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Biocompatibility in low-density lipoprotein apheresis and plasma separation

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Contents

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List of articles

Article I

Hovland, A., Hardersen, R., Sexton, J., Mollnes, T. E., Lappegard, K. T. Different inflammatory responses induced by three LDL-lowering apheresis columns. J Clin Apher. 2009;24(6): 247-253.

Article II

Hovland, A., Hardersen, R., Nielsen, E. W., Enebakk, T., Christiansen, D., Ludviksen, J. K. Mollnes, T. E., Lappegard, K. T. Complement profile and activation mechanisms by different LDL apheresis systems. Acta Biomater. 2012;8(6): 2288-2296.

Article III

Hardersen, R., Enebakk, T., Christiansen, D., Ludviksen J. K., Mollnes, T. E., Lappegard, K. T., Hovland, A. Comparison of cytokine changes in three different lipoprotein apheresis systems in an ex vivo whole blood model. J Clin Apher. 2020;35(2): 104-116.

Article IV

Hardersen, R., Enebakk, T., Christiansen, D., Bergseth, G., Brekke, O. L., Mollnes, T. E. Lappegard, K. T., Hovland, A. Granulocyte and monocyte CD11b expression during plasma separation is dependent on complement factor 5 (C5) - an ex vivo study with blood from a C5-deficient individual. APMIS. 2018;126(4): 342-352.

Abbreviations

LDL: Low-density lipoprotein

HELP: Heparin-induced LDL precipitation

MDF: Membrane differentiated filtration

DFPP: Double filtration plasmapheresis

VLDL: Very low-density lipoprotein

Lp(a): Lipoprotein (a)

PCSK9: Proprotein convertase subtilisin/kexin type 9

FH: Familial hypercholesterolemia

HoFH: Homozygous familial hypercholesterolemia

HeFH: Heterozygous familial hypercholesterolemia

miRNA: micro Ribonucleic acid

mRNA: messenger Ribonucleic acid

C3: Complement factor 3

C5: Complement factor 5

CD62P: Cluster of differentiation 62 Platelet

IL-6: Interleukin - 6

IL-1ra: Interleukin -1 receptor antagonist

VEGF: Vascular endothelial growth factor

CRP: C- reactive protein

TNF: Tumor necrosis factor

ICAM-1: Intracellular adhesion molecule -1

VCAM-1: Vascular cell adhesion molecule -1

Nox-NADPH: Nitrogen oxides - nicotinamide adenine dinucleotide phosphate

ACD-A: Acid citrate dextrose - A

C5D: Complement factor 5 deficient

C5DR: Complement factor 5 deficient reconstituted

CTR: Control

Bb: Complement factor Bb

TCC/ C5b-9: Terminal complement complex

C1rs - C1-inh: Complex of complement factor 1 and complement factor 1 inhibitor

CR3 and CD11b/CD18: Complement receptor 3.

FITC: fluorescein isothiocyanate

LDS-751: Label double-stranded - 751

SSC: Side scatter flowcytometry

PDGF: Platelet-derived growth factor

RANTES: Regulation upon activation, normal T-cell expressed, and secreted

GM-CSF: Granulocyte macrophage - colony stimulating factor

PS1: Plasma sample 1/ position after/ post plasma separation column

PS2: Plasma sample 2/ position after/ post LDL apheresis column

BS1: Blood sample 1/ position before/ pre plasma separation column

BS2: Blood side 2/ position after/ post LDL apheresis where red cell and plasma were combined

1. Introduction

1.1 Apheresis in a historical perspective

The word apheresis comes from the late Greek word *apherein* which means "*to remove".* One medical understanding of the word apheresis used today is: "*A procedure in which blood is temporarily withdrawn, one or more components selectively removed, and the rest of the blood is reinfused into the donor. "The process is used in treating various disease conditions in the donor and for obtaining blood elements for the treatment of other patients or for research" (1).* The first experimental plasmapheresis was described by Abel, after performing the procedure on uremic dogs (2). Apheresis was developed for medical purposes in the 1950s when Cohn et al developed a method for separating Albumin from human whole blood using a centrifuge (3). This centrifugal version went through modifications and became commercially available as a single use centrifugal device for platelet donation and therapeutical apheresis procedures (4). In 1968 Judson et al published an article about a continuous-flow centrifugal device and argued that this could be used for the collection of cells and particles such as macromolecules, e.g. chylomicrons, immunoglobulins and cryoglobulins (5). This continuous flow or membrane apheresis as it's called today was developed from the technique used in hemofiltration with modified column membrane characteristics (6). From the mid-1970`s membrane plasmapheresis was invented for the removal of specific plasma solutes without the need for discharge of the complete plasma volume. Diseases where plasma exchange or therapeutical apheresis was either tried for or existed as established treatment at that time, included Waldenstroms macroglobulinemia, multiple myeloma, myasthenia gravis, hypercholesterolemia, hypertriglyceridemia and toxin removal (7).

1.2 Low-density lipoprotein (LDL) apheresis systems

The first published treatment using plasma separation for hypercholesterolemia was done in Paris, 1967 (8). In 1975 Thompson et al described therapeutic apheresis for treatment of familial hypercholesterolemia (FH). Centrifugal methodology modified for the removal of plasma rather than leukocytes was applied, and the authors noted a reduction in plasma cholesterol, and both patients were relieved from their angina pectoris symptoms (9). Selective LDL apheresis techniques have since developed and several systems exist today (10), as will be discussed below.

1.2.1 Plasma separation LDL apheresis systems

The Heparin-induced LDL precipitation (HELP) separation apheresis system uses low pH mediated by the use of acetate and heparin in precipitating cholesterol before its removal from plasma. This technique requires bicarbonate dialysis for correction of electrolytes and pH, before returning plasma to the patient and is as such a complex procedure (11). Heparin is used as anticoagulation. Lipid filtration (membrane differentiated filtration (MDF) or double filtration plasmapheresis (DFPP) is also a plasma separation LDL apheresis system and extracts LDL cholesterol from plasma based on the three-dimensional structure and molecular weight and pore size in the column. Plasma proteins with a diameter larger than 15 nm are captured by the column, smaller proteins pass through the column and are returned to the patient (12). Heparin is used as anticoagulation. The third method used is lipid adsorption from plasma. The method is based on electrostatic binding between cellulose beads covered with negatively charged dextran sulphate groups that bind to positively charged Apolipoprotein B100 incorporated in the membrane of LDL and very low-density lipoprotein (VLDL) particles (13-15). The adsorbing beads are regenerated during the procedure, making

the system capable of treating large volumes of plasma, hence removing large quantities of LDL cholesterol. In this model as well, heparin is used as anticoagulation.

1.2.2 Whole blood LDL apheresis systems

LDL cholesterol absorption systems adsorbing directly from whole blood use modified polyacrylate ligands immobilized on polyacrylamide matrix (16) or modified dextran sulphate cellulose, both negatively charged, with larger particle size beads for LDL adsorption (17). Positively charged Apolipoprotein B100 included in LDL and VLDL particles binds to the negatively charged beads upon perfusion. Heparin as a bolus dose and citrate as a continuous infusion is used as anticoagulation in both whole blood lipoprotein apheresis systems. The columns cannot be regenerated. Angiotensin converting enzyme inhibitors should not be used when performing dextran sulphate cellulose or polyacrylate LDL apheresis due to the accumulation of bradykinin. Bradykinin can cause an anaphylactoid reaction in patients. However, angiotensin II receptor antagonists can safely be used (18).

Performing whole blood LDL apheresis is a simpler procedure compared to the plasma separation systems (19). There are differences in adverse effects according to the system and anticoagulation used for LDL apheresis, the precipitation system showing more mild adverse effects, and the double filtration plasmapheresis systems showing more moderate adverse effects (20). Patient experiences indicate that double filtration plasmapheresis has fewer side effects than dextran sulphate plasma separation and whole blood LDL apheresis (21), however, the treatment systems are in general well tolerated. A new membrane has also been developed using polysulfone as the basis on which negatively charged sodium alginate sulphate was attached for capturing LDL particles through the principle of electrostatic binding (22).

The filtration and adsorption LDL apheresis systems also adsorb Lipoprotein (a) (Lp (a)) efficiently (13, 23). Further and possibly connected to the removal of Lp (a), lipoprotein apheresis also removed oxidized lipoproteins (24, 25). Triglycerides are also reduced with whole blood LDL apheresis (26).

The reduction of LDL cholesterol and Lp(a) for all systems is in the range of 30-70 % during one session, depending on the system used, and the blood or plasma volume treated (10, 27, 28).

In Europe, LDL apheresis is mainly performed within dialysis units and nephrologists are involved in the treatment. In the United Kingdom, the favoured system used is whole blood dextran sulphate cellulose adsorption (29). In the USA, there are separate apheresis centers in addition to dialysis and blood bank units performing LDL apheresis treatment, and also endocrinology departments perform this treatment. Specialists in preventive cardiology and nephrologists are involved in conducting this treatment. The HELP and plasma separation dextran sulphate cellulose system are reimbursed and widely used. In Japan, cardiology units are those most involved in the treatment, and the lipid filtration and both the whole blood and plasma separation dextran sulphate adsorption systems are widely used (30). Table 1 summarizes the most widely used systems for LDL apheresis used today.

System/Commercial Instrument	Principle of LDL Cholesterol Removal	Anticoagulation	Blood component perfused
HELP: Heparin-induced LDL precipitation.	Use low pH for precipitation of LDL with heparin and other molecules	Heparin	Plasma
Liposorber system Filter: LA15®	Negatively charged dextran sulphate beads on cellulose adsorb Apo B-100 containing lipoproteins	Heparin	Plasma
Lipid filtration Filter: EC50®	Filtration of LDL based on molecular weight. Membrane retains LDL (MW 2300000 Dalton)	Heparin	Plasma
DALI® system	Negatively charged polyacrylate beads on polyacrylamide adsorb Apo B-100 containing lipoproteins	Heparin bolus and citrate continuous infusion	Whole blood
Liposorber system Filter: Liposorber D®	Negatively charged dextran sulphate beads on cellulose adsorb Apo B-100 containing lipoproteins.	Heparin bolus and citrate continuous infusion	Whole blood
Immune adsorption	Perfusion through sepharose columns coated with LDL antibodies	Heparin bolus and citrate continuous infusion	Plasma

Table 1. LDL apheresis systems

1.3 Familial hypercholesterolemia

Heterozygous familial hypercholesterolemia (HeFH) is a common autosomal dominant inherited disease, which leads to reduced LDL receptor activity, defects in apolipoprotein B, or increased levels of proprotein convertase subtilisin/kexin type 9 (PCSK9) and hence increased level of LDL cholesterol in affected individuals (31). Mutation testing is considered the gold standard for diagnosing the condition, however, the use of tests varies widely across the world, and hence clinical criteria are often used instead (e.g. The Dutch Lipid Clinic Network Diagnostic Criteria) (32). High levels of LDL cholesterol in FH are associated with premature risk of disabling cardiovascular disease and cardiovascular death (33). On a worldwide base, the prevalence of FH is estimated to 1/313 (32). It has been estimated that approximately 23000 people in Norway could be affected, giving a prevalence of 1/228. (34). The homozygous form of familial hypercholesterolemia (HoFH) has an estimated prevalence of 1:1000000, but studies from the Dutch surveillance program indicate a prevalence of 1:300000 (35). At this time in Norway, there are 11 known patients with HoFH (Martin Prøven Bogsrud, OUS, personal communication). This form of FH includes severely increased levels of LDL cholesterol and extremely early cardiovascular disease, including myocardial infarction in children (36). Initial treatment of FH consists of statins and ezetimibe supplemented with the use of PCSK9 inhibitors, or PCSK9 inhibitors alone when adverse events are experienced with the use of statins. LDL apheresis is a supplement to the initial treatment if adverse effects occur or target cholesterol levels are not met, especially in HoFH (37). In many countries, including Norway, the use of PCSK9 inhibitors was limited by strict rules for reimbursement by the governments or insurance companies.

1.4 The use of LDL apheresis systems beyond the reduction of lipoproteins

Even if the columns used in LDL apheresis are selective for capture and removal of LDL cholesterol and Lp(a), these systems were also shown to affect several other molecules related to several human biological systems. Alterations in markers of vascular function include a decrease in Endothelin-1 (38) and an increase in prostaglandin-2 (39), bradykinin and nitric oxide, depending on the anticoagulation used in the treatment (40). Effect on markers of coagulation and fibrinolysis has been documented by the reduction of thrombin, coagulation factors V, VII, XI, XII and fibrinogen (27, 28, 41-43). Studies have documented reduced blood viscosity during LDL apheresis treatment partly by the favourable alteration of red cell aggregation and deformability and reduced concentration of fibrinogen, however, this effect occurred immediately after treatment and was not long-lasting (44). Molecules in the inflammatory network were also shown to be altered to various degrees by LDL apheresis, mostly with reduced concentrations (45, 46). Furthermore, studies have shown decreased concentrations of markers of endothelial dysfunction (47). These studies were performed with different LDL apheresis systems, and also using different anticoagulation during treatment, and it is hence difficult to generalize about the effects of different LDL apheresis systems on markers of human biological systems (48). In addition to adsorption documented by proteomic studies of columns used for LDL apheresis (49), there are indications that removal of LDL through adsorption columns can co-precipitate extracellular vesicles containing certain pro-coagulant proteins (50). A possible effect on gene expression and signaling has also been indicated and is another mechanism in which LDL apheresis possibly affects biological systems in long term treatment. Studies have described reduction of micro ribonucleic acid (miRNA) and other gene expression products, with a possible role in regulation of lipid homeostasis after LDL apheresis (51). Micro RNA of pentraxin 3 was reduced and messenger RNA coding for IL-1α, IL-6 and TNF was reduced during LDL

apheresis treatment (52, 53). There are also indications that LDL apheresis reduces the soluble form of activated leukocyte cell adhesion molecules which are recognized as pattern recognition receptors and hence markers of inflammation (54).

1.5 The Complement system (Fig. 1)

The complement system is part of the innate immune system. It consists of about 50 membrane-bound and soluble proteins. The complement system is a self-amplifying cascade and its potency is illustrated by the fact that it contains only one regulatory protein, properdin, that increases activity, but > 10 soluble and membrane-bound proteins that act as inhibitors. The amplification loop is essential for the short activation time of the complement system when needed in host defence (55). The complement system can be activated by three different pathways: classical, lectin and the alternative pathway. The classical pathway responds to antibodies and pentraxins, the lectin pathway responds to carbohydrates trough mannose-binding lectin, ficolins, collectins and IgM antibodies, and the alternative pathway responds to damaged self or non-self (as in artificial surfaces). The three pathways converge in the C3 convertase which activates C3 and forms C3a, C3b and iC3b. C3b connects with the C3 convertase and forms the C5 convertase which cleaves C5 into C5a and C5b. The anaphylatoxins C3a and C5a induce the production of inflammatory mediators and also work as chemotaxins. C5b binds to other complement proteins to form the terminal complement complex and C3b is cleaved into iC3b which promotes inflammation, phagocytosis and oxidative burst.

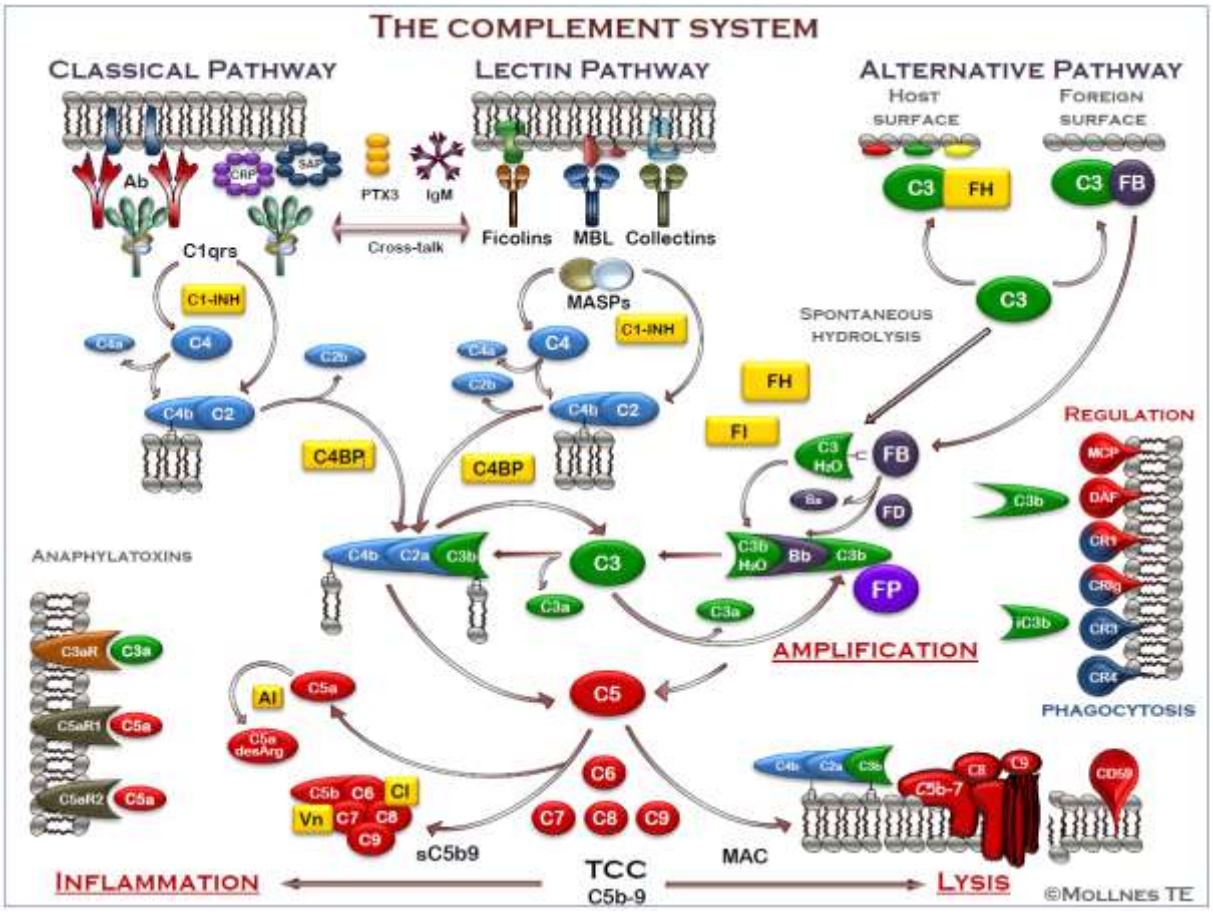

Fig. 1. There are three main activation pathways in the complement system (top). The classical (left), the lectin (middle) and the alternative pathway (right) The classical pathway is mainly activated by antibodies. The lectin pathway is mainly activated by carbohydrates through mannose-binding lectin. The alternative pathway is typically activated by recognition of damaged cells of foreign or self origin, and can also be activated by artificial materials. The classical and lectin pathways merge and form the classical and lectin pathway C3 convertase (C4bC2a) and the alternative pathway forms the alternative pathway convertase (C3bBbP).

The alternative pathway convertase is also formed by continuous low-speed hydrolysis of C3(H2O) to C3b(H2O)BbP.

Properdin (FP) stabilizes the C3 convertase providing an amplification loop potentiating the response upon activation trough all three initial pathways.

The C3 convertases add another C3b molecule and convert into the C5 convertases (C4bC2aC3b and C3bBbC3bFP)) which cleaves C5 into C5a and C5b. C5b is incorporated as a part of the terminal complement complex (TCC, C5b-9) which contributes to cell lysis (bottom right). C3b takes part in regulation (middle right). C3a together with C5a act as an anaphylatoxin in the inflammation response (bottom left).

The only positive stimulating molecule in the complement system is properdin (FP) which stabilizes the C3 convertase. Negative regulators are factor H (FH), C1 inhibitor (C1-INH), C4 binding protein (C4BP), factor I (FI), anaphylatoxin inhibitor (AI), vitronectin (Vn) and clusterin (Cl). The figure is used with permission from prof. Tom Eirik Mollnes.

