) In accordance with this, a recent paper has demonstrated that high levels of C3a and C5a increase the risk of lumen reduction after implantation of drug eluting stents in the coronary arteries.\(^{103}\)

Bicompatibility

When utilizing artificial surfaces during blood-biomaterial interaction as a part of LDL apheresis, it is important that other constituents of the blood are not affected in an adverse manner as iterated in the new definition of biocompatibility (section 1.6.4, page 19). Because complement activation precedes other inflammatory responses, we chose complement as the read-out. We found that the whole blood column DL-75 is an inert column with regard to complement activation, and is thus more complement compatible than the other two columns. The filtration column EC-50W is the least complement compatible column.
5.2 Sensitive CRP and cytokines (paper II)

Some data indicate that CRP could be causative with regard to atherosclerosis, but data are conflicting and a consensus has yet to be established. The JUPITER Trial clearly demonstrated that clinical end-points were reduced along with reductions in CRP and LDL-cholesterol in healthy persons with LDL-cholesterol below 3.4 mmol/L and CRP below 2 mg/L. Previous studies on LDL apheresis have demonstrated reductions in CRP levels. We found a decrease for all columns, especially for DL-75 and least for EC-50W. Whether or not reduction in CRP during apheresis is beneficial regarding clinical endpoints remains to be determined in larger clinical trials.

Several cytokines correlate with risk of atherosclerosis, and previous studies on apheresis have indicated that LDL apheresis columns affect cytokine levels. In our clinical study (paper I) we found a significant decrease in the amount of pro-inflammatory cytokines; TNF-α and RANTES, and an increase in the amount of anti-inflammatory cytokines IL-1ra and IP-10. The adsorption columns DL-75 and LA-15 had a more beneficial profile than the filtration column EC-50W, with regards to the pro- and anti-inflammatory cytokines. According to this; DL-75 and LA-15 were more biocompatible than EC-50W with regard to cytokine response. Adsorption of cytokines has been characterized in sepsis and Stefanutti et al. later reported on the cytokine profiles before and after LDL apheresis by means of plasma adsorption columns in patients with hoFH. They found a decrease in TNF-α, and a decrease in IL-10 with no change in IL-1ra. Therefore the reduction in TNF-α is similar to our results, while changes in the anti-inflammatory cytokines differ, as do the results when comparing with LA-15 (plasma separation and adsorption). It is possible that some of these changes could be explained by different methodology, and the fact that our study observed heFH while Stefanutti et al. examined hoFH. Recent reviews have proposed that some of the beneficial effects of LDL apheresis on clinical endpoints may be due in part to modulation of pro-inflammatory biomarkers.

5.3 Markers of hemostasis (paper II)

Previous studies have established that LDL apheresis lowers fibrinogen, despite some differences between the columns. We found that all columns lowered fibrinogen, especially for the filtration column EC-50W. However, the level of
fibrinogen is closely tied to other risk markers for atherosclerosis\textsuperscript{[119]}, so the net effect of fibrinogen lowering in LDL apheresis should be further tested.

A recent study has shown that PAI-1 is reduced in LDL apheresis\textsuperscript{[120]}, and a pilot study showed that TAT was suppressed during apheresis, while post apheresis values were elevated compared to baseline.\textsuperscript{[121]} We noted a decrease in PAI-1 for all columns, the largest reduction were in the adsorption columns DL-75 and LA-15, and the least reduction was observed in the filtration column, whereas the least increase in TAT was found in the adsorption column LA-15. Few studies have systematically compared the effect of different apheresis columns on these hemostatic markers, and our findings indicate that the adsorption column, LA-15, is the more biocompatible column with regard to hemostatic and fibrinolytic parameters. Recent data indicate that PAI-1 is pivotal in development of coronary thrombus, underlying acute coronary events.\textsuperscript{[122]} However, the prognostic value of this marker is uncertain.\textsuperscript{[123]} It has also been shown that formation of TAT complexes is increased in patients with acute myocardial infarction when compared to stable coronary artery disease.\textsuperscript{[124]} It has also been demonstrated that previous cholesterol lowering by means of statins reduces formation of TAT complexes in ST-segment elevation myocardial infarctions.\textsuperscript{[125]}
5.4 Lipoprotein (a) (paper III)

Reduction of Lp(a) with lipid apheresis has consistently reduced clinical endpoints in patients with coronary artery disease and elevated levels of Lp(a)\textsuperscript{[126]}. Studies examining the effect of LDL apheresis on Lp(a) levels have typically reduced Lp(a) by 60 to 80 percent\textsuperscript{[81,127]}. We found a similar 70-75\% reduction in Lp(a) and there were no inter-column differences. This is within threshold reduction (70\%) when treating patients with FH and elevated Lp(a) as recommended by Borberg et al.\textsuperscript{[127]}

In addition to the level of Lp(a) in each patient, the size of the Lp(a) particle is also of importance regarding the risk of atherosclerotic complications.\textsuperscript{[75,76]} The Lp(a) particle is a complex of a hydrophobic LDL-particle and a hydrophilic apolipoprotein(a) [Apo(a)] -particle, and furthermore the isoforms, and thus the size of Apo(a), depend on the number of so-called kringle 4 (K 4) repeats.\textsuperscript{[73]} The isoform, and hence size of the Apo(a) particle, is associated with risk of cardiovascular disease.\textsuperscript{[76]} Therefore, our finding of equal reduction of Lp(a) in heFH patients with small Lp(a) particles may be of clinical importance. At the present time, with regard to clinical endpoints, no randomized study has been conducted with LDL apheresis on patients with heFH and elevated Lp(a). Also, a recent review on this subject concludes that apheresis is an important treatment modality in both FH and elevated Lp(a).\textsuperscript{[128]}
6.1 Main Conclusions

- LDL-cholesterol was lowered significantly and to the same extent by the LDL apheresis columns DL-75, LA-15 and EC-50W in a group of patients with heterozygous familial hypercholesterolemia.
- The clinical study showed that the complement cascade was activated through the alternative pathway, while the ex vivo study indicated activation through the classical pathway as well. The adsorption columns DL-75 and LA-15 cleared the anaphylatoxins C3a and C5a more effectively than the filtration column EC-50W in the clinical study. This finding was reproduced in the ex-vivo study, and thus the adsorption columns DL-75 and LA-15 exhibit greater complement compatibility.
- The adsorption columns DL-75 and LA-15 were less pro-inflammatory regarding cytokines and sensitive CRP than the filtration column EC-50W.
- EC-50W reduced fibrinogen the most, while LA-15 increased TAT the least, and DL-75 reduced PAI-1 the most, thus the least hemostatic column could not be identified in this study.
- All columns reduced Lp(a) significantly and to the same extent in this group of patients with heFH and elevated levels of Lp(a).

6.2 Unresolved questions/directions of future research

- The findings of the clinical study should be reproduced in a larger number of heFH patients with a multi-center design if possible.
- The magnitude of adsorptive properties of the different columns could be studied ex vivo after stimulation of complement and cytokine production. We have planned such an experiment.
- The “up-stream” effect of complement activation with regard to inflammatory responses should be further explored, and we are currently conducting such a study.
- The effects of altered immunological and hemostatic responses with the different LDL columns should be studied with regard to clinical endpoints.
• The clinical relevance of activation of the complement system should be further explored in patients with FH.
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