1.6 System biocompatibility

The current definition of biocompatibility is formulated as "*the ability of a material to perform with an appropriate host response in a specific application*" (56). The term biocompatibility does not only include the properties of the biomaterial. It is of importance to recognize that biocompatibility is the interaction between the biomaterial and the biological system it is located in and that the interaction is bidirectional. A call for changing the nomenclature and not to use the phrase *"biocompatibility of a biomaterial",* but rather use the phrase *"biocompatibility of the system"* was put forward and is advantageous for emphasizing the bidirectional interactions taking place (57). The general understanding describing the interaction is that the biomaterial is first exposed to molecular adsorption and/or mechanical, physical and chemical factors. Secondly, this leads to cell responses of a defensive, targeting or interfering way (57). On a molecular level, there are arguments to view system biocompatibility as an inflammation. The biomaterial represents or induces the formation of danger associated molecular patterns being recognized by pattern recognition receptors. This leads to activation of the immune system (56), sterile inflammation, and at the end, interactions, effects or interference that give a wanted or unwanted outcome for the patient. Mechanotransduction is one of the main perspectives on how system biocompatibility processes are initiated. In this view, any mechanic effect on the interface between the biological system and the biomaterial converts into biological signals leading to a sterile inflammatory reaction and the biocompatibility reaction within the biological system where the biomaterial is placed (58, 59).

Another view on system biocompatibility is the activation of the cascade systems as the initial step leading to inflammation, secondly leading to the biocompatibility reaction. The binding of plasma proteins on to the biomaterial surface is believed to be the first step (60, 61). The proteins can go through conformational changes and together with the biomaterial itself act as

binding seats for proteins of the innate immune system initiating an inflammatory response (62). The reaction includes activation of the complement system through both classical, lectin and alternative pathway, and the kinin-kallikrein system as well as activation of the coagulation system including platelet activation. All the cascade systems are involved in this crosstalk reaction termed thromboinflammation (63-65). The activation of the complement system initiates the production of C3a and C5a and other proteins that activate the cellular components of the immune system. Monocytes, granulocytes and endothelial cells induce the production of cytokines further enhancing the immune response (66, 67). The flow of blood through needles, tubing and columns included in extracorporeal treatment systems including apheresis, exerts flow stress and shear stress on the contents of the blood. Platelets are vulnerable to shear stress and can be activated with increased expression of CD62P and CD42 binding to von Willebrand factor which can lead to clot formation. Increased expression of the CD41/CD61 integrin receptor on platelets can bind to the CD11b/CD18 integrin receptor on activated leukocytes leading to the formation of leukocyte-platelet conjugates (68). Shear stress is also known to increase the formation of extracellular vesicles. The content of these vesicles depends on which cell they are derived from and can in plasma separation include platelet-derived extracellular vesicles containing CD62P and activated CD41/CD61 complexes. The generation of extracellular vesicles is also recognized as a potential marker of adverse events in treatments, including extracorporeal circulation (69). Activation of the leukocytes is also recognized by exposure to shear stress (70), indicating contribution to the system compatibility reaction.

1.7 Clinical perspectives

The initial use of LDL apheresis was for the reduction of LDL cholesterol in patients with FH when the medication was not tolerated or the target for LDL reduction was not met (71). The arrest of progression of coronary atherosclerosis during treatment with LDL apheresis in combination with cholesterol lowering drugs was documented in the LAARS and L-CAPS studies (72, 73). Prospective randomized trials on endpoints are not documented, however, Thompson used plasmapheresis for LDL reduction in siblings with HoFH. Siblings treated regularly with plasma exchange survived their siblings treated with medical treatment of the time by 5,5 years (9). A non-randomized study by Mabuchi and co-workers compared patients treated with LDL apheresis and cholesterol lowering drugs with a group of patients receiving only medical treatment for six years. They found a significant reduction in nonfatal myocardial infarction, and death from coronary heart disease, in the group also treated with LDL apheresis compared to the drug treated patient group (74). Results showing improvement of left ventricular ejection fraction in LDL apheresis treated patients, independent of concomitant statin treatment, have also been published in a small study (75). LDL apheresis has a role in cardiovascular risk reduction (74, 76), and there are also indications that Lp (a) reduction with LDL apheresis treatment may further prevent coronary events (77-80). Recently LDL apheresis was used as lipid lowering treatment in acute coronary syndrome, as a supplement to statins, and performed within 72 hours of percutaneous coronary intervention (81). The authors conclude that the procedure is safe and that there is a trend towards early coronary plaque regression.

Lipoprotein apheresis is also used for other medical indications besides lowering LDL cholesterol and Lp(a). A guideline document was published by The American Society for Apheresis indicating that lipoprotein apheresis could be of use in the treatment of phytanic acid storage disease, sudden sensorineural hearing loss and focal segmental

glomerulosclerosis (82). Using lipoprotein apheresis in the treatment of nephrotic syndrome of various etiologies (83-87) and nephropathy in diabetes mellitus (88, 89) has also shown positive results. It has also been suggested that lipoprotein apheresis can have favourable effects in patients with critical limb ischemia due to below-knee arterial lesions (90, 91) and can reduce the risk of in-stent coronary restenosis in the early post-implantation period (47). Wu et al have also indicated that LDL apheresis can have an immediately positive effect on coronary microvascular dysfunction (92). Whole blood LDL apheresis has been used in severe cases of hypertriglyceridemia with recurrent pancreatitis (26, 93). Reduction of LDL cholesterol and oxidized cholesterol (94), with the theoretical potential of reduction of foam cell formation, and modification of the endothelial damage and inflammatory mechanisms, with subsequent favourable clinical effects, have been suggested as possible mechanisms. In addition, the possible removal of a putative, yet still unknown, soluble factor in nephrotic syndrome, and improvement of hemorheology, possibly trough cholesterol and/or fibrinogen removal, reduction of proinflammatory cytokines and adhesion molecules were suggested as mechanisms (47, 95-98). In these studies, double filtration plasmapheresis, dextran sulphate plasma adsorption and heparin-induced extracorporeal LDL precipitation were used as lipoprotein apheresis systems. There were also indications that LDL apheresis can be used in preeclampsia for prolongation of pregnancy. The mechanism is not clear, whether it is because of reducing cholesterol or because of the removal of placental soluble fms-like tyrosine kinase-1 (99). One study has also described that LDL apheresis can reduce the concentration of extracellular microparticles shredded by platelets in FH patients. The finding was also positively correlated to a reduction in thrombin formation, indicating another possible effect of LDL apheresis beyond the reduction of LDL cholesterol (100).

With the introduction of PCSK9 inhibitors (101, 102) the use of LDL apheresis for treatment of FH has decreased (103). However, it is still a treatment option for some patients with high

cardiovascular risk scores as well as in HoFH (104). In addition, LDL apheresis is at present the only treatment option that effectively reduces elevated Lp (a) (80, 105), but with new medications in the pipeline (106), the use of LDL apheresis may be reduced also for this group of patients.

A rationale for using LDL apheresis in the treatment of Alzheimer's disease was put forward in a recent article (107) with the intention being a decrease of what is called "metabolic inflammation". This article points to the capability not only to reduce LDL cholesterol and Lp(a) but also removal of circulating cytokines, immune complexes, proinflammatory metals and toxic chaperones. As the Covid-19 pandemic is evolving, articles have been published about the possible association between elevated concentrations of Lp (a), risk of coronary complications during Covid-19 infection and the possible role of LDL apheresis (108). Furthermore, the use of extracorporeal treatments like LDL apheresis, therapeutic plasma exchange and immune adsorption has been discussed, the intention being cytokine removal to dampen the cytokine release syndrome seen in patients critically ill from Covid-19 infection (109-111).

LDL apheresis can be used with a favourable clinical effect in medical conditions other than hypercholesterolemia. The mechanisms of action are not clarified. It is the treatment of choice in HoFH patients and patients with elevated Lp(a) and is still a supplement in the treatment of HeFH.

2. Aims of the study

2.1 General aims

Many studies describe the effects of different LDL systems on cholesterol removal, plasma proteins and other inflammation markers and mediators (48, 112, 113).

Authors	Year	Apheresis systems used	Inflammation marker: system and effect
Otto et al. (27)	2007	DL75, DALI	DL75: IL-6 ↑, CRP ↓ DALI: IL-6 ↔, CRP ↓
Stefanutti et al. (48)	2011	LA15	TNF-α ↓, IL-1ra ↑, VEGF ↓
Utsumi et al. (114)	2007	LA15	IL-1β ↓, TNF ↔.
Dihazi et al. (115)	2008	EC50, DALI, HELP	EC50/DALI/HELP: Complement factor B ↓.
Tishko et al. (47)	2017	EC50	ICAM-1 ↓, VCAM- 1 ↓
Kopprasch et al (116)	2015	LA15 vs EC50	LA15 vs EC50: ↓ Nox-NADPH oxidase activity, ↓ opsonin receptor activity

Table 2: Studies describing concentration change in inflammation markers in different lipoprotein apheresis systems used in this study focusing mainly on cytokine and complement system effects. ↔ Unchanged. ↓ Reduction. ↑ Increase.

However systematic comparisons of multiple systems including the whole blood system Liosorber D® are infrequent. The primary aim of the study was to perform a systematic investigation of how the whole blood lipoprotein apheresis column Liposorber D®, the plasma separation LDL adsorption apheresis column LA15® and the plasma separation LDL filtration column EC50® affect the immune system, including the complement cascade, the cytokine network and the activation of the cell fraction of the immune system under *in vivo* and *ex vivo* conditions. Secondly, to describe the effect of a commonly used plasma separation column OP-05®, and finally, if possible, to indicate differences between the systems that can be favourable in a clinical and biocompatibility perspective. The reason for the choice of these systems was that they represent different principles, both whole blood and plasma separation lipoprotein apheresis systems. They apply different types of anticoagulation known to affect

activation of the immune, coagulation and complement systems differently. Including the LA15 system in the study, which was well described for its effect on the immune system, gave a basis for comparison of the different systems (48). These systems have for years been clinically used in our hospital, as well as in numerous hospitals worldwide.

2.2 Specific aims

Article I

The first article investigated how three different LDL apheresis systems (one whole blood adsorption, one plasma separation-adsorption, and one plasma separation lipid filtration LDL apheresis system) affected the immune system represented by the complement and the cytokine network in an *in vivo* clinical treatment situation.

Article II

The second article investigated how three different LDL apheresis systems (one whole blood adsorption, one plasma separation-adsorption and one plasma separation lipid filtration LDL apheresis system) affected the complement system in an *ex vivo* situation using blood from healthy donors. The investigation also included the ability to explore temporal differences between the three LDL apheresis systems during treatment, and to differentiate between the effect on the plasma separation column and the LDL apheresis column in the two plasma separation LDL apheresis systems.

Article III

The third article was a follow-up of the second article and investigated how three different LDL apheresis systems (one whole blood adsorption, one plasma separation-adsorption and one plasma separation lipid filtration LDL apheresis system) affected the cytokine network under *ex vivo* conditions.

Article IV

The fourth article explored to which extent a number of the reactions seen in the plasma separation column used in the plasma separation LDL apheresis systems were dependent on the complement system. For this we used blood from a complement factor 5 deficient donor and blood from healthy blood donors. Readouts were CD11b/CD18 (CR3) upregulation on leukocytes, leukocyte-platelet conjugate formation, changes in concentration of the terminal complement complex and changes in platelet counts. The investigation also included the ability to explore temporal differences for the chosen readouts.

2.3 Hypothesis

Article I

H0: Complement and cytokine concentrations are equally affected by three LDL apheresis treatment systems under *in vivo* conditions.

HA: Complement and cytokine concentrations are differently affected by three different LDL apheresis treatment systems under *in vivo* conditions

Article II

H0: Complement factor concentrations are equally affected by three LDL apheresis treatment systems under *ex vivo* conditions.

HA: Complement factor concentrations are differently affected by three LDL apheresis treatment systems under *ex vivo* conditions.

Article III

H0: Cytokine concentrations are equally affected by three LDL apheresis treatment systems under *ex vivo* conditions.

HA: Cytokine concentrations are differently affected by three LDL apheresis treatment systems under *ex vivo* conditions.

Article IV

H0: Granulocyte and monocyte CD11b expression and platelet-leukocyte conjugate generation are not dependent on complement factor C5 during *ex vivo* plasma separation

HA: Granulocyte and monocyte CD11b expression and platelet-leukocyte conjugate generation are dependent on complement factor C5 during *ex vivo* plasma separation

3. Materials and Methods

3.1 Article I

Article I was a prospective crossover clinical study. The blood sampling was done in a regular patient LDL apheresis treatment situation. Anticoagulation was performed as described in the user manual for each system. Heparin was used in the LA15 and EC50 system and acid citrate dextrose (ACD-A) was used in the DL75 system. The study participants were three HeFH patients who had received LDL apheresis treatment with either the plasma filtration LDL adsorption (LA15) or the double filtration plasmapheresis (EC50) system for more than two years. Random selection order was chosen for the first two LDL apheresis systems used for each patient. Six consecutive weekly treatments were done with each of the three LDL apheresis systems (LA15, EC50, and DL75). The total number of samples for each LDL apheresis system was 18. Blood samples were taken from the arteriovenous fistula immediately before the treatment started and immediately after the treatment stopped. All treatments were performed at the department of nephrology/dialysis unit and a limited number of nurses and doctors handled the treatments and blood samples. The study was approved by the regional ethics committee and was performed following the Helsinki declaration. A signed, written informed consent was obtained from all participants.

Fig. 2. The plasma separation column is shown to the left and the LDL apheresis column LA15 on the right. In the hand, the blood sample site pre LDL apheresis column.

Fig. 3. The lab engineers Judith Krey Ludviksen to the left and Dorthe Christiansen to the right, and Randolf Hardersen in the middle.

3.2 Articles II and III

The results in article I indicated differences in concentration of complement activation products and cytokines and thus prompted us to investigate the selective effect of the columns in each system during treatment. An *ex vivo* study was set up for individual system comparison also with the capability to enlighten temporal concentration changes during treatment. The blood donors were six healthy volunteers. A blood bag was used as a blood reservoir and as a control. Lepirudin was used as anticoagulation in order not to affect complement activation during treatment (117). The study was performed at the Research Laboratory, Nordland Hospital Trust, Bodø. The biological samples for articles II and III were obtained in the same study. The study was approved by the regional ethics committee and was performed in accordance with the Helsinki declaration. A signed, written informed consent was obtained from all participants.

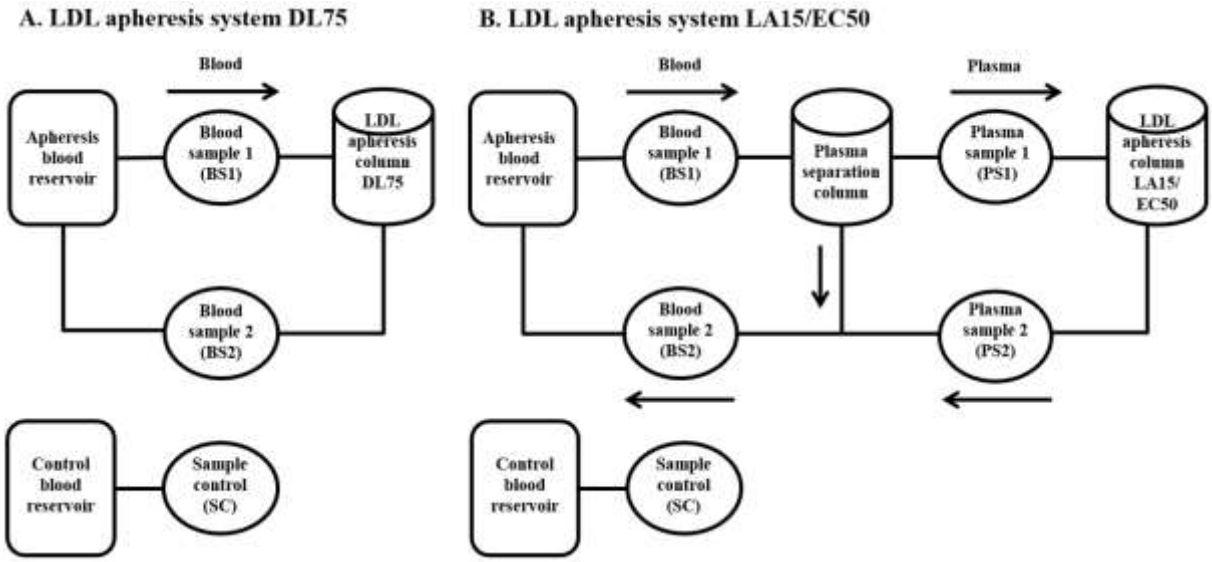

Fig 4. Schematic drawing of the *ex vivo* study model used in articles II and III.

A. Schematic drawing of the DL75 *ex vivo* model with blood sample positions. The blood reservoir denotes the whole blood bag to which the thrombin specific inhibitor lepirudin was added. Blood samples were obtained from a position after the reservoir (BS1) and then after the lipoprotein apheresis column (BS2).

B. The columns LA15 and EC50 required plasma separation before lipoprotein apheresis. The sample sites were before plasma separation (BS1), after plasma separation (PS1), after lipoprotein apheresis (PS2), and after the cell fraction (from plasma separation) and plasma (after lipoprotein apheresis) were combined (BS2). BS2 indicates the position where the treated blood would be returned to the patient in a clinical setting. The arrows show the direction of flow in the system.

The control blood reservoir was kept on the test tube rotator and samples (SC) were drawn directly from the bag.

3.3 Article IV

Article number II and III indicated that the plasma separation column used in the plasma separation LDL apheresis systems induced complement activation. An *ex vivo* study was set up to investigate the influence of this column separately on complement activation and granulocyte and monocyte activation. As the complement system is of importance in system biocompatibility, blood from a, by nature, complement 5 deficient (C5D) donor was used without and with added purified complement factor 5. Purified C5 was added to C5-deficient (C5DR) blood to give a final plasma concentration of 80 μg/mL, corresponding to the concentration of C5 in normal individuals (118). As a control, blood from two healthy individuals was used (CTR). As a control to the plasma separation system blood was kept in a blood bag under constant movement and temperature. Lepirudin was used as anticoagulation. The study was approved by the regional ethics committee and was performed following the Helsinki declaration. A signed, written informed consent was obtained from all participants.

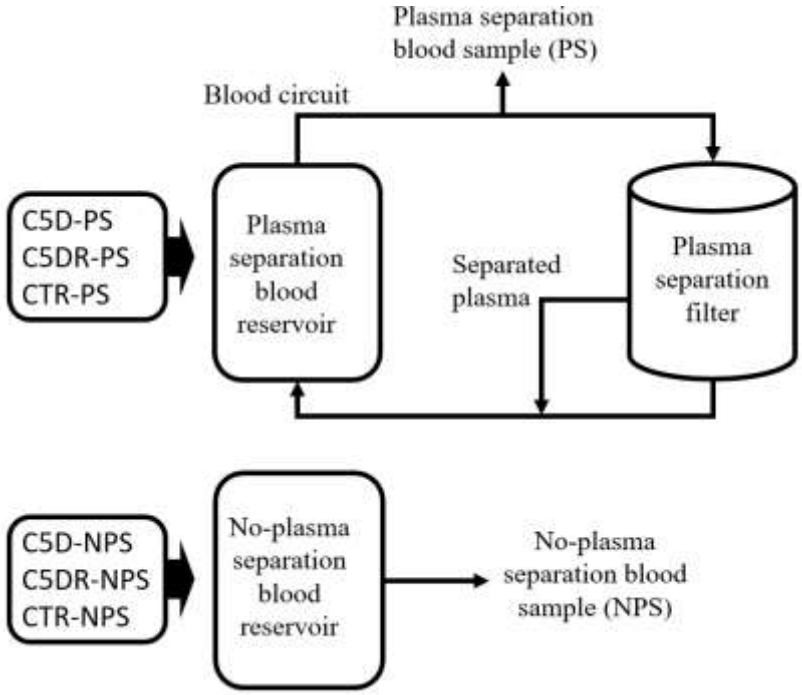

Fig. 5. Schematic drawing of the ex vivo model used in article IV.

The plasma separation blood bag served as the reservoir for the plasma separation circuit. Plasma separation blood samples (C5D, C5DR, CTR-PS) were obtained from the tubing blood sample outlet after the plasma separation blood reservoir. The arrows show the direction of blood flow and plasma flow in the system. The no-plasma separation blood reservoir was kept at 37°C on the test tube rotator next to the plasma separation blood reservoir. No-plasma separation blood samples (C5D, C5DR, CTR-NPS) were drawn directly from the no-plasma separation blood reservoir.

3.4 Complement analysis (articles I, II and IV)

The complement samples were anticoagulated with EDTA and centrifuged. The plasma was frozen in aliquots at -70 °C for later analysis. The complement activation products C4d, C3a, C3bc, Bb and C5a were measured using enzyme immunoassays based on capture antibodies reacting with neoepitopes exposed selectively in the activation product. TCC was analyzed using a monoclonal antibody attaching to a neoantigen on the C9 exposed selectively in the activation product. C1rs - C1-inh was quantified using an enzyme immunoassay analysis, previously described, based on a neoepitope of the molecule (119).

3.5 Cytokine analysis (articles I and III)

The cytokine samples were anticoagulated with EDTA and centrifuged. The plasma was frozen in aliquots at -70 °C for later analysis. Samples were analyzed using a multiplex cytokine immunoassay (Bio-Plex Pro Human cytokine Grp I Panel 27-Plex; Bio-Rad Laboratories Inc., Hercules, CA) containing the following 27 analytes: Interleukin (IL)-1β (IL-1β), IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-9, IL-10, IL- 12, IL-13, IL-15, IL-17, eotaxin (CCL11), basic fibroblast growth factor, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), Interferon-γ (IFN-γ), chemokine (C-X-C motif) ligand 10 (IP-10 or CXCL10), monocyte chemoattractant protein 1 (MCP-1 or CCL2), macrophage inflammatory protein (MIP) -1α (MIP-1α or CCL3), MIP-1β (or CCL4), platelet-derived growth factor (PDGF), regulated on activation T-cell expressed and secreted (RANTES or CCL5), tumor necrosis factor (TNF), and vascular endothelial growth factor (VEGF). The analysis was performed according to the manufacturer's instructions (120).

3.6 Routine biochemistry analysis

Hematocrit, Hemoglobin, leukocytes, and platelets were analyzed using a Siemens ADVIA® 2120 Hematology System (Siemens Healthcare Diagnostics Ltd., Camberly, UK). Total protein, albumin, C4, IgG, IgM, and IgA were analyzed in an ADVIA®1800 system (Siemens Medical Solutions Diagnostics, Japan) with reagents from Siemens Healthcare Diagnostics Ltd. Cholesterol parameters and high-sensitivity C-reactive protein (hs-CRP) were immediately measured by standardized laboratory tests in the hospital laboratory. Routine biochemistry analysis was done immediately after sample collection.

3.7 Flow cytometric analysis (article IV)

Flow cytometric analysis was used to detect upregulation of CR3 on granulocytes and monocytes. Flow cytometry differentiates granulocytes and monocytes at the X-axis based on cell shape and at the Y-axis based on granularity and complexity. Cells were labeled with anti CD14 for gating purpose and phycoerythrin labeled anti-human CD11b specific for the alpha subunit of the CD11b/CD18 integrin before gating in an anti-CD14 dot plot, and mean fluorescent intensity (MFI) for anti-CD11b was calculated (121).

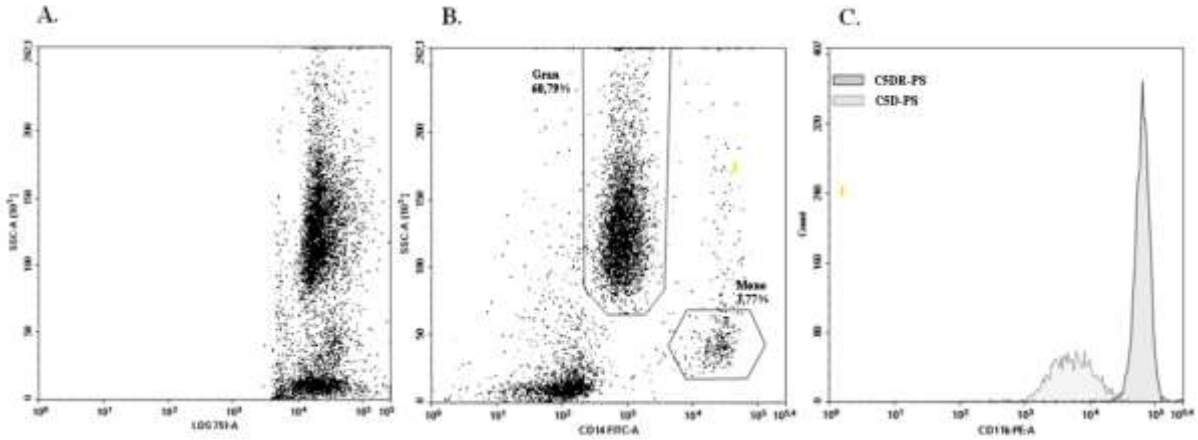

Fig. 6. Flow cytometric readouts (MFI) at 30 min of granulocyte and monocyte CD11b. During acquisition, the threshold was set on the nuclear stain LDS 751 (A). Granulocytes and monocytes were gated for in a CD14 FITC/SSC dotplot (B). Granulocyte CD11b expression in samples C5DR-PS and C5D-PS shown in a histogram (C).

3.8 Leukocyte - platelet conjugate analysis (article IV)

For detection of leukocyte - platelet conjugates fixed blood cells were stained with anti-CD14 PE (anti-CD14 phycoerythrin), anti-CD61 FITC (fluorescein isothiocyanate), LDS (label double-stranded) -751. Granulocytes and monocytes were gated in an SSC (side scatter flowcytometric)/anti-CD14-dotplot, and the mean fluorescent intensity values for CD61 were calculated.

3.9 Correction for dilution (articles II, III and IV)

A priming solution was used to prepare the tubing and columns before the *ex vivo* and in vivo loop which could affect the concentration. Hematocrit was used to correct the concentrations of the read-outs according to a standardized formula (122).

3.10 Statistical analysis

In article I mean ± SD of standard laboratory tests were compared with a t-test. A two-tailed P-value < 0.05 was considered statistically significant. Because of inter-individual variation, cytokine and complement activation product concentrations were normalized for evaluation of column effect and changes are in percent. The column effects on complement activation products and cytokines were furthermore studied using a (blocked) one-way ANOVA. This is a one-way analysis of variance correcting for the pre-treatment level of the inflammatory parameter. As responses from different individuals are likely to differ, the analysis treats the patients as block effects. Statistical analysis was performed with SPSS® 16.0 software for Windows. In article II mean concentrations of complement activation factors with the standard error of the mean (SEM) were calculated. A correlation was calculated according to Spearman. A two-tailed P value of <0.05 was considered statistically significant. Calculations were performed with Prism® 5.0, GraphPad software for Windows. In article III results are presented as mean and SEM. For calculation of differences in SC between baseline (T0) and after 240 minutes perfusion (T240), a two-tailed unpaired student's t-test was used. The significance level was set at < 0.05. For pairwise comparison of the lipoprotein apheresis systems, a regular two-way ANOVA analysis of the covariance, with Sidak's multiple comparison modification was used. The significance level was set at < 0.05. Statistical calculations presented were performed with Prism® 7.05, GraphPad software for Windows.

3.11 The Candidate`s contribution

The candidate was the second author in articles I and II and the first author of articles III and IV. The candidate participated in the planning of all the studies before the start and throughout the study period together with the co-authors. He also participated in setting up and conducting of the *ex vivo* studies at the laboratory. Flow cytometric and enzyme immunosorbent analysis were done by co-authors and other colleagues at the research laboratory. The candidate actively participated in the writing of articles I and II, including approving the final drafts and made the first draft of articles III and IV which then were reviewed by the co-authors. The candidate made changes to the manuscript in line with feedback from fellow authors who approved the final drafts.

4. Summary of main results

4.1 Article I

This *in vivo* investigation showed an equal concentration increase in TCC for all LDL apheresis systems. Complement activation factor Bb (Bb) representing alternative pathway complement activation showed an increase in concentration for all systems, however, results indicated that factor Bb concentration increased more in the LA15 and EC50 systems compared to the DL75 LDL apheresis system. Complement factor 4d (C4d), representing classical pathway complement activation, increased the most in the DL75 system and showed only a marginal change in the LA15 and EC50 systems. Complement factor 3a (C3a) concentration increased for all LDL apheresis systems, most pronounced in the EC50 system. Complement factor 5a (C5a) concentration was reduced in the DL75 and LA15 systems and showed no concentration change in the EC50 system. Eight of the 27 cytokines showed marked concentration changes during LDL apheresis. IL-1ra, IP-10 and MCP-1 were increased in all systems, IP-10 more so in the DL75 and LA15 system. IFN- γ, TNF, RANTES, VEGF, PDGF-BB were reduced in concentration, TNF and RANTES more so in the DL75 and LA15 system. All LDL apheresis systems reduced LDL cholesterol to the same extent.

4.2 Article II

This case-control *ex vivo* investigation indicated that the factors of the complement system were differently influenced by the three LDL apheresis systems. In the sample control, TCC, Bb and C3a showed a small concentration increase, C4d and C5a did not change in concentration during 240 minutes. The concentration of TCC did not change during treatment in the DL75 system whereas the concentration increased initially in the LA15 and EC50 LDL

apheresis systems and was reduced towards baseline at the end of the treatment. At position post LDL apheresis column the concentration of TCC stayed at baseline during the whole treatment in the LA15 and EC50 LDL apheresis system. For the DL75 system, concentrations of C4d, Bb and the anaphylatoxins C3a and C5a stayed at baseline during the whole treatment. In the LA15 and EC50 systems, there was an initial increase in the concentration of C4d, Bb, C3a and C5a in position post plasma separation. The LA15 system reduced the concentration of C4d, Bb, C3a and C5a towards baseline at the end of the treatment, and in position post LDL apheresis the concentration of the anaphylatoxins stayed close to baseline during treatment. In the EC50 system C4d, Bb, C3a and C5a concentration was increased from T15-T30 and throughout the entire treatment period in all positions.

In the DL75 system, C1rs-C1-inh stayed at the concentration level of the sample control during the 60 minutes sample period. In the LA15 and EC50 systems there was an initial increase in concentration at position post plasma separation however the concentration stayed at a baseline level in position post LDL apheresis.

4.3 Article III

This case-control *ex vivo* investigation showed that also the concentrations of cytokines are differently influenced by the three LDL apheresis systems. In the sample control bag, there was a marked increase of cytokines IFN-γ, IL-8, IL-1ra, TNF, PDGF-BB, RANTES and MIP-1β. The other biomarkers stayed on the baseline for 240 minutes. In the DL75 and LA15 LDL apheresis system IFN-γ, IL-8, IL-1ra, eotaxin, TNF, MCP-1, PDGF-BB, MIP-1β, and IP-10 were reduced in concentration, but only the DL75 system reduced RANTES concentration. IL-17 was unchanged in both systems and RANTES was unchanged in the LA15 system. VEGF and GM-CSF were increased in both systems. The EC50 system reduced the concentration of IFN-γ, IL-1ra, TNF, MIP-1β, and IP-10. IL-8, eotaxin, IL17, and PDGF-BB

were unchanged, and MCP-1, VEGF, GM-CSF, and RANTES were increased in concentration. Temporal patterns of concentration change included early increase and reduction, early reduction and increase, and late reduction and late increase during 240 minutes of treatment. Eotaxin concentration was reduced in position post plasma separation from the start of the treatment, in the LA15 and EC50 systems, indicating that the plasma separation column possibly can be a barrier to filtration into the plasma fraction in the plasma separation LDL apheresis systems.

4.4 Article IV

This is a case-control *ex vivo* investigation comparing blood from healthy individuals with blood from a C5 deficient individual and blood from the C5 deficient individual reconstituted with purified C5.

In the blood sample from the healthy individuals, granulocyte CD11b expression increased in plasma separation but not in the no-plasma separation sample. Monocyte CD11b expression increased in plasma separation and to a lesser extent in no-plasma separation. In the C5 deficient blood sample, granulocyte CD11b expression increased neither in plasma separation nor in the no-plasma separation sample. Monocyte CD11b expression also remained on the baseline in the plasma and no-plasma separation sample. In the C5 deficient blood sample reconstituted with purified C5, granulocyte and monocyte CD11b expression showed a substantial increase both in the plasma separation and no-plasma separation sample.

In the sample from the healthy individuals, granulocyte-platelet and monocyte-platelet conjugate formation increased to the same extent both in the plasma separation and no plasma separation. In the C5 deficient sample, granulocyte-platelet conjugate formation showed slightly less increase compared to the sample from C5 deficient reconstituted with purified C5

in plasma separation. Monocyte-platelet conjugate formation was equal in the C5 deficient and C5 reconstituted sample both in plasma separation and no-plasma separation sample. Platelet count, total protein and complement factor 4 (C4) concentrations were all reduced in the sample from the healthy individuals, the C5 deficient and in the C5 deficient sample reconstituted with purified C5 in plasma separation, but remained on the baseline in all the no-plasma separation samples. Leukocyte count stayed on the baseline in the sample from the healthy individuals, the C5 deficient sample and in the sample from the C5 deficient reconstituted with purified C5 both in plasma separation and no-plasma separation. Complement factor 3bc (C3bc) was increased in the sample from the healthy individuals, the C5 deficient and in the sample from C5 deficient reconstituted with purified C5 both in plasma separation and no-plasma separation. TCC concentration increased in the sample form the healthy individuals both in plasma separation and no-plasma separation. TCC stayed on the baseline in the C5 deficient sample both in plasma separation and no-plasma separation. In the sample reconstituted with purified C5, the TCC concentration increased in the plasma separation and the no-plasma separation samples above the concentration level in the control sample from the healthy individuals.

5. Discussion

5.1 Methodological considerations

In article I the blood donors were patients treated with LDL apheresis for more than two years before the investigation took place. Studies have indicated that LDL apheresis can alter gene expression of inflammatory markers (53) and this may have influenced our readouts. Blood was taken from the AV fistula immediately after the end of treatment and this did not allow for equalization of concentrations between the body fluids before blood sampling which could affect our results. Blood samples were collected from individuals with genetically verified HeFH and established cardiovascular disease in article I. In articles II and III, six healthy volunteers were used as blood donors. In article IV a C5 deficient individual and two healthy volunteers were used as blood donors. There are differences in inflammatory status and hemorheology between individuals with and without cardiovascular disease or high levels of cholesterol and this might have an impact on inflammatory responses on an individual level (123), and the fact that we used both healthy individuals and individuals with cardiovascular disease and HeFH as donors implies a methodological difference between our, and others, *in vivo* and *ex vivo* investigations. In article IV, C5 deficient blood was reconstituted with purified human C5. Purified human C5 has been described to be hyper-reactive compared to native C5. We have experienced this with the purified C5 in our laboratory (non-published observations). This probably had an impact in this study as TCC from the reconstituted samples was elevated compared to TCC from the healthy individuals. Heparin is a highly negatively charged molecule and interacts with a number of proteins (124). Interactions between heparin and blood proteins can possibly contribute to the differences seen in the *in* vivo and *ex vivo* study. In the *ex vivo* investigations (articles II-IV) lepirudin was chosen for anticoagulation, because of its ability not to influence the complement system (117, 125).

5.2 Biocompatibility

5.2.1 Complement system

The *in vivo* study indicated complete activation of the complement system by the elevation of TCC in all systems. Factor Bb, a marker of the alternative pathway activation, was markedly elevated in all systems, and one can argue that the alternative pathway activation was of most importance in accordance with former studies (55). C4d, a marker of classical pathway activation was to some degree elevated in the whole blood LDL apheresis system but showed only minor concentration changes in the plasma separation-LDL apheresis systems indicating either activation of the complement system in the whole blood column or adsorption in the other systems. Activation of the classical pathway by artificial surfaces is recognized in other studies (126). The *ex vivo* investigation indicated that all three LDL columns were able to adsorb TCC keeping it at baseline values. The DL75 system adsorbed TCC from start to end of treatment. The LA15 and EC50 systems showed a first pass increased production of TCC before adsorption reduced the concentration to baseline at the end of treatment. This indicates either better adsorption capabilities of the DL75 column or initially increased activation of the complement system by the two plasma separation LDL apheresis systems in the *ex vivo* study. In the ex vivo study, factor Bb was completely adsorbed by the DL75 column as opposed to in the *in vivo* study. In the LA15 system, there was an initial high generation of Bb and late complete adsorption. In the EC50 system, Bb was initially generated but not to the same extent as in the LA15 system and the concentration level remained increased throughout the treatment. This indicates reduced removal capabilities in the EC50 column compared to the LA15 column and may be caused by different materials and principles of action between the two columns as described in the introduction. C3a and C5a were increased in all three systems in the *in vivo* study, however, the *ex vivo* study showed the same pattern of production and adsorption as factor Bb, exemplifying the first pass effect on activation of the complement system in the two plasma separation based systems and the adsorption capabilities of dextran-

sulphate based adsorption columns. One can argue that in the first minutes of circulation the foreign material surface is exposed to the blood proteins in a way that activates the complement system vigorously. Later during treatment, the surfaces are covered with proteins that either enhance or reduce this cascade, balancing out the generation of complement components making the relative removal capability supersede the production. Proteomic studies were done investigating the eluate from LDL apheresis columns after treatments of patients with FH. Several complement proteins were identified indicating adsorption and filtration by the LA15 and EC50 columns (49, 115). It is known that heparin influences the complement system in a biphasic pattern. At low concentration complement alternative pathway is enhanced and at high concentration the level of factor Bb and TCC reduced (127). This might contribute to the differences seen as we used heparin as anticoagulation in the *in vivo* study.

5.2.2 Cytokines

There were also differences in the concentration changes of cytokines between the *in vivo* and *ex vivo* studies. IFN-γ, TNF, and PDGF-BB had the same direction of concentration change in all LDL systems, *in vivo* and *ex vivo*. IL-1ra and IP-10 were increased in the *in vivo* study and reduced in the ex vivo study. The *ex vivo* investigation excluded the total body blood volume and organs that may respond to biocompatibility reactions. This may have consequences for regulatory mechanisms, and to some degree contribute to the difference between the *in vivo* and *ex vivo* study. Furthermore, heparin and ACD-A were used as anticoagulation according to the description by the manufacturer of the equipment in the *in vivo* study. Citrate, as in ACD-A, not only inhibits the coagulation system, it also reduces cytokine secretion and activation of the complement system (128). A recent study also documented that heparin and citrate anticoagulation also influence which proteins that are bound to the membrane in

dialysis columns (129) and different anticoagulation used in our studies could affect the results and should be taken into account in the interpretation of the results.

5.2.3 Temporal concentration changes

Temporal changes in concentration (article III) showed that some cytokines are immediately adsorbed (eotaxin, IP-10) and some are adsorbed after a first pass induction of expression (PDGF, RANTES) in the whole blood column DL75. The LA15 column showed adsorption capacities at the same level as the DL75 column except for RANTES where the concentration increased from treatment start to finish even though the LA15 beads seemed to adsorb RANTES keeping the concentration at a low level at position post LDL apheresis column. This illustrated the difference in the two systems and points to the possible effect of the plasma separation column which increased the production of RANTES. The temporal change of eotaxin concentration is an example of a cytokine not easily filtered through the plasmapheresis column as the concentration of eotaxin was reduced immediately after the treatment started, in position post plasma separation in the LA15 and EC50 system. The LA15 bead removed the small amount of eotaxin present in the plasma and decreased the concentration during the treatment. The reduced filtration capabilities of eotaxin in the plasma separation column might be caused by the chemical structure of eotaxin as this protein has a disordered N-terminus compared to e.g. RANTES (130, 131). The EC50 column did not decrease the eotaxin concentration beyond the first pass reduction. The immediate fall in position post plasma separation, with no further temporal removal, is in alignment with the demonstrated initial coating of foreign material with proteins when contact with blood is established (61).

5.2.4 Shear stress in artificial systems

GM-CSF and VEGF showed a clear increase in the LDL apheresis systems compared to the control bag (article III) and there was also a clear reduction of platelet count in the plasmapheresis circuit compared to the control bag (article IV). Shear stress in artificial systems circulating blood induces expression of platelet glycoprotein Ib -IX-V receptor complex which can induce agglutination (132, 133). Biomaterial surface interaction also induces platelet activation through conformational changes of the platelets (134), expression of surface P-selectin and glycoprotein IIb/IIIa receptors (135) and microparticle formation and release (136), which can induce cytokine release and cloth formation.

5.2.5 CD11b expression and conjugate formation.

The results in article number IV indicated C5 dependent upregulation of CD11b expression on granulocytes and monocytes and is supported by former studies (137, 138). The results also indicated a possible dependence on C5 for the formation of granulocyte-platelet conjugates. Monocytes-platelet conjugate formation was C5 independent and there was no difference in leukocyte-conjugate formation between plasma separation and no-plasma separation samples. This is not in line with former studies (137, 138), where partial C5 dependent monocyte-platelet conjugate formation was demonstrated. Leukocyte-platelet conjugates are primarily created by binding between P- selectin (CD62P) and P-selectin glycoprotein ligand-1 (PSGL-1)/CD15 and integrin-mediated ligations via glycoprotein (GP) IIb/IIIa - fibrinogen - CD11b/CD18. Platelet glycoprotein 4 (GPIV, CD36) - thrombospondin also contributes (139). It was described that the integrin binding via glycoprotein (GP) IIb/IIIa - fibrinogen - CD11b/CD18 is of more importance when shear force increased (139). It is also known that under certain shear stress conditions the CD11b/CD18 integrin can be downregulated (70). Other studies have concluded a certain C5 dependence of monocyte - platelet conjugate

formation (138), and the lack of difference with and without C5 demonstrated in article IV despite the difference in expression of CR3 can be explained by the contribution of other mechanisms forming conjugates under the conditions used in this study.

5.3 Clinical consideration

The LDL apheresis systems equally reduced LDL cholesterol and the choice of which system to use for LDL lowering can be made according to the physicians' preferences and possible adverse effects from the treatment. The use of LDL apheresis in the treatment for other indications, e.g. nephrotic syndrome or improved peripheral circulation can possibly be beneficial to the patient, however, the different systems need to be tested for each indication independently, as each system had its own profile on how the immune system was modulated and how different mediators and markers of inflammation were changed in concentration also during the treatment time applied.

6. Main Conclusions

In vivo, the EC50 LDL apheresis system showed the least impact on inflammatory markers pre and post treatment, indicating the EC50 LDL apheresis system being the most biocompatible system. However, the DL75 and LA15 LDL apheresis systems showed a more beneficial inflammatory profile concerning concentration changes in complement factors and cytokines profile.

The *ex vivo* experiments showed that both the classical and alternative pathways of the complement were activated in the systems tested. There were differences in how the three LDL apheresis systems affected the different complement factors. All the three LDL apheresis columns reduced TCC. The cytokines were differently affected by the three LDL apheresis systems, the DL75 column being the most effective in reducing cytokines.

The plasma separation column increased CD11b/CD18 integrin upregulation on granulocytes and monocytes in a C5 dependent manner. Formation of monocyte - platelet aggregates was C5 independent in *ex vivo* plasma separation indicating several possible mechanisms for the formation of leukocyte - platelet conjugates. The plasma separation system induced platelet agglutination.

As LDL apheresis systems were used for other indications than the removal of lipoproteins, partly to favourably modulate the immune system by altering concentrations of cytokines, it is of importance to acknowledge that the cytokines and the complement factors were differently affected by the three LDL apheresis systems herein tested, both under *ex vivo* and *in vivo* conditions. This is a fact that underlines the need to test every system independently for each medical indication where it is used, and as close to the clinical setting as possible. Temporal

differences also ought to be taken into account. It is not recommended to solely rely on methods and results from similar studies with comparable systems.

7. Future perspectives

The traditional indications for the use of LDL apheresis will probably be reduced as new treatment options regarding LDL-cholesterol reduction evolve. However, patients with HoFH and patients who do not tolerate LDL-cholesterol lowering therapy may still require this treatment option.

There is evidence that LDL apheresis may improve microcirculation and can reduce extracellular vesicles, indicating a possible effect in the treatment of acute coronary syndromes which should be further explored.

The possible beneficial effect of LDL apheresis in the treatment of nephrotic syndrome and other diseases needs further investigation in larger studies.

Mechanisms of platelet aggregation in extracorporeal treatments are also a field requiring further investigation (139-141).

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9. Articles I-IV

Article I

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Different inflammatory responses induced by three LDL-lowering apheresis columns.

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Different Inflammatory Responses Induced by Three LDL-Lowering Apheresis Columns

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Low-density lipoprotein (LDL) apheresis is well-established in selected patients with uncontrolled LDL levels. As such treatment affects biomarkers important in atherosclerosis and acute coronary syndromes, we systematically compared the inflammatory response induced by three LDL apheresis columns. Three patients with heterozygous familial hypercholesterolemia participated in a cross-over study with six consecutive treatments with three different LDL apheresis columns: DL-75 (whole blood adsorption), LA-15 (plasma adsorption), and EC-50W (plasma filtration). Biochemical parameters and inflammatory biomarkers, including complement activation products and 27 cytokines, chemokines, and growth factors were measured before and after treatment. Complement was activated through the alternative pathway. The final end product sC5b-9 increased significantly ($P < 0.01$) and equally with all devices, whereas the anaphylatoxins C3a and C5a were lower by use of the adsorption columns. Hs-CRP was reduced by 77% (DL-75), 72% (LA-15), and 43% (EC-50W). The cytokines were consistently either increased (IL-1ra, IP-10, MCP-1), decreased (IFN-γ, TNF-α, RANTES, PDGF, VEGF), or hardly changed (including IL-6, IL8, MIP-1αβ) during treatment. The changes were in general less pronounced with the adsorption columns. All columns reduced LDL significantly and to the same extent. In conclusion, three LDL-apheresis devices with equal cholesterol-lowering effect differed significantly with respect to the inflammatory response. J. Clin. Apheresis 24:247–253, 2009.

Key words: LDL cholesterol; inflammation; cytokines; apheresis; familial hypercholesterolemia

INTRODUCTION

Low-density lipoprotein (LDL) apheresis is a well-established treatment modality in hypercholesterolemia when target LDL levels cannot be achieved by diet and/or lipid lowering drug therapy or because of intolerance to the drugs [1]. LDL apheresis is often used in patients with homozygous familial hypercholesterolemia, but also in heterozygosity when LDL cholesterol cannot be adequately controlled. Familial hypercholesterolemia carries a high-risk of premature coronary artery disease if not treated [2]. Clinical effects of cholesterol reduction with drugs and/or LDL apheresis include reduced mortality and morbidity in coronary heart disease [3] as well as functional improvement and arrested progression of coronary stenoses [4]. LDL cholesterol can be removed from plasma by adsorption devices, precipitation devices, or filtration devices, or more recently from whole blood. All methods are effective in reducing LDL levels [1], but superiority regarding prevention of coronary events has so far not been demonstrated for any of the columns. The complement system is postulated to be of pathogenetic importance for development of atherosclerotic lesions [5–8] and there is evidence that apheresis treatment activates complement and thus increases the amount of systemic complement activation products [9]. Chemokines (chemotactic cytokines) are important in many diseases including atherosclerotic coronary heart disease [10] and several chemokines correlate with cardiovascular risk [11]. Some studies have compared different apheresis columns regarding immunoglobulins, coagulation, and some inflammatory markers [12–16], but a systematic investigation of how three different columns affect a whole range of biomarkers such as chemokines and complement activation products has

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not previously been performed. Different column effects could be of great importance for patients facing many years of LDL apheresis and possible column dependent effects on the immune system. The aim of the present study was therefore to examine and compare the inflammatory response induced by LDL apheresis treatment utilizing three different apheresis columns.

METHODS

The study was designed as a cross-over study in which three patients already established in long-term LDL apheresis treatment underwent six consecutive treatments with three different LDL apheresis columns. The order of the columns was random for each patient.

Patients and Ethics

Three patients with familial hypercholesterolemia who were established in LDL apheresis for more than 24 months were included. They were heterozygous for the C210G mutation in the LDL receptor gene and had coronary artery disease. There were two women and one man ranging from 41 to 47 years of age. They were all intolerant to statins due to myalgia. Vascular access was obtained by arterio-venous (AV) fistulas. The study was approved by the local ethic committee and all patients gave their written, informed consent.

LDL Apheresis

The following devices were compared as follows: Liposorber® D DL-75 (DL-75; Kaneka Corporation, Osaka, Japan), Liposorber® LA-15 (LA-15; Kaneka Corporation), and Cascadeflo EC-50W (EC-50W; Asahi Kasei, Medical Europe). Treatment with the DL-75 and LA-15 columns was conducted with the Kaneka MA-03 (Kaneka Corporation) machine. The OctoNova (MeSys Gmbh, Hannover, Germany) machine was used in the treatment with the EC-50W filter.

DL-75 is a whole blood adsorption column and part of the Liposorber D system. This column utilizes dextran sulphate cellulose beads for adsorption of LDL. It is modified with regard to the particle size and allows for perfusion and adsorption of LDL cholesterol directly from whole blood [17,18].

LA-15 is a dextran sulfate cellulose adsorption column, removing LDL from plasma. It is based on electrostatic binding between positively charged Apo B and the negative charges of dextran [19].

The Cascadeflo EC-50W is a lipid filtration system eliminating LDL on the basis of molecular weight and three-dimensional structure [20].

Anticoagulation is mandatory during the apheresis treatment. In whole blood apheresis (DL-75) acid citrate dextrose-A was used, and in the two plasma-systems (LA-15 and EC-50W) heparin was used.

Treatment volume averaged 4500 ml plasma for both plasma columns (LA-15 and EC-50W) and 8500 ml whole blood for the DL-75 column. The treatment was performed weekly.

Blood Samples and Analyses

Blood samples were drawn from the AV-fistulas immediately before and after the LDL apheresis treatment. Analyses included routine biochemical markers, lipid parameters, and a number of inflammatory markers. The patients underwent six weekly treatments with each apheresis system, adding up to 18 treatments per patient and 36 sets of samples per patient.

LDL cholesterol and high-sensitivity C-reactive protein (hs-CRP) were measured by standardized laboratory tests in the hospital laboratory immediately after collection. For complement and cytokine analyses, blood was anticoagulated with EDTA and centrifuged for 15 min, 3220*g* at 4°C. The plasma was frozen in aliquots at −70°C until analyzed.

Complement Activation Products

C4d, C3a, and Bb were analyzed according to the manufacturer's specifications in kits delivered by Quidel Corporation (San Diego, CA). C5a was analyzed by a kit from BD Biosciences Pharmingen (San Diego, CA). The fluid-phase terminal sC5b-9 complex (TCC) was analyzed according to a method previously described [21].

Cytokines

Plasma samples were analyzed using a multiplex cytokine assay (Bio-Plex Human Cytokine 27-Plex Panel; Bio-Rad Laboratories Inc., Hercules, CA) containing the following 27 analytes: IL (interleukin) 1 beta (IL-1β), IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin (CCL11), basic fibroblast growth factor (FGF), G-CSF, GM-CSF, IFN-γ, chemokine (C-X-C motif) ligand 10 (IP-10 or CXCL10), monocyte chemoattractant protein 1 (MCP-1 or CCL2), MIP-1α (or CCL3), MIP-1β (or CCL4), platelet derived growth factor (PDGF), regulated on activation T cell expressed and secreted (RANTES or CCL5), TNF-α, and vascular endothelial growth factor (VEGF). The analysis was performed according to the manufacturer's instructions.

Statistics

Mean ± SD of standard laboratory tests were compared with *t*-tests. A two-tailed *P*-value < 0.05 was considered statistically significant. Because of inter-individual variation, cytokine and complement activa-

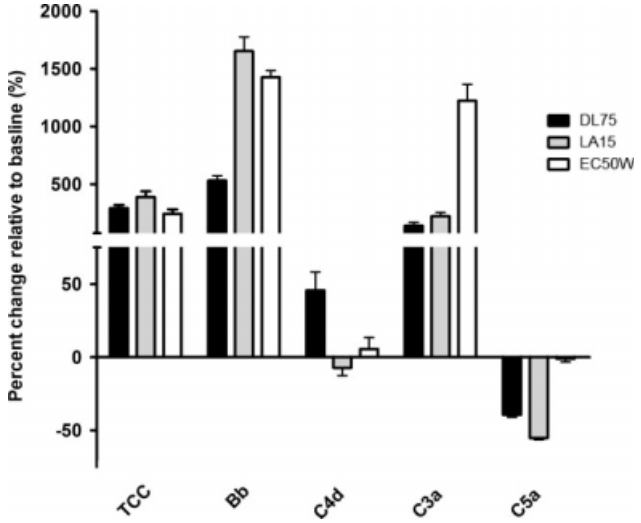

Fig. 1. Complement activation: Percent change in complement activation products relative to baseline (mean, standard error) with the columns DL-75, LA-15, and EC-50W. TCC, the soluble C5b-9 complement complex; Bb, split product, alternative pathway; C4d, split product, classical/lectin pathways; C3a, C5a, anaphylatoxins from cleavage of C3 and C5; respectively.

tion product concentrations were normalized for evaluation of column effect and changes are in percent.

The column effects on complement activation products and cytokines were furthermore studied using a (blocked) one-way ANCOVA (analysis of covariance). This is a one way analysis of variance (ANOVA) correcting for the pretreatment level of the inflammatory parameter. As responses from different individuals are likely to differ, the analysis treats the patients as block effects. All statistical analyses were performed with SPSS for Windows (SPSS 16.0, Chicago, IL).

RESULTS

Complement

All columns induced complement activation as revealed by a significant ($P < 0.01$) and similar increase (approximately 500%) in the soluble C5b-9 terminal complement complex (TCC) indicating that, the cascade was activated to completion (Fig. 1). A substantial increase in fragment Bb, reflecting alternative pathway activation, was observed for all columns. The increase in Bb for the whole blood column (DL-75) was markedly less than for the plasma columns (LA-15 and EC-50W). C4d, reflecting classical and lectin pathway activation, changed only marginally. The anaphylatoxins C3a and C5a varied substantially between the columns. C3a increased to the same extent in the adsorption columns (DL-75 and LA-75) and substantially more in the filtration device (EC-50W), whereas C5a was reduced in the adsorption columns and unchanged in the filtration device, consistent with

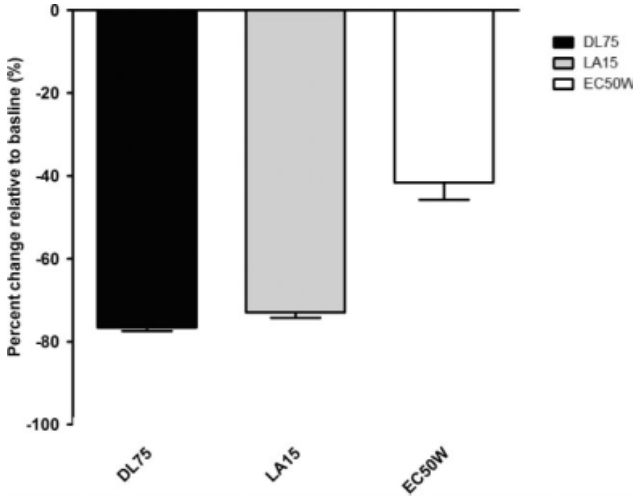

Fig. 2. Hs-CRP: Percent change relative to baseline in hs-CRP for the three different LDL apheresis columns as reported in Figure 1.

the well-known adsorptive properties of the anaphylatoxins. When the columns were compared with ANCOVA there were significant differences before and after apheresis for complement activation products for the three columns ($P < 0.05$).

Hs-CRP

Mean hs-CRP was lowered by all columns and to the greatest extent by the adsorption columns (Fig. 2). Thus, DL-75 reduced hs-CRP from 1.2 ± 0.4 mg/l to 0.3 ± 0.1 mg/l (77%) and LA-75 reduced hs-CRP from 1.4 ± 0.7 mg/l to 0.4 ± 0.2 mg/l (72%), whereas the filtration device EC-50W reduced hs-CRP from 1.3 ± 0.5 mg/l to 0.7 ± 0.3 mg/l (43%) ($P < 0.001$ for all).

Cytokines

Eight of the 27 cytokines, chemokines, and growth factors examined showed substantial changes during treatment and could be grouped according to whether they increased or decreased (Fig. 3). IL-1ra, IP-10, and MCP-1 were consistently increased (approximately doubled) after apheresis, whereas IFN-γ, TNF-α, RANTES, VEGF, and PDGF consistently decreased. Notably, the whole blood adsorption column DL-75 had no effect on the levels of the chemokine MCP-1. Other cytokines including IL-6, IL-8, eotaxin, FGF-basic, G-CSF, and GM-CSF showed less changes (0.5- to 1.5-fold) during treatment; e.g., IL-6 changed by 10, 40, and −10% with columns DL-75, LA-15, and EC-50W, respectively (data not shown). The statistical significances between the three columns for the eight biomarkers that changed substantially during treatment are shown in Table I. Interestingly, the two adsorption columns LA-15 and DL-75 behaved similarly without significant differences for six of the eight markers

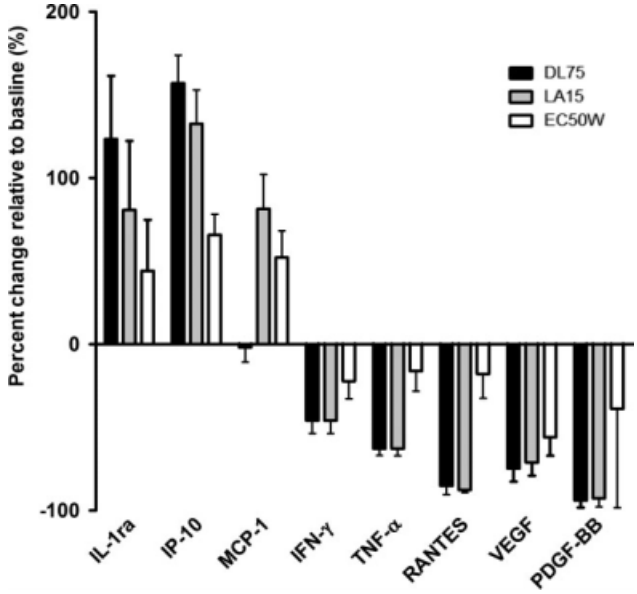

Fig. 3. Cytokines: Percentage change in cytokines (columns indicated in Fig. 1). IL-1ra, interleukin 1 receptor antagonist; IP-10, interferon-induced protein 10; MCP-1, monocyte chemotactic protein-1; IFN-γ, interferon gamma; TNF-α, tumor necrosis factor-alpha; RANTES, regulated on activation normal t cell expressed and secreted; VEGF, vascular endothelial growth factor; PDGF-BB, platelet-derived growth factor-BB.

TABLE I. Pair-Wise Comparison of Three LDL Apheresis Columns with *P*-Values Indicating Differences for Selected Inflammatory Parameters Using ANCOVA Statistics

Biomarker	DL-75 vs. LA-15[a]	DL-75 vs. EC-50W	LA-15 vs. EC-50W
IL-1ra	0.557	***0.037***[b]	0.137
IP-10	0.307	***0.007***	***0.031***
MCP-1	***0.003***	***0.002***	0.809
IFN-γ	0.767	0.120	***0.015***
TNF-α	0.809	***0.004***	***0.001***
RANTES	0.588	***<0.001***	***<0.001***
PDGF-BB	0.297	***<0.001***	***<0.001***
VEGF	***<0.001***	***0.012***	***0.035***

Abbreviation used for the biomarkers: IL-1ra, Interleukin 1 receptor antagonist; IP-10, Interferon-inducible protein 10; MCP-1, Monocyte chemotactic protein-1; IFN-γ, Interferon gamma; TNF-α, Tumor necrosis factor-alpha; RANTES, Regulated on activation normal t cell expressed and secreted; PDGF-BB, Platelet-derived growth factor-BB; VEGF, Vascular endothelial growth factor.

[a]The following columns were used: DL-75: whole blood adsorption column; LA-15: plasma adsorption column; EC-50W: Plasma filtration device.

[b]Statistically significant differences indicated in bold italics.

(Table I, left column), whereas the plasma filtration device differed significantly from the adsorption columns LA-15 and DA-75 for six and seven of the eight markers, respectively (Table I, middle and right columns).

Lipid Parameters

All three columns efficiently reduced LDL cholesterol. Thus, mean LDL cholesterol was lowered from 5.6 ± 0.4 mmol/l to 1.9 ± 0.3 mmol/l (67%) for DL-75 ($P < 0.001$), from 5.3 ± 1.3 mmol/l to 1.3 ± 0.3 mmol/l (75%) for LA-15 ($P < 0.001$), and from 5.6 ± 0.9 mmol/l to 1.8 ± 0.3 mmol/l (68%) for EC-50W ($P < 0.001$). There were reductions in HDL-cholesterol of 20%, 12%, and 18% for the columns DL-75, LA-15, and EC-50W, respectively (all $P < 0.001$).

DISCUSSION

The present study is the first to systematically compare the effects of three different LDL apheresis columns on the inflammatory network during treatment of familial hypercholesterolemia. Although only marginal changes in general biochemical markers were observed, the effect on inflammation was distinct and should be taken into account when evaluating the overall biocompatibility since inflammatory biomarkers might give adverse long-term vascular effects. The whole blood adsorption column DL-75, the plasma adsorption column LA-15, and the plasma filtration device EC-50W all reduced LDL cholesterol efficiently and to the same extent. All columns reduced hs-CRP significantly, the two adsorption columns being most effective. Inflammatory biomarkers from the complement system and the cytokine network were differently affected by the devices in a complex manner as discussed in detail later.

Complement

Both plasma separation columns and LDL apheresis columns may activate the complement system [9]. Kobayashi et al. found that C3a was reduced during a single pass through an LDL adsorbent column ex vivo whereas C5a was unchanged [17]. This is apparently in contrast to our findings, as we observed a marked increase in C3a with all columns. The increase was, however, substantially less pronounced in the adsorption columns. Furthermore, our study was performed in vivo, in which plasma or blood was passed continuously through the columns in a true clinical setting. Complement activation by apheresis columns cannot merely be evaluated by measuring single intermediate activation products; e.g., the anaphylatoxins C3a and C5a are subject to adsorption to various surfaces because they are highly positively charged (pI of C3a is 11.3). Our finding of high levels of the neutrally charged TCC, induced equally by all columns, proves large scale complement activation with formation of C5a, although plasma levels of this marker actually decreased after apheresis. Thus, our findings indicate that complement is activated similarly by all columns,

but C3a and C5a are adsorbed to the surface of the adsorption columns and thereby show less increase than the filtration device. Of clinical importance, however, is the fact that even if the anaphylatoxins are attached to an adsorption column they may still be biologically active. In a recent proteomic study, LDL apheresis columns were shown to remove various components of the complement system, and in line with our findings there were differences between the columns [22]. C4d and Bb do not have the characteristic positive charges as the anaphylatoxins, which may in part explain the different patterns displayed by the various activation products in the present study.

It should be kept in mind that the concentration of the various complement activation products in the blood leaving the columns is the net result of activation by the artificial surface, biological degradation and adsorption by the column. Furthermore, the concentration measured does not reflect the systemic concentration, as blood entering the patient will be thoroughly mixed in the venous circulation. Epidemiological studies have shown that serum levels of complement component C3 are associated with cardiovascular risk [23] and complement activation products have been identified in tissue samples of recent myocardial infarctions [24]. Speidl et al. reported that the circulating level of C5a seems to be associated with increased cardiovascular risk in patients with advanced atherosclerosis.25 Thus, our finding that C3a increased the most with the filtration device EC-50W and that C5a was reduced by the adsorption columns DL-75 and LA-15 may be of clinical importance. However, any direct causal role of complement activation in atherosclerosis has not been fully established. Therefore, our preliminary results should be confirmed in a larger group of patients with different end points. Although the activation products may be biologically active after adsorption as discussed earlier, it is reasonable to suggest that the adsorption effect is overall beneficial for the individual because the total load of biologically active products released to the circulation is reduced.

Hs-CRP

Several studies have shown that LDL apheresis with different apheresis devices lowers hs-CRP [12,15]. Hershcovici et al. systematically compared plasmapheresis, dextran sulfate attached cellulose (DSA), and the whole blood DALI system in four patients and found a 47.5%, 36.1%, and 22% reduction in hs-CRP, respectively [14]. We found the largest reduction in hs-CRP for the whole blood column DL-75. This is in contrast to Hershcovicis findings. However, they used a different whole blood column that may have had less adsorptive effects than DL-75. Notably, the plasma adsorption column LA-15 reduced hs-CRP virtually to the same extent as the whole blood column in our study, whereas the reduction in the filtration device EC-50W was less pronounced. Hs-CRP is now thought to take part in the inflammatory response [26] and recently, in a large clinical statin trial with rosuvastatin, both CRP and clinical end-points were reduced in the treatment arm [27]. Thus, how the LDL apheresis columns affect this biomarker may be of clinical importance and our data imply a favorable effect of the adsorption columns in this regard.

Cytokines

A few previous studies have addressed how LDL apheresis affects biomarkers including cytokines. Kobayashi et al. performed LDL apheresis in patients with peripheral artery disease and found a nonsignificant increase in MCP-1 after treatment with the LA-15 column [15], in line with our findings. Kojima et al. noted a 70% increase in IL-6 during dextran-sulfate LDL apheresis even if hs-CRP was reduced [19]. We found less changes in IL-6 than Kojima, with a 10, 40, and −10% differences in IL-6 levels with columns DL-75, LA-15, and EC-50W, respectively. A recent article from Otto et al. describes a significant 40% increase in IL-6 after treatment with Liposorber D compared with another whole blood system (DALI) [28]. We could not demonstrate such a relationship using the whole blood adsorption column DL-75, finding a nonsignificant increase.

Cytokines are classified as proatherogenic (for instance TNF-α, MCP-1, RANTES) or antiatherogenic (for instance IL-1ra and IL-10) [29]. Serum levels of RANTES are very recently associated with coronary artery disease risk [30]. We found significantly higher levels of the antiatherogenic IL-1ra with the adsorption columns DL-75 and LA-15 than with the filtration device EC-50W. Regarding proatherogenic chemokines, DL-75 had no effect on levels of MCP-1, whereas LA-15 and EC-50W induced a modest increase. For both RANTES and TNF-α, there was a significantly more pronounced decrease after treatment with the DL-75 and the LA-15, than with the EC-50W filter, where only minor changes were observed. This illustrates that various LDL apheresis columns affect proatherogenic and antiatherogenic cytokines differently, and from our data it is tempting to speculate that the adsorption columns display a more beneficial inflammatory profile. As this kind of treatment is given for a long time, these differences could be of importance for progression of the atherosclerotic disease. At the present, it is not possible from our results to determine whether differences in cytokine levels between the columns reflect differences in adsorption to the columns, but the net effect of a lower load of inflammatory mediators released to the circulation would probably be beneficial as discussed

for the anaphylatoxins earlier. In an in vitro model of biocompatibility, we have previously shown that inhibition of complement activation blocked the release of certain cytokines and chemokines indicating that complement activation may also affect cytokine release in the present setting [31].

Lipid Parameters

A comparison of several studies has documented that apheresis treatment significantly reduces LDL-cholesterol [1], and we correspondingly found a 67% to 75% decrease. HDL-cholesterol is typically reduced from 10% to 20% [1] as we also noted. There are few studies comparing columns head-to-head, but Julius et al. found a more pronounced decrease in LDL cholesterol in whole blood apheresis than in LDL plasma apheresis, however stating that both methods are clinically effective and that LDL reduction partly is a function of treated volume [32]. Poli and Busnach compared two different whole blood apheresis systems (direct adsorption with a polyacrylic acid column *vs.* adsorption with a modified dextran sulfate column) and found LDL reductions of 62% and 59%, respectively, in accordance with our findings [33]. Thus, the intended therapeutic effect was equal with the three columns studied, but as discussed earlier differences in inflammatory profile should be taken into account when comparing columns for long-term clinical use.

Limitations

A limitation of the present study is the sample size, and due to this our study might function as a pilot study. The small sample size is partly counteracted by treatment with the different columns in a randomized cross-over design attenuating the significance of possible carry-over effects. Furthermore, as the study was performed in only one center, sample collection and analyses were highly standardized. The different lab tests and biomarkers were sampled immediately before and after apheresis treatment. The kinetics of the changes occurring before the next apheresis treatment is not known. The fact that none of the patients received statin therapy might have influenced levels of the inflammatory biomarkers compared to statin users. However, statin intolerance is a frequent indication for LDL apheresis in patients with familial hypercholesterolemia (FH) meaning that the present combination is clinically relevant and fairly common.

CONCLUSIONS

Several complement activation products and cytokines with importance for progression of vascular atherosclerosis as well as plaque stability, including C3a, C5a, IL-1ra, MCP-1, and TNF-α were differently affected by the three apheresis columns DL-75, LA-15, and EC-50W even if LDL cholesterol was reduced equally by all of them. However, as LDL apheresis is a long-term treatment, differences between apheresis columns with respect to their inflammatory profile could be of clinical significance for patients with or at high risk for atherosclerotic diseases. For most of the inflammatory markers studied, EC-50W was the filter with the smallest changes between pretreatment and post-treatment levels, but the differences between the two adsorption columns were minor. In general, the adsorption columns showed an apparently more beneficial inflammatory profile than the filtration device. Larger studies are needed to confirm our findings and also to establish whether the different changes in inflammatory biomarkers are due to unequal activation by the various columns, whether the columns have different adsorptive properties or both. Furthermore, clinical end points related to changes in biomarkers should be addressed.

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Article II

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Complement profile and activation mechanisms by different LDL apheresis systems

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ABSTRACT

Extracorporeal removal of low-density lipoprotein (LDL) cholesterol by means of selective LDL apheresis is indicated in otherwise uncontrolled familial hypercholesterolemia. During blood–biomaterial interaction other constituents than the LDL particles are affected, including the complement system. We set up an ex vivo model in which human whole blood was passed through an LDL apheresis system with one of three different apheresis columns: whole blood adsorption, plasma adsorption and plasma filtration. The concentrations of complement activation products revealed distinctly different patterns of activation and adsorption by the different systems. Evaluated as the final common terminal complement complex (TCC) the whole blood system was inert, in contrast to the plasma systems, which generated substantial and equal amounts of TCC. Initial classical pathway activation was revealed equally for both plasma systems as increases in the C1rs–C1inh complex and C4d. Alternative pathway activation (Bb) was most pronounced for the plasma adsorption system. Although the anaphylatoxins (C3a and C5a) were equally generated by the two plasma separation systems, they were efficiently adsorbed to the plasma adsorption column before the "outlet", whereas they were left free in the plasma in the filtration system. Consequently, during blood–biomaterial interaction in LDL apheresis the complement system is modulated in different manners depending on the device composition.

1. Introduction

Heterozygous familial hypercholesterolemia is common and, due to high levels of low-density lipoprotein (LDL) cholesterol, carries a high risk of premature atherosclerosis if not treated [1]. In most cases the disease is controlled by lipid lowering medication, but in some instances extracorporeal treatment by means of LDL apheresis is necessary [2]. This treatment is highly effective in reducing LDL cholesterol and clinical end-points [2,3]. The artificial surfaces may, however, affect other constituents of the blood in an adverse manner. Studies on blood–biomaterial interaction during extracorporeal treatment have demonstrated that complement activation may be triggered by biomaterial surfaces [4], and studies in hemodialysis have shown that hemodialysis membranes trigger the complement system [5]. The biocompatibility of dialysis membranes is also linked to clinical end-points [6]. Studies indicate that the alternative pathway (AP) of complement activation is important when foreign surfaces interact with blood [7,8]. The alternative pathway can be activated directly by the surface or amplified after initial activation by classical or lectin pathway activation [4,9], in both cases playing a pivotal role in the degree of activation beyond C3. Notably, even if the biomaterial surfaces induce complement activation, the membranes may also adsorb complement factors such as C3a and C5a [10]. Consequently, it is the net result after extracorporeal treatment that is of clinical importance. This is in accordance with the definition of biocompatibility as being "the ability of a material to perform with an appropriate host response in a specific application" [11], recently revised to "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" [12].

Complement activation may be of particular clinical importance for patients undergoing long-term, potentially lifelong, LDL apheresis treatment as the complement system plays a role in the development of atherosclerosis [13]. Whereas activation of the initial

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phase (C1–C3) might contribute to the clearance of plaque debris, the later phase (C5–C9) may enhance inflammation and lead to plaque destabilization. Our previous work has indicated that three LDL apheresis columns affect biomarkers, including complement activation products, differently [14,15]. This prompted us to set up an ex vivo study investigating in detail the complement compatibility of these LDL apheresis columns by repeatedly circulating whole blood from healthy donors through the systems while sampling at sites prior to and after plasma separation and column passage. Total complement activation was evaluated by measuring the fluid phase terminal C5b–9 complex (TCC). The initial pathway activation mechanisms were revealed by determining the levels of C1rs–C1 inhibitor complexes (C1rs–C1inh) for the classical pathway, C4d for the classical and the lectin pathways, and Bb for the alternative pathway. The "inflammatory load" from the common activation of all pathways was evaluated as the amounts of the potent anaphylatoxins C3a and C5a.

2. Materials and methods

2.1. Ethics

The local ethics committee approved the study and all blood donors signed an informed consent.

2.2. Donors

Blood was drawn from six healthy donors (three males and three females). Each individual donated 450 ml of blood three times at approximately one month intervals.

2.3. LDL apheresis

The following devices were compared: Liposorber1 D DL-75 (DL-75) (Kaneka Corp.n, Osaka, Japan); Liposorber1 LA-15 (LA-15) (Kaneka Corp.); Cascadeflo EC-50W (EC-50W) (Asahi Kasei Medical Europe). Treatment with the DL-75 and LA-15 columns was conducted using a Kaneka MA-03 (Kaneka Corp.) machine. The OctoNova (MeSys Gmbh, Hannover, Germany) machine was used in treatment with the EC-50W column.

DL-75 is a whole blood adsorption column and part of the Liposorber D system. This column utilizes dextran sulfate cellulose beads for adsorption of LDL cholesterol. It is modified with regard to the particle size and allows for perfusion and adsorption of LDL cholesterol directly from whole blood. The flow rate was 30 ml min^{-1}. LA-15 is a dextran sulfate cellulose adsorption column which removes LDL cholesterol from plasma. It is based on electrostatic binding between positively charged apolipoprotein B (ApoB) and the negative charges of dextran. The flow rate was 100 ml min^{-1}, the plasma flow rate 20 ml min^{-1}. Cascadeflo EC-50W is a lipid filtration system eliminating LDL cholesterol on the basis of molecular weight and three-dimensional structure. The flow rate was 100 ml min^{-1}, the plasma flow rate 20 ml min^{-1}.

The plasma separation column used in both LA-15 and EC-50W is a PlasmaFlo OP-05 W column (Asahi Kasei Medical Europe) and hence the column is identical for both the plasma separation based systems.

Anticoagulation is mandatory during clinical apheresis treatment. In whole blood apheresis (DL-75) acid citrate dextrose-A is used, while in the two plasma systems (LA-15 and EC-50W) heparin is commonly used. In the current ex vivo model lepirudin was used, as it has been demonstrated that lepirudin does not affect the complement system [16].

A total of 18 ex vivo LDL apheresis treatments were performed, for six donors on the three different LDL apheresis columns (DL-75, LA-15 and EC-50W). Treatment time was 240 min for each column.

2.4. Ex vivo model

25 mg lepirudin (Refludan, Celgene, Marburg, Germany) in 50 ml of 0.9% NaCl was added to a 600 ml Blood-Pack Unit without anticoagulant (Fenwal, Lake Zürich, IL). The freshly donated whole blood (450 ml) was immediately transported to the research laboratory (transportation time <5 min). 50 ml was transferred to a control bag (sample control (SC)) similar to that mentioned above (Fig. 1). The main bag served as a reservoir for the closed circuit. The blood reservoir and the control bag were then placed in a temperature controlled heater (Binder, Binder GmBH, Tuttlingen, Germany) set at 37 °C, with constant movement by means of a modified test tube rotator (Rock 'n Roller, Labinco BV, Breda, The Netherlands). The reservoir was attached to the LDL apheresis system (DL-75, Fig. 1A, LA-15 and EC-50W, Fig. 1B).

Blood samples were obtained from the control bag (SC) before LDL apheresis (T_0), and then during apheresis at 15, 30, 60, 120, 180 and 240 min at the positions shown in Fig. 1. All blood samples from the different positions were drawn simultaneously (within a time frame of 1 min) for each sample time.

2.5. Blood samples and analyses

Samples were drawn into tubes containing EDTA (to block any further complement activation) to a final concentration of 10 mM

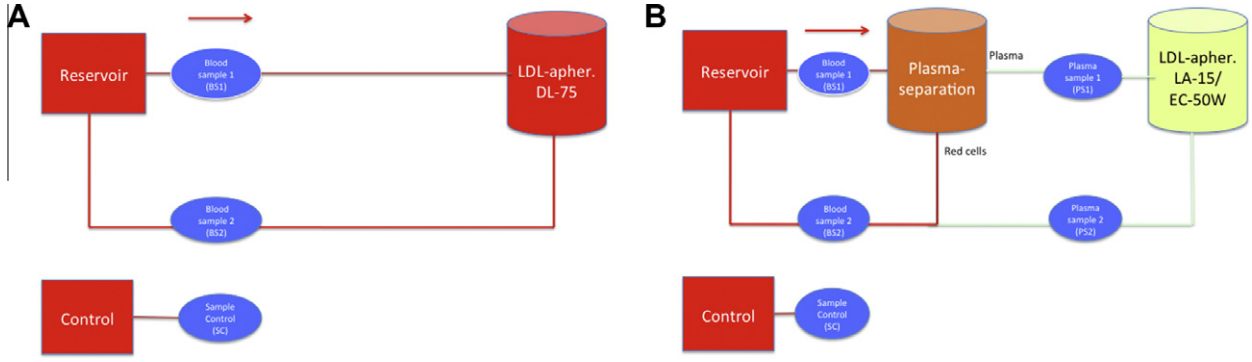

Fig. 1. Schematic drawing of the ex vivo model with blood sample postitions. The reservoir denotes the whole blood bag to which leipuridin was added. (A) Blood samples were obtained from a position after the reservoir (BS1) and then after the LDL apheresis column DL-75 (BS2). A red arrow shows the direction of flow in the system. The control bag was kept on the test tube rotator next to the reservoir and samples were drawn directly from this bag (SC). (B) The columns LA-15 and EC-50W, which required plasma separation before LDL apheresis. The sample sites were before plasma separation (BS1), after plasma separation (PS1), after LDL apheresis (PS2), and after red cells (from plasma separation) and plasma (after LDL apheresis) were combined (BS2), indicating the position where the treated blood would be returned to the patient in a clinical setting. Again, the direction of flow is denoted with a red arrow. As for the whole blood system, samples from the control bag are indicated by SC.

and then placed on ice before centrifugation for 15 min at 3220*g* at 4 °C. The plasma was frozen in aliquots at −70 °C until analysis in batches.

2.6. Complement activation products

The complement activation products were measured using enzyme immunoassays based on capture antibodies reacting with neoepitopes exposed selectively in the activation product and not in the native component. C4d, C3a, Bb (Quidel Corporation, San Diego, CA) and C5a (BD Biosciences, San Jose, CA) were analyzed according to the manufacturer's specifications. The fluid phase TCC and C1rs–C1-inh were analyzed according to methods previously described in detail [17,18].

2.7. Correction for dilution

A small amount of priming solution (isotonic saline) was used to prepare the tubing and columns before the ex vivo loop was started. Hematocrit was used to correct the concentrations of the complement activation products according to a standardized formula [19].

2.8. Statistics

Mean concentrations of complement activation factors, corrected for dilution as described above, with the standard error of the mean (SEM) were calculated. Correlation was calculated according to Spearman. A two-tailed *P* value of <0.05 was consid-

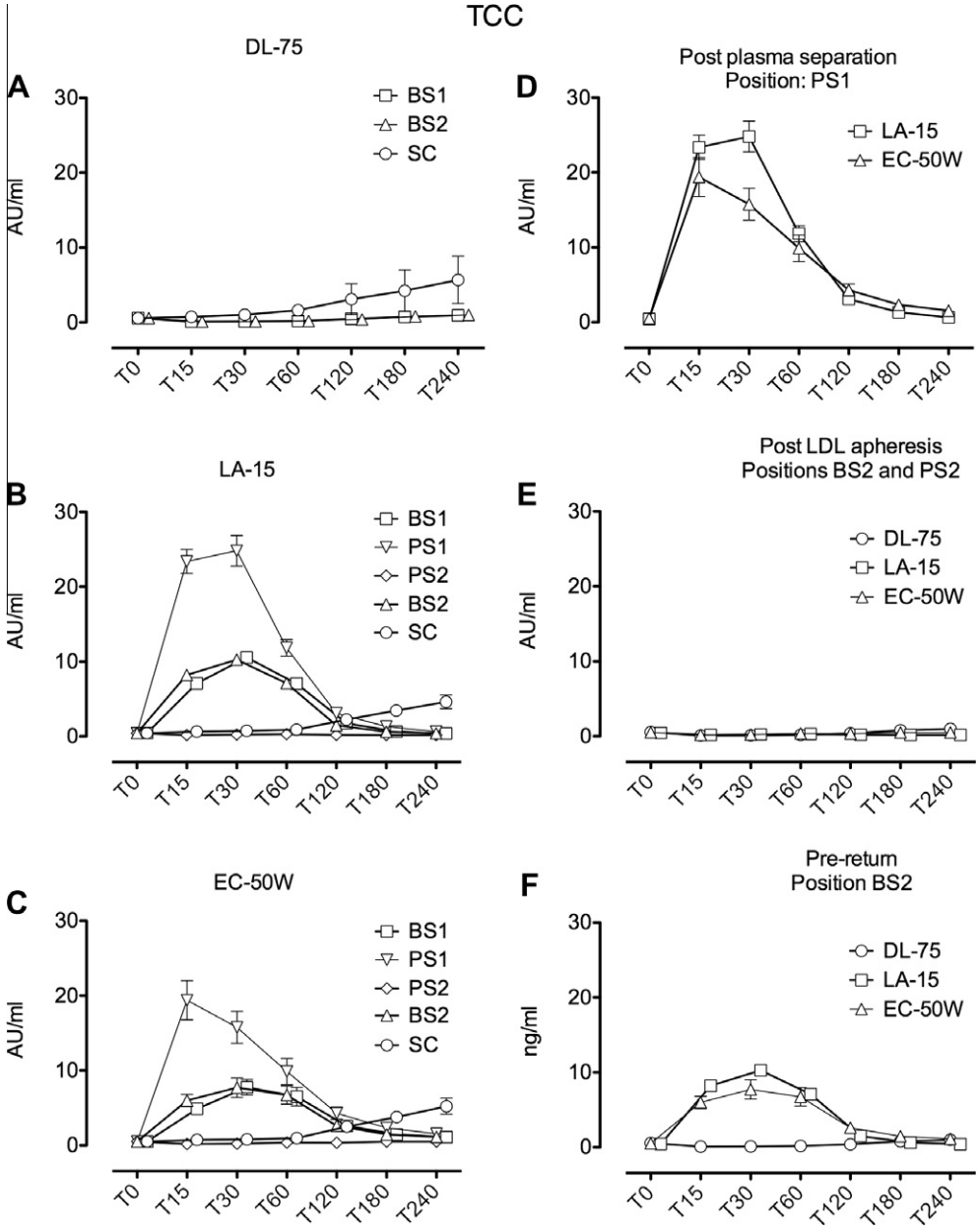

Fig. 2. Formation of TCC. The three left panels (A–C) show the concentrations of the terminal C5b-9 complex (TCC) for the three LDL apheresis columns, DL-75 (whole blood adsorption), LA-15 (plasma adsorption) and EC-50W (plasma filtration). The sampling positions are as indicated in Fig. 1. Times of sampling are indicated on the *x*-axes. Concentrations are corrected for dilution and shown as means with the standard error of the mean for each sample site. The right panels compare post plasma separation values for columns LA-15 and EC-50W (D), TCC concentrations measured immediately after the LDL apheresis columns for all three columns (E) and, finally, a pre-return value for the three columns (F), indicating the position where the treated blood would return to the patient in a clinical setting.

ered statistically significant. All calculations were performed with Prism 5.0 for Mac, Graphpad software (San Diego, USA).

3. Results

3.1. Terminal common pathway activation (Fig. 2)

TCC is the final common activation product of complement and was used to indicate the degree of total complement activation. In the whole blood system DL-75 there was no change in TCC before or after LDL apheresis (Fig. 2A). In contrast, TCC was markedly increased (>40-fold) after passage through the plasma separation columns in LA-15 and EC-50W (Fig. 2B and C), with the increase being slightly less for EC-50W than for LA-15 (Fig. 2D). The TCC concentration gradually decreased with time. Notably, TCC concentrations were reduced to background after passage through the LDL apheresis columns in both plasma systems (Fig. 2E). There was a slight and similar increase in TCC in all control bags (SC), reflecting spontaneous in vitro activation (data not shown). This increase was not seen in post-apheresis samples in any of the three systems (BS2 for DL-75 and PS2 for the plasma systems), consistent with adsorption of the plasma separation-induced TCC by the apheresis column. In the BS2 sample (corresponding to "return to patient") there was increased levels of TCC in both plasma systems, but not in the whole blood system, during the first hour of apheresis (Fig. 2F).

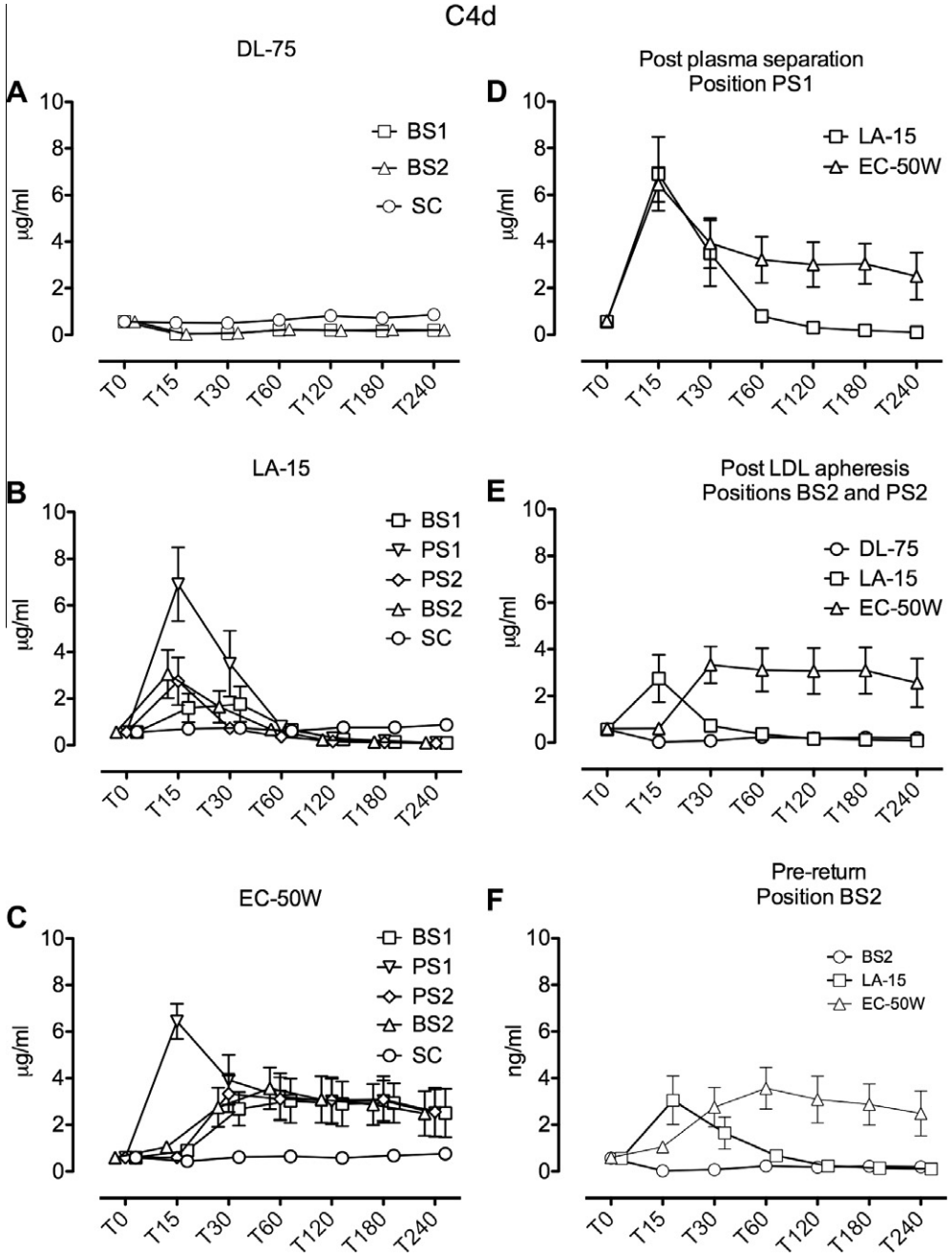

Fig. 3. Formation of C4d. The three left panels show the concentrations of C4d for the sample positions described in Fig. 1A and B. Sample times are given on the *x*-axes, with the columns being as described in Fig. 2. The three right panels denote the comparison described in Fig. 2.

3.2. Mechanisms of initial pathway activation (Figs. 3–5)

C4d is a marker for both classical and lectin pathway activation. In the whole blood system DL-75 there was no change in C4d before or after LDL apheresis (Fig. 3A). In contrast, C4d was markedly increased (>10-fold) after passage through the plasma separation columns LA-15 and EC-50W (Fig. 3B and D). The LDL apheresis columns LA-15 and EC-50W reduced C4d. After 30 min the concentration of C4d was back to the background level in the LA-15 system, whereas it remained elevated throughout in the EC-50W system (Fig. 3C and E). Furthermore, the pre-return values of C4d remained more than 15-fold higher after 240 min in the closed EC-50W circuit compared with the other two systems. C4d remained stable in the control bags (data not shown). Samples from two donors at selected time points were analyzed for C1rs–C1inh, which is specific for the classical pathway. In the whole blood system DL-75 there was no change in C1rs–C1inh before or after LDL apheresis (Fig. 4A). In contrast, C1rs–C1inh was markedly increased (>100-fold) after passage through the plasma separation columns (Fig. 4B and C), providing evidence for classical pathway activation. Accordingly, there was a highly positive correlation between C4d and C1rs–C1inh, with $r^2 = 0.77$ ($P < 0.0001$).

Bb is a marker for the alternative pathway. In the whole blood system DL-75 there was no change in Bb before or after LDL apheresis (Fig. 5A). In contrast, Bb was markedly increased (>10-fold) after passage through the plasma separation columns (Fig. 5B and C), substantially more for LA-15 than for EC-50W (Fig. 5D). After passage through the LA-15 and EC-50W LDL apheresis columns the Bb levels were reduced (Fig. 5E), with the levels of Bb at BS2 (“return to patient”) being consistently higher for LA-15. Similarly to TCC, there was a slight increase in all control bags (data not shown).

3.3. Anaphylatoxins C3a and C5a

(Figs. 6 and 7) C3a and C5a represent biologically highly active fragments of similar size and physico-chemical properties. They behaved strikingly similarly in the LDL apheresis study (Figs. 6 and 7). In the whole blood system DL-75 there were no changes in C3a and C5a before or after LDL apheresis (Figs. 6A and 7A). In contrast, C3a and C5a were markedly increased (100–1000-fold) after passage through the plasma separation columns (Figs. 6 and 7B and C). C3a and C5a were reduced to baseline levels after passage through the LDL apheresis column LA-15. The EC-50W system only slightly reduced the anaphylatoxin levels, which remained at stable high levels from 30 min apheresis. The pre-return values of C3a and C5a were about 1000-fold higher in the filtration system EC-50W than in the other two systems (Figs. 6F and 7F). Similarly to TCC and Bb the C3a and C5a concentrations increased with time in the control bags (data not shown).

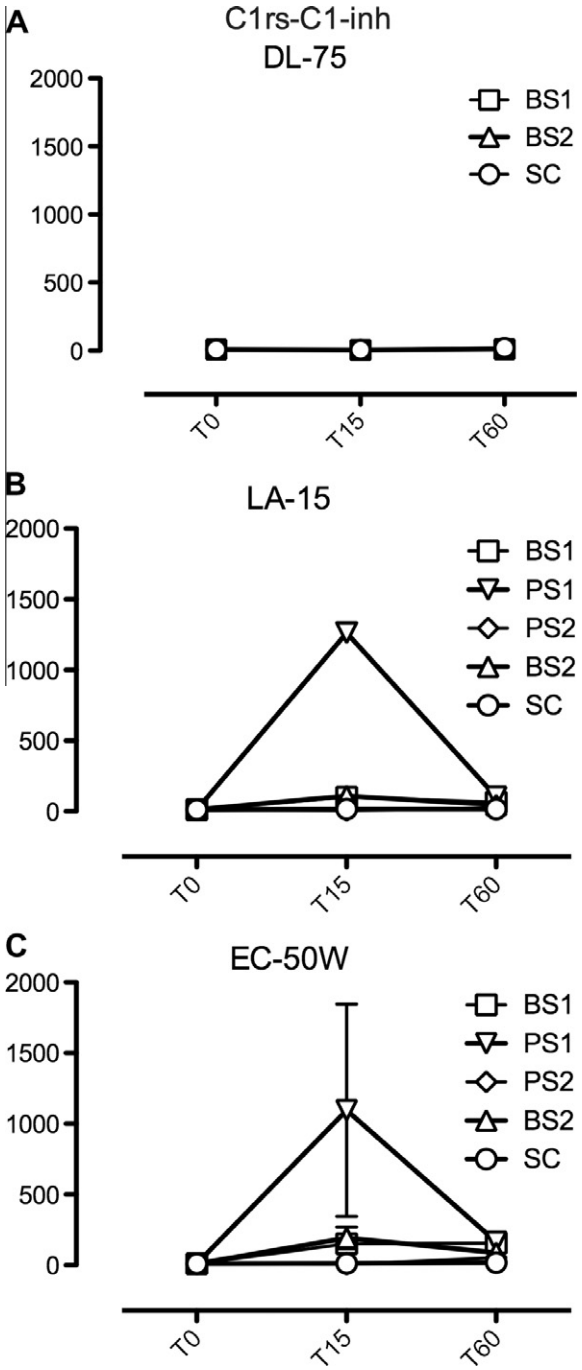

Fig. 4. Formation of C1rs–C1inh. The three panels show concentrations of C1 rs–C1inh, for two selected donors, for the sample positions described in Fig. 1A and B. Sample times are given on the *x*-axes, with the columns being as described in Fig. 2.

4. Discussion

In a previous in vivo study of LDL apheresis we showed that the complement cascade is activated to completion, predominantly through the alternative pathway [14]. In the current ex vivo study the experimental set-up has allowed us to demonstrate that the initial complement activation is through the classical pathway. Subsequently the alternative pathway amplifies complement activation.

4.1. Terminal common pathway activation

In the in vivo study we found that TCC increased approximately three times for the tested columns DL-75, LA-15 and EC-50W [14]. Fadul et al. studied different types of plasma purification therapies, including LDL apheresis, and found that TCC was increased after the plasma separation column and decreased after the LDL apheresis column [20]. Our findings are consistent with these observations; in the whole blood system (DL-75) TCC was unchanged, while in both plasma separation systems (LA-15 and EC-50W) TCC was markedly increased after the plasma separation columns, but subsequently removed in the LDL apheresis columns. The composition of the biomaterial may also be of importance and Ferraz et al. have demonstrated that the pore size of a biomaterial is of importance for activation of the complement system and formation of soluble TCC [21]. The fact that TCC is a large molecule

may contribute to the observation that the filtration column EC-50W is also able to clear this molecule due to the filtration pore size. With time the effects of the plasma separation columns on complement activation were reduced and the concentration of TCC approached baseline values. It is likely that this was due to coating of the biomaterials used in the columns and diminished activation of the complement system with time, analogous to the clinically known "first use syndrome" in which allergic reactions to hemodialysis membranes are attenuated with repetitive use [22], in addition to adsorption and/or degradation of activated complement factors.

It should be noted that when studying complement activation in blood–biomaterial interactions the choice of anticoagulant is crucial. We have previously shown that the direct thrombin inhibitor lepirudin is at present the best candidate for this purpose as it has no effect on complement activation in doses sufficient for adequate anti-coagulation. Both heparin and calcium-binding agents such as EDTA may affect complement activation and are thus unsuitable for the purpose [16]. This should be kept in mind when comparing our results with previous studies.

4.2. Mechanisms of initial pathway activation

Experimental studies have demonstrated that the alternative pathway is important for complement activation in blood–biomaterial interactions [7,8], and a recent review has also underlined the importance of the alternative pathway as an amplification loop for the classical and lectin pathways [23]. Interestingly, we found no changes in the alternative pathway marker Bb for the DL-75 columns, while there was an increase in Bb after the plasma

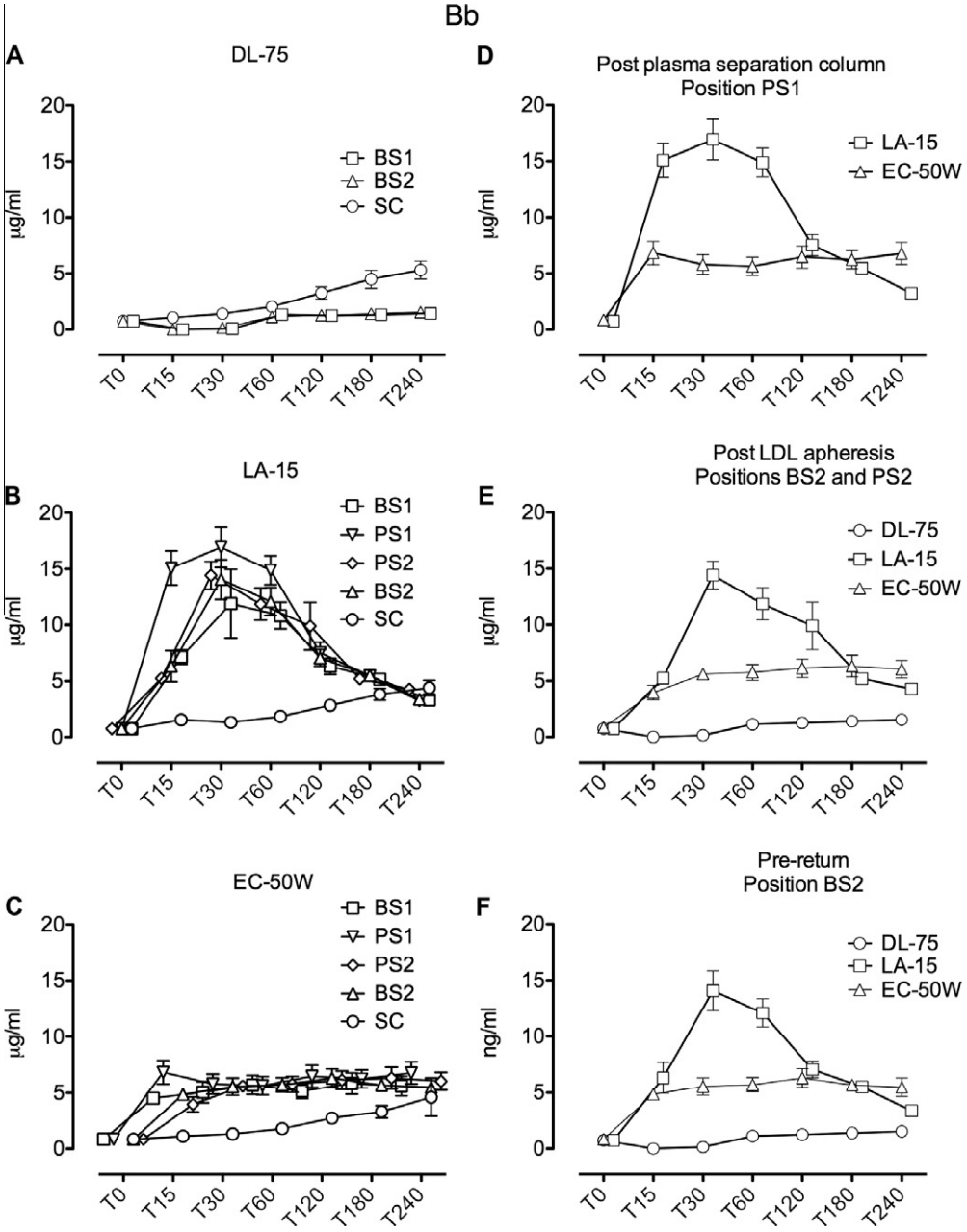

Fig. 5. Formation of Bb. The three left panels show concentrations of Bb for the sample positions described in Fig. 1A and B. Sample times are given on the *x*-axes, with the columns being as described in Fig. 2. The three right panels denote the comparison described in Fig. 2.

separation columns for LA-15 and EC-50W. With time Bb was reduced in the closed circuit of the LA-15 column, suggesting adsorption by the LDL apheresis column. In contrast, the filtration column EC-50W did not clear Bb. Therefore, during LDL apheresis the biomaterial of the plasma separation column activated complement by the alternative pathway, as expected. The classical pathway, however, is thought to be of less importance for complement activation during blood–biomaterial interaction [4]. Complement factor C4d is a marker of activation via the classical and/or lectin pathways. We found no increase after the DL-75 column. However, there was an increase after both plasma separation columns, indicating activation other than by the alternative pathway. The LA-15 column removed C4d, while the EC-50W column did not. The fact that C1rs–C1inh was increased in parallel with the increase in C4d, and the fact that these biomarkers were highly correlated, are further evidence of activation through the classical pathway as well as the alternative pathway in the blood–biomaterial interaction during LDL apheresis, even if additional activation through the lectin pathway cannot be completely ruled out. Unfortunately, at present there is no specific marker available to indicate isolated lectin pathway activation. Accordingly, we have proved that in addition to activation through the alternative pathway there is direct evidence that the complement cascade is activated through the classical pathway during LDL apheresis.

4.3. Anaphylatoxins C3a and C5a

The anaphylatoxins C3a and C5a are small molecules. A previous apheresis study has indicated that C3a is increased after the plasma separation column and decreased after the LDL apheresis column [20]. Takeda et al. demonstrated that the anaphylatoxins C3a, C4a and C5a were inactivated by cellulose acetate beads [24]. Würzner et al. studied complement activation in heparin-induced extracorporeal elimination of low density lipoproteins (HELP) and found induction of C3 and C5a in the plasma separation column. Furthermore, in their study C3 was removed by the LDL

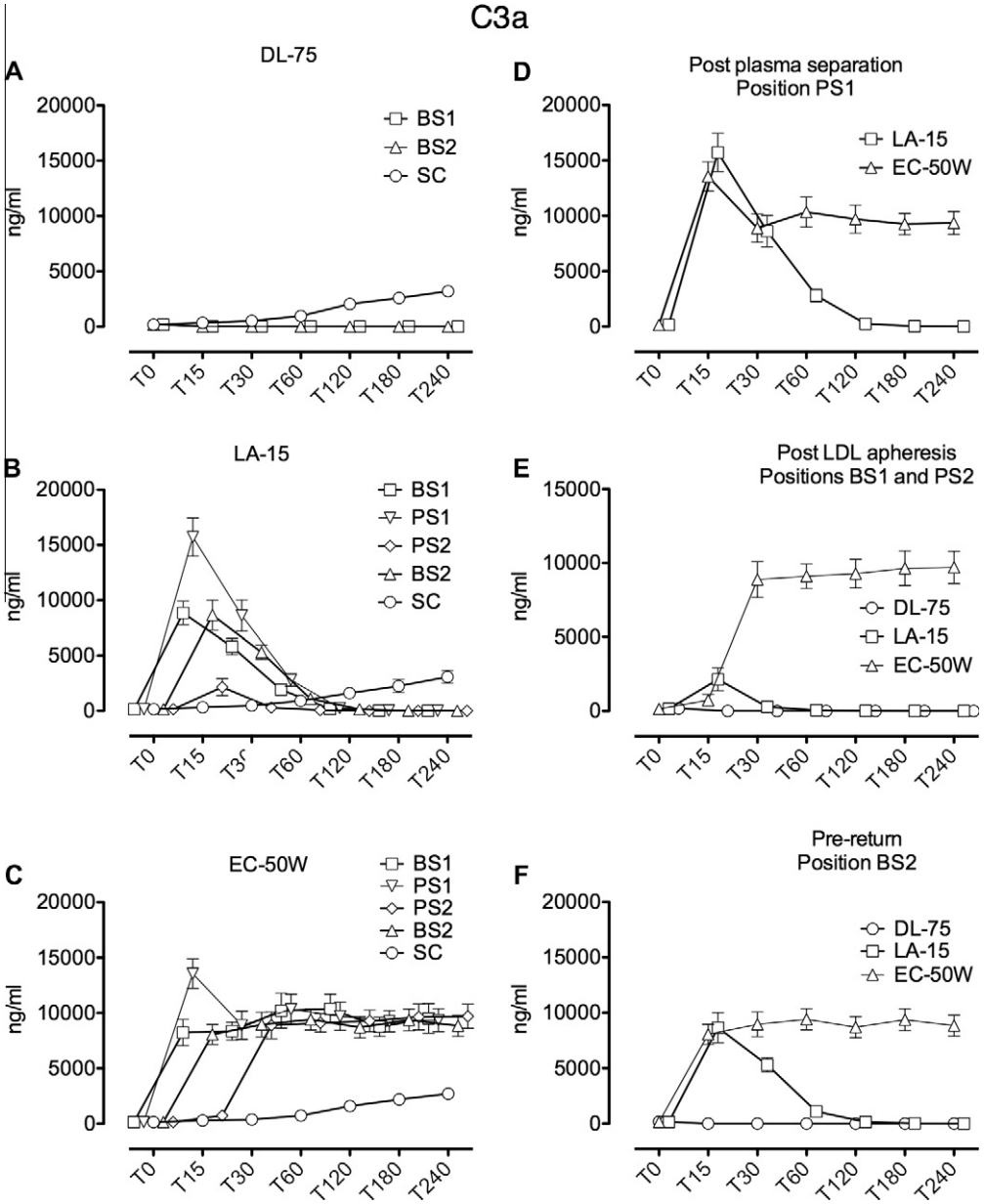

Fig. 6. Formation of C3a. The three left panels show concentrations of C3a for the sample positions described in Fig. 1A and B. Sample times are given on the *x*-axes, with the columns being as described in Fig. 2. The three right panels denote the comparison as described in Fig. 2.

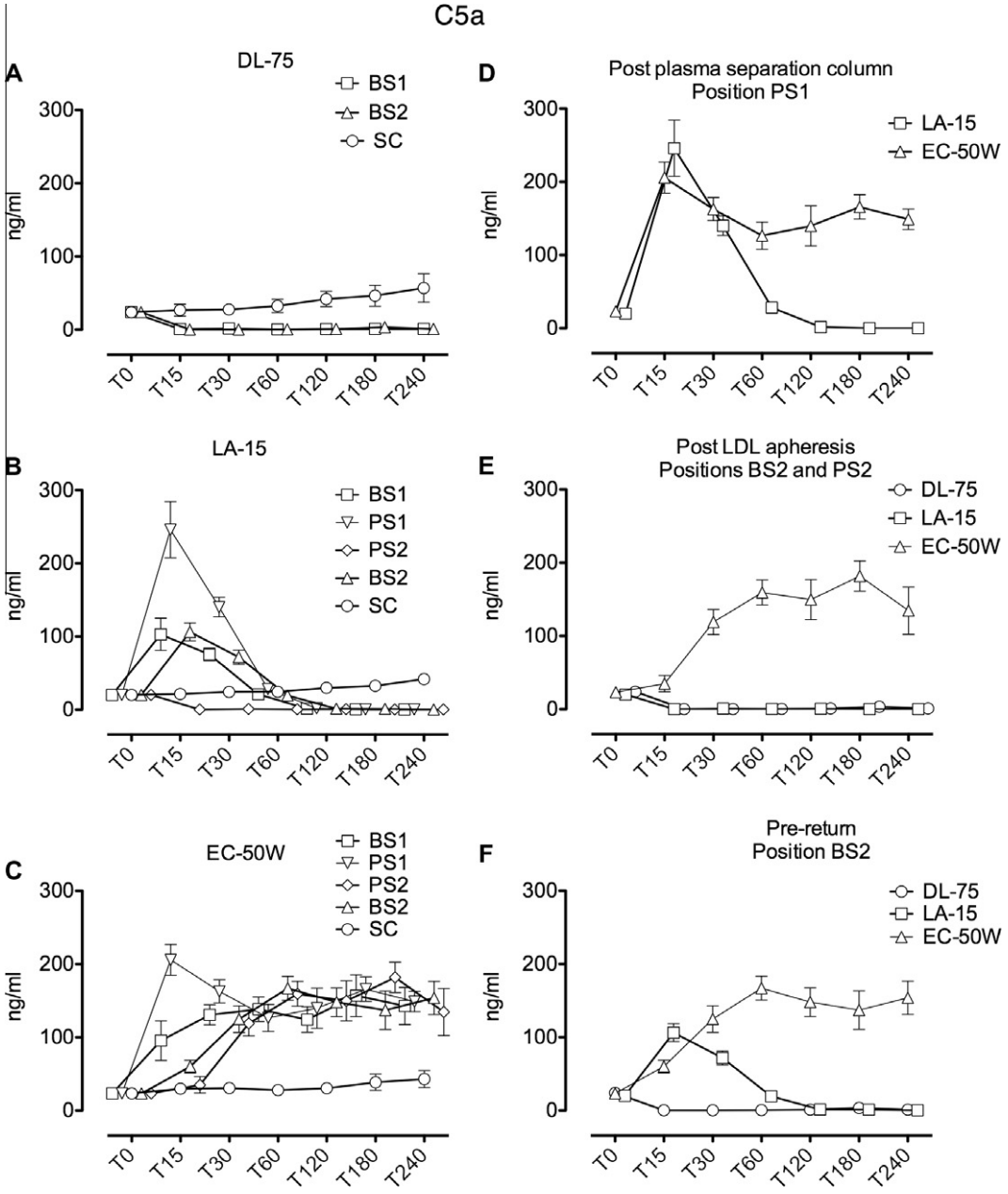

Fig. 7. Formation of C5a. The three left panels show concentrations of C5a for the sample positions described in Fig. 1A and B. Sample times are given on the *x*-axes, with the columns being as described in Fig. 2. The three right panels denote the comparison described in Fig. 2.

apheresis column, but C5a was not. It was suggested that clearance of complement was associated with the ability to bind LDL cholesterol [25]. We found only minor changes in the anaphylatoxins C3a and C5a for the whole blood column DL-75. There were marked increases in both of these anaphylatoxins for both plasma separation systems, indicating activation. With time C3a and C5a were adsorbed by the LDL apheresis column LA-15, but not by the filtration column EC-50W, underlining the inability of the latter column to remove the anaphylatoxins being formed. We have previously established that the ability to lower LDL cholesterol was equal for the three columns [14], even though there were differences between the same columns regarding complement activation. This shows that clearance of complement factors cannot solely be dependent on the ability of columns to bind LDL cholesterol.

Several reviews have pointed out the importance of the complement system for atherosclerosis [13,26]. Complement activation through the alternative pathway seems to be proatherogenic due to pro-inflammatory effects, whereas activation through the classical or lectin pathways seems to be protective [13,27]. C3a and C5a are pro-inflammatory and, indeed, C5a is associated with plaque ruptures leading to acute cardiovascular events [28]. The terminal pathway with formation of TCC is associated with atherosclerosis [13] and Wu et al. have demonstrated that inhibiting formation of the complement membrane attack complex protects against atherosclerosis [29].

We have shown that the whole blood system DL-75 is biocompatible and that the complement system is not activated by this system, and thus in this setting is "complement compatible". The two plasma separation-based systems both induced the pro-atherogenic factors TCC, C3a and C5a, however, LA-15 was able to clear C3a and C5a, but the plasma filtration column EC-50W was not and thus was less complement compatible. These findings

could be of importance for patients facing possible lifelong treatment with extracorporeal removal of LDL cholesterol. The current study was performed ex vivo and thus in a closed system different from the clinical setting, in which complement factor production and removal may take place in the body. Evidently, the in vitro and in vivo half-lives of the proteins and their activation products also differ between these conditions. Furthermore, in the clinical setting anticoagulants are different from lepirudin, which may explain differences in the read-outs. The flow rate was also lower for DL-75 than in an ordinary clinical setting, which may also affect the findings. In a clinical study we found increased levels of TCC and C3a at the end of the treatment (compared with baseline), while C5a was reduced [14]. In the ex vivo study we extended these findings to a continuous time axis, demonstrating that TCC, C3a and C5a were increased after the plasma separation columns, and that LA-15 adsorbed these factors during time while EC-50W could not.

5. Conclusions

There are marked differences in biocompatibility between different LDL apheresis columns with regard to complement activation. Plasma separation columns induced the formation of pro-atherogenic complement factors, and there were marked differences in the ability of the LDL apheresis columns to clear these factors. Furthermore, the complement cascade was activated through both the classical and the alternative pathway during blood–biomaterial interaction in LDL apheresis.

Acknowledgements

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Fig. 1, is difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.actbio.2012.02.017.

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Article III

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RESEARCH ARTICLE

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Comparison of cytokine changes in three different lipoprotein apheresis systems in an ex vivo whole blood model

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Abstract

Introduction: Even if proprotein convertase subtilisin/kexin type 9 inhibitors have replaced lipoprotein apheresis in many patients, lipoprotein apheresis still is an important option in homozygous familial hypercholesterolemia, progressive atherosclerosis or when removal of lipoprotein(a) is indicated. Additional possible favorable effects beyond lipid lowering could include changes in the concentration of cytokines and improvement of hemorheology.

Methods: We evaluated how whole blood adsorption, dextran sulfate plasma adsorption, and double filtration plasmapheresis lipoprotein apheresis systems affected cytokine concentrations, using a human whole blood ex vivo model differentiating the effect of the lipoprotein apheresis and plasma separation columns and describing temporal changes.

Results: Compared to the control bag, the whole blood adsorption system reduced Interferon-γ (IFN-γ), IL-8, IL-1ra, eotaxin, tumor necrosis factor (TNF), monocyte chemoattractant protein 1 (MCP-1), platelet derived growth factor (PDGF)-BB, regulated on activation T cell expressed and secreted (RANTES), macrophage inflammatory protein-1β (MIP-1β), and IP-10 ($P < .05$). The dextran sulfate plasma adsorption system reduced IFN-γ, IL-8, IL-1ra, eotaxin, TNF, MCP-1, PDGF-BB, MIP-1β, and IP-10 ($P < .05$). Vascular endothelial growth factor (VEGF) and granulocyte macrophage colony stimulating factor (GM-CSF) were increased in the whole blood and dextran sulfate plasma adsorption systems ($P < .05$). The double filtration plasmapheresis system reduced IFN-γ, IL-1ra, TNF, MIP-1β, and IP-10 ($P < .05$), while MCP-1,-VEGF, GM-CSF, and RANTES were increased ($P < .05$). The plasma

separation column increased concentration of RANTES, and was a barrier to reduction of eotaxin. Temporal patterns of concentration change indicated first pass increase of PDGF-BB and first pass reduction of IP-10.

Conclusion: There were marked differences in how the three systems affected total and temporal cytokine concentration changes in this in vitro model, as well as compared to former in vivo studies.

KEYWORDS

biocompatibility, cytokine, ex vivo, lipoprotein apheresis

1 | INTRODUCTION

Lipoprotein apheresis has traditionally been used in cardiovascular risk reduction, when lipid lowering therapy was not tolerated or the therapeutic target was not achieved, in particular in patients with familial hypercholesterolemia.[1-3] Beneficial effect on clinical endpoints was documented in the LAARS and L-CAPS study.[4,5] As new types of lipid lowering therapy have emerged, in particular proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors, the use of lipoprotein apheresis has diminished. However, it is still an option in homozygous familial hypercholesterolemia and for other selected high-risk patients when pharmacological lipid lowering therapy fails to reduce low-density lipoprotein (LDL) cholesterol sufficiently. It can furthermore be a treatment option when progression of atherosclerosis is not inhibited, or when the concentration of lipoprotein (a) is high,[2,6] since lipoprotein apheresis supersedes PCSK9 inhibition in reducing levels of lipoprotein (a).[7] Lipoprotein apheresis can be performed with columns designed for perfusion of whole blood or plasma and LDL cholesterol removal from plasma depends on plasma separation prior to LDL cholesterol removal. The mechanisms involved in lipoprotein apheresis are either adsorption or capture through filtration or precipitation.[8]

Promising results have been reported when using lipoprotein apheresis for other purposes than LDL cholesterol reduction. The American Society for Apheresis has published a guideline document indicating that lipoprotein apheresis could be of use in focal segmental glomerulosclerosis, sudden sensorineural hearing loss, and phytanic acid storage disease.[9] Lipoprotein apheresis has also shown promising results in treatment of nephropathy in diabetes mellitus[10,11] and nephrotic syndrome of various etiologies.[12-14] It has been suggested that lipoprotein apheresis can reduce the risk of in-stent coronary restenosis in the early postimplantation period,[15] and also have favorable effects in patients with critical limb ischemia due to below-knee arterial lesions.[16,17] In these studies, double filtration plasmapheresis, dextran sulfate plasma adsorption, and heparin-induced, extracorporeal LDL precipitation were used as lipoprotein apheresis systems. Possible mechanisms for the effects include improvement of hemorheology, possibly trough cholesterol and/or fibrinogen removal, reduction of pro-inflammatory cytokines, adhesion molecules and lipoprotein (a), and the possible removal of a putative, yet still unknown, soluble factor in nephrotic syndrome. It has also been suggested that the reduction in LDL cholesterol itself reduces foam cell formation, modifying endothelial damage and inflammatory mechanisms with subsequent favorable clinical effects.[15,18-20]

It is well known from a biocompatibility perspective that lipoprotein apheresis and plasma separation columns modify the complement system and induce changes in the levels of cytokines.[21] Furthermore, activation of immune cells and platelets occurs in lipoprotein apheresis, as in all forms of contact between blood and artificial surfaces.[8,22] Our group has previously shown that different types of lipoprotein apheresis systems have different impact on the cytokine concentration and the complement system in vivo.[23,24] Others have also studied changes of cytokines during lipoprotein apheresis, but the results are not entirely consistent.[25]

The aim of the present study was to investigate how different lipoprotein apheresis columns affect cytokines, including chemokines and growth factors. We used an ex vivo model with three commercially available lipoprotein apheresis systems. The model allowed for differentiation of effects between the plasma separation and the lipoprotein apheresis column, as well as evaluation of temporal changes during perfusion.

2 | METHODS

2.1 | Ethics

The local ethics committee approved the study and all blood donors signed an informed consent. Blood was drawn from six healthy donors (three males and three

females). Each individual donated 450 mL of blood three times at approximately 1 month intervals.

2.2 | Lipoprotein apheresis

The experimental setup has, beyond below stated, previously been described in detail.[24] A short summary is given here. The blood pack used as both the sample control (SC) bag and the apheresis blood reservoir was made from polyvinylchloride copolymer plasticized with di-2-ethylhexyl phthalate. Lepirudin (Refludan, Celgene, Marburg, Germany) was used as sole anticoagulant in all tree lipoprotein apheresis systems and in the SC bag, in this study. Blood flow in the whole blood adsorption lipoprotein apheresis system (DL75) (Filter DL75, Kaneka Corp., Osaka, Japan) was 30 mL/min. In the dextran sulfate plasma adsorption (LA15) (Filter LA15; Kaneka Corp.) and double filtration plasmapheresis (EC50) (Filter EC50; Asahi Kasei Medical, Europe) lipoprotein apheresis systems blood flow was 100 mL/min and the plasma flow 20 mL/min. Six treatments were performed with each lipoprotein apheresis system. The same plasma separation column (PlasmaFlo OP05; Asahi Kasei Medical) was used in the two plasma separation lipoprotein apheresis systems. The three experimental apheresis models are illustrated in Figure 1, showing the whole blood adsorption lipoprotein apheresis system DL75 (A) and the dextran sulfate plasma adsorption and the double filtration plasmapheresis systems LA15 and EC50 (B).

2.3 | Cytokines

Plasma samples were analyzed using a multiplex cytokine assay (Bio-Plex Pro Human Cytokine Grp I Panel 27-Plex; Bio-Rad Laboratories Inc., Hercules, California) containing the following 27 analytes: Interleukin (IL)-1β (IL-1β), IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-9, IL-10, IL- 12, IL-13, IL-15, IL-17, eotaxin (CCL11), basic fibroblast growth factor, granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), Interferon-γ (IFN-γ), chemokine (C-X-C motif) ligand 10 (IP-10 or CXCL10), monocyte chemoattractant protein 1 (MCP-1 or CCL2), macrophage inflammatory protein (MIP) -1α (MIP-1α or CCL3), MIP-1β (or CCL4), platelet derived growth factor (PDGF), regulated on activation T cell expressed and secreted (RANTES or CCL5), tumor necrosis factor (TNF), and vascular endothelial growth

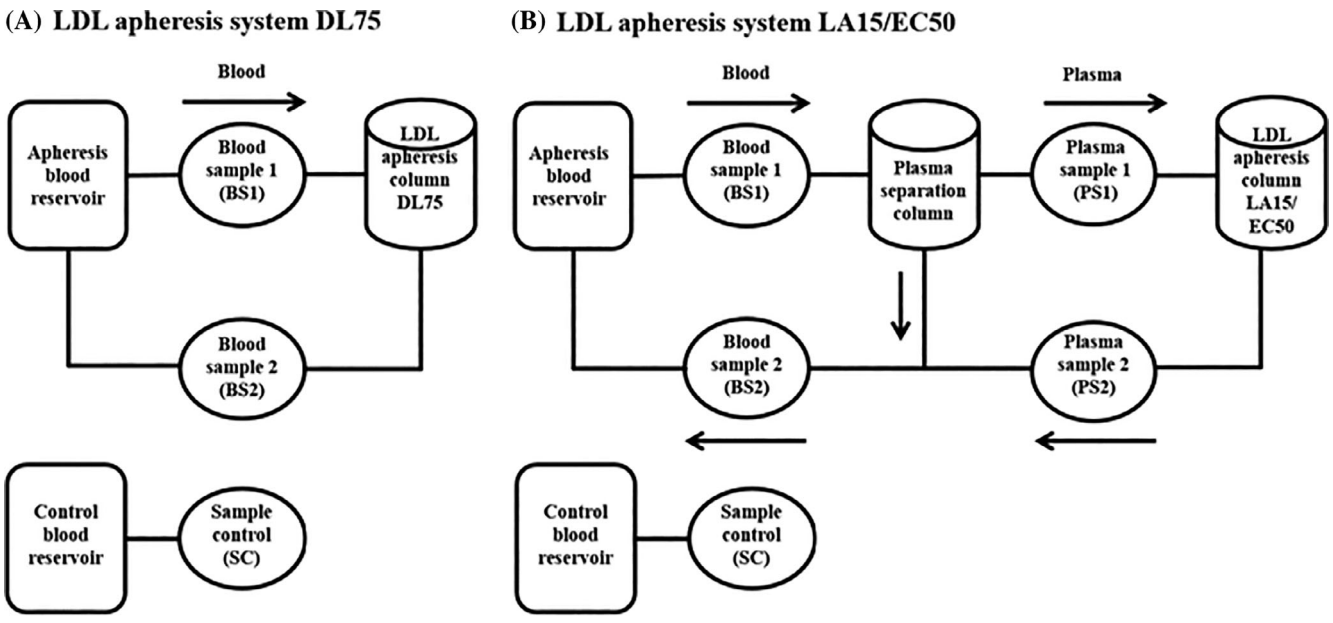

FIGURE 1 A, Schematic drawing of the DL75 ex vivo model with blood sample positions. The apheresis blood reservoir denotes the whole blood bag to which the thrombin specific inhibitor lepirudin was added. Blood samples were obtained from a position after the reservoir (BS1) and then after the lipoprotein apheresis column (BS2). B, The columns LA15 and EC50 required plasma separation before lipoprotein apheresis. The sample sites were before plasma separation (BS1), after plasma separation (PS1), after lipoprotein apheresis (PS2), and after the cell fraction (from plasma separation) and plasma (after lipoprotein apheresis) were combined (BS2). Position BS2 indicates the position where the treated blood would be returned to the patient in a clinical setting. The arrows show the direction of flow in the system. The control blood reservoir (sample control [SC]) was kept on the test tube rotator and samples were drawn directly from the bag

factor (VEGF). The analysis was performed according to the manufacturer's instructions.

2.4 | Calculations and statistics

Results are presented as mean and SEM. Statistical calculations presented were performed with Prism 7.05 for Windows, GraphPad software (San Diego, California). For calculation of differences in SC between baseline (T0) and after 240 minutes perfusion (T240) a two-tailed unpaired student's *t* test was used. Significance level was set at <.05. For pairwise comparison of the lipoprotein apheresis systems, a regular two-way analysis of covariance model with Sidak's multiple comparison modification was used. Significance level was set at <.05.

3 | RESULTS

3.1 | Overall concentration changes in the SC bag and the lipoprotein apheresis systems

The rationale for selecting the 27 cytokines was both to be able to compare results with formerly published data from our and other groups, and because these cytokines are included in a reliable test kit. Thirteen of the 27 cytokines analyzed in the multiplex cytokine assay gave qualitatively acceptable readouts within the limits of the assay used. The other cytokines were out of range and not usable for analysis. The results are presented in Table 1 and Figure 2. Figure 2 is divided into A and B to discriminate between small (A) and large (B) concentration changes.

3.2 | SC bag

In SC, IFN-γ, IL-8, IL-1ra, TNF, PDGF-BB, RANTES, and MIP-1β increased in concentration at 240 minutes (T240) compared to baseline (T0) ($P < .05$) (Figure 2A,B, white bars). The other biomarkers did not differ in concentration in SC at T240 compared to baseline.

3.3 | DL75 lipoprotein apheresis system

In the DL75 system IFN-γ, IL-8, IL-1ra, eotaxin, TNF, MCP-1, PDGF-BB, RANTES, MIP-1β, and IP-10 were reduced ($P < .05$), IL-17 was unchanged and VEGF and GM-CSF were increased ($P < .05$) in position blood sample 2 (BS2), before the apheresis blood reservoir, compared to the SC at T240 (Figure 2A,B, black bars). RANTES was reduced only in the DL75 lipoprotein apheresis system. The other parameters did not differ between the DL75 and LA15 systems at T240. IL-8, eotaxin, MCP-1, PDGF-BB, RANTES, MIP-1β, and IP-10 were reduced in the DL75 system compared to the EC50 lipoprotein apheresis system ($P < .05$). The other biomarkers did not

TABLE 1 The cytokines are listed with main biological property or action (p, pro-inflammatory; a, anti-inflammatory; gf, growth factor), and pairwise comparison of adsorption (DL75 and LA15) vs filtration (EC50) lipoprotein apheresis systems

Biomarker	Action	DL75 vs LA15	DL75 vs EC50	LA15 vs EC50
IFN-γ	p	↔	↔	↔
IL-8	p	↔	↓	↓
Eotaxin	p	↔	↓	↓
TNF	p	↔	↔	↔
IL-17	p	↔	↔	↔
MCP-1	p	↔	↓	↓
RANTES	p	↓	↓	↓
MIP-1β	p	↔	↓	↓
IP-10	p	↔	↓	↓
IL-1ra	a	↔	↔	↔
VEGF	gf	↔	↔	↔
GM-CSF	gf	↔	↔	↔
PDGF-BB	gf	↔	↓	↓

Note: ↓: reduction ($P < .05$). ↔: unchanged.
Abbreviations: GM-CSF, granulocyte macrophage colony stimulating factor; IFN-γ, Interferon-γ; MIP-1β, macrophage inflammatory protein-1β; MCP-1, monocyte chemoattractant protein 1; PDGF, platelet derived growth factor; RANTES, regulated on activation T cell expressed and secreted; VEGF, vascular endothelial growth factor; TNF, tumor necrosis factor.

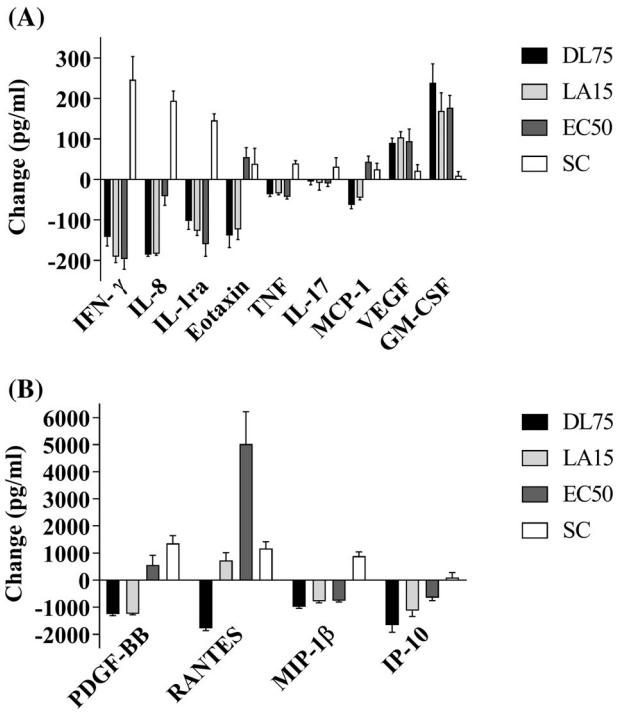

FIGURE 2 Concentration change (pg/mL ± SEM) in lipoprotein apheresis system DL75, LA15, and EC50 relative to the sample control (SC) bag from T0 to T240 at position BS2. The figure was divided into two parts for better visualization of small (A) and large (B) concentration differences

differ between the DL75 and EC50 systems at T240 (Table 1).

3.4 | LA15 lipoprotein apheresis system

In the LA15 system, IFN-γ, IL-8, IL-1ra, eotaxin, TNF, MCP-1, PDGF-BB, MIP-1β, and IP-10 were reduced ($P < .05$), IL17 and RANTES were unchanged and VEGF and GM-CSF were increased ($P < .05$) in position BS2 compared to the SC at T240. (Figure 2A,B, light gray bars). IL-8, eotaxin, MCP-1, PDGF-BB, RANTES, MIP-1β, and IP-10 were reduced in the LA15 system compared to the EC50 system ($P < .05$). The other biomarkers did not differ between the LA15 and EC50 systems at T240 (Table 1).

3.5 | EC50 lipoprotein apheresis system

In the EC50 system, IFN-γ, IL-1ra, TNF, MIP-1β, and IP-10 were reduced ($P < .05$), IL-8, eotaxin, IL17, and PDGF-BB were unchanged and MCP-1,VEGF, GM-CSF, and RANTES were increased ($P < .05$) in position BS2 compared to the SC at T240 (Figure 2A,B, dark gray bars).

3.6 | Temporal patterns of concentration change

The biomarkers eotaxin, RANTES, PDGF-BB, and IP-10 were chosen to describe temporal patterns of concentration change in lipoprotein apheresis systems. Detailed figures of the cytokines not described below are available as supplementary material.

3.7 | Eotaxin

Eotaxin concentration was unchanged from baseline to T240 in SC (Figure 3A-C).

In the DL75 system, position BS2, eotaxin showed a marked reduction from T0 to T15 and remained on this level until T240 (Figure 3A,F). In the LA15 system, plasma sample 1 (PS1), position post plasma separation, concentration of eotaxin was reduced from 158.3 pg/mL ± 44.8 at T0 to 46.2 pg/mL ± 14.9 at T15 (Figure 3B,D). The concentration remained on this level until T240, indicating only minor filtration of eotaxin into plasma in the plasma separation column. In position BS2, eotaxin concentration was reduced gradually from 158.3 pg/mL ± 44.9 at T0 to 55.1 pg/mL ± 20.2 at T240 indicating reduction in the LA15 column (Figure 3B,F). In the EC50 system, position PS1, a similar pattern was seen as for the LA15 system (Figure 3C,D); however, in position PS2, eotaxin increased slightly from T15 until T240 (Figure 3C,E).

3.8 | Platelet derived growth factor-BB

PDGF-BB concentration increased 25-fold from baseline to T240 in SC (Figures 4 and 5A-C). In the DL75 system, position blood sample 1 (BS1), after the blood reservoir, PDGF-BB increased from 42.4 pg/mL ± 8.9 at T0 to 1563.7 pg/mL ± 246.4 at T15. From T15 PDGF-BB was reduced to 276.5 pg/mL ± 70.9 at T30 (Figure 5A), indicating a pattern of first pass increase and reduction of concentration. In the LA15 system, position PS1, there was a 30-fold increase in concentration from T0 to T120 (Figure 5A,D). From T120 until T240, there was a decrease in concentration toward baseline (Figure 5B,F). In position PS2, the concentration stayed at baseline from T0 to T240 (Figure 5B,E) indicating effective reduction in the LA15 column. In the EC50 system, positions BS1, BS2, PS1, and PS2, there was a 40-fold gradual increase in PDGF-BB concentration from T0 to T240 (Figure 5C-F).

3.9 | Regulated on activation T cell expressed and secreted

RANTES concentration increased from baseline to T240 in SC (Figure 5A-C). In the DL75 system, position BS1,

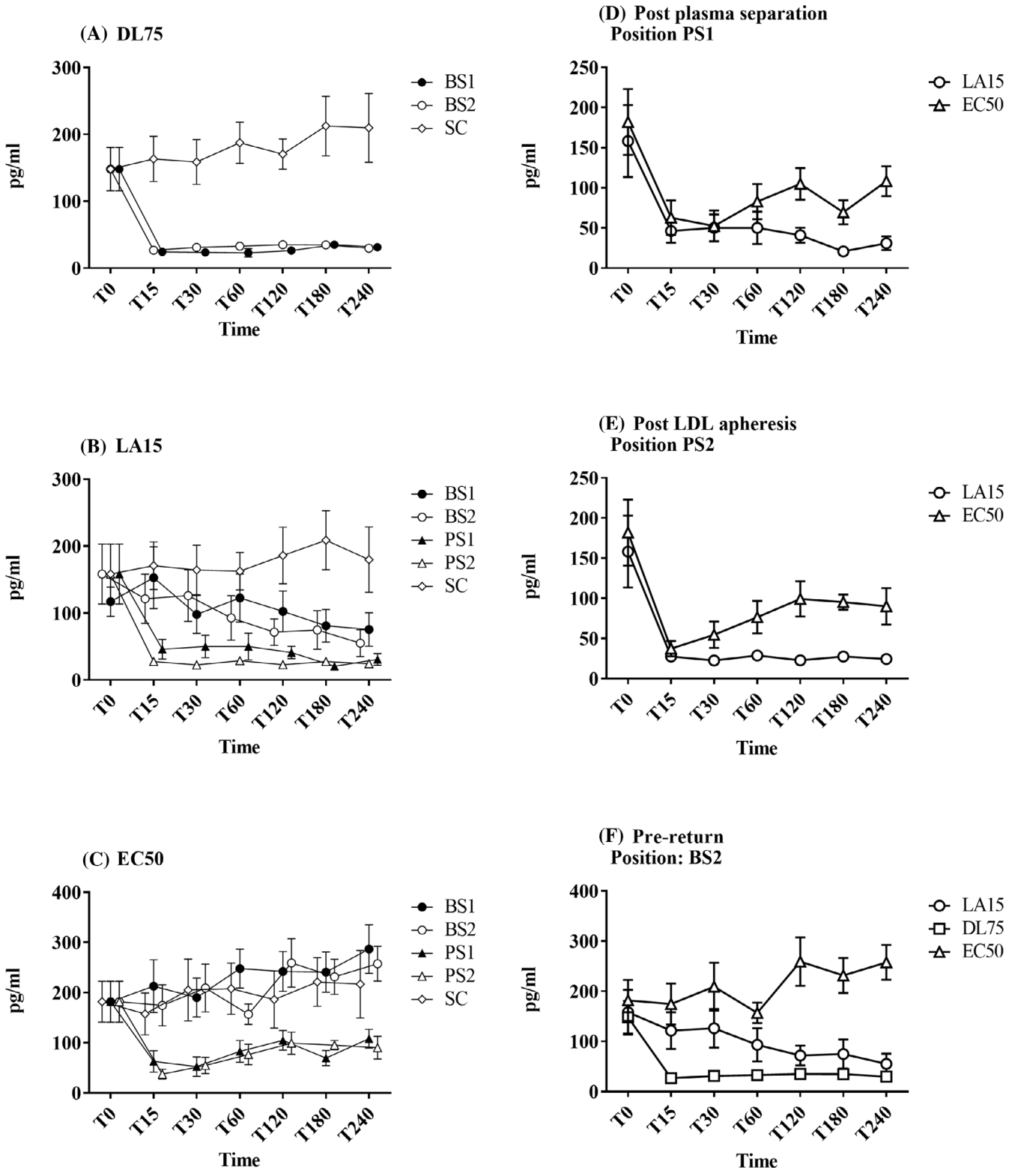

FIGURE 3 Eotaxin. Panels A-C: Change in eotaxin concentration (pg/mL ± SEM) in positions BS1, BS2, PS1, PS2 and sample control (SC) for systems LA15, EC50, and DL75 during 240 minutes. Panels D-F: Change in eotaxin concentration (pg/mL) at selected positions in the LA15, EC50, and DL75 systems during 240 minutes

RANTES increased fourfold until T15 before a gradual reduction to below baseline at T240 (Figure 5A). In the LA15 system, position BS2, concentration increased form baseline to T240 (Figure 5B,F) indicating that the LA15 system as a whole increased the concentration of RANTES. In position PS1, the same pattern was seen as

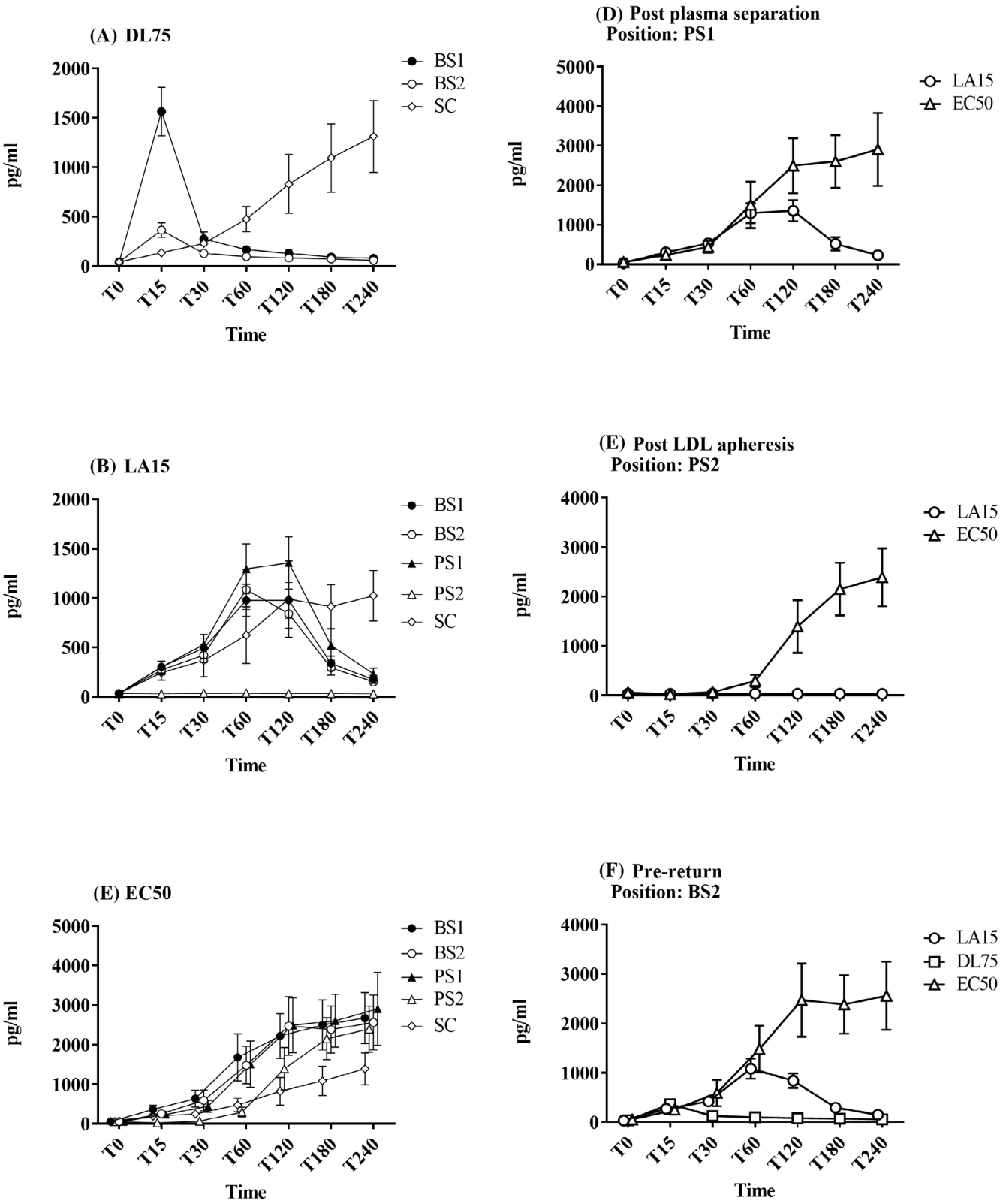

FIGURE 4 Platelet derived growth factor (PDGF)-BB. Panels A-C: Change in PDGF-BB concentration (pg/mL ± SEM) in position BS1, BS2, PS1, PS2, and sample control (SC) for systems LA15, EC50, and DL75 during 240 minutes. Panels D-F: Change in PDGF-BB concentration (pg/mL) at selected positions in the LA15, EC50, and DL75 systems during 240 minutes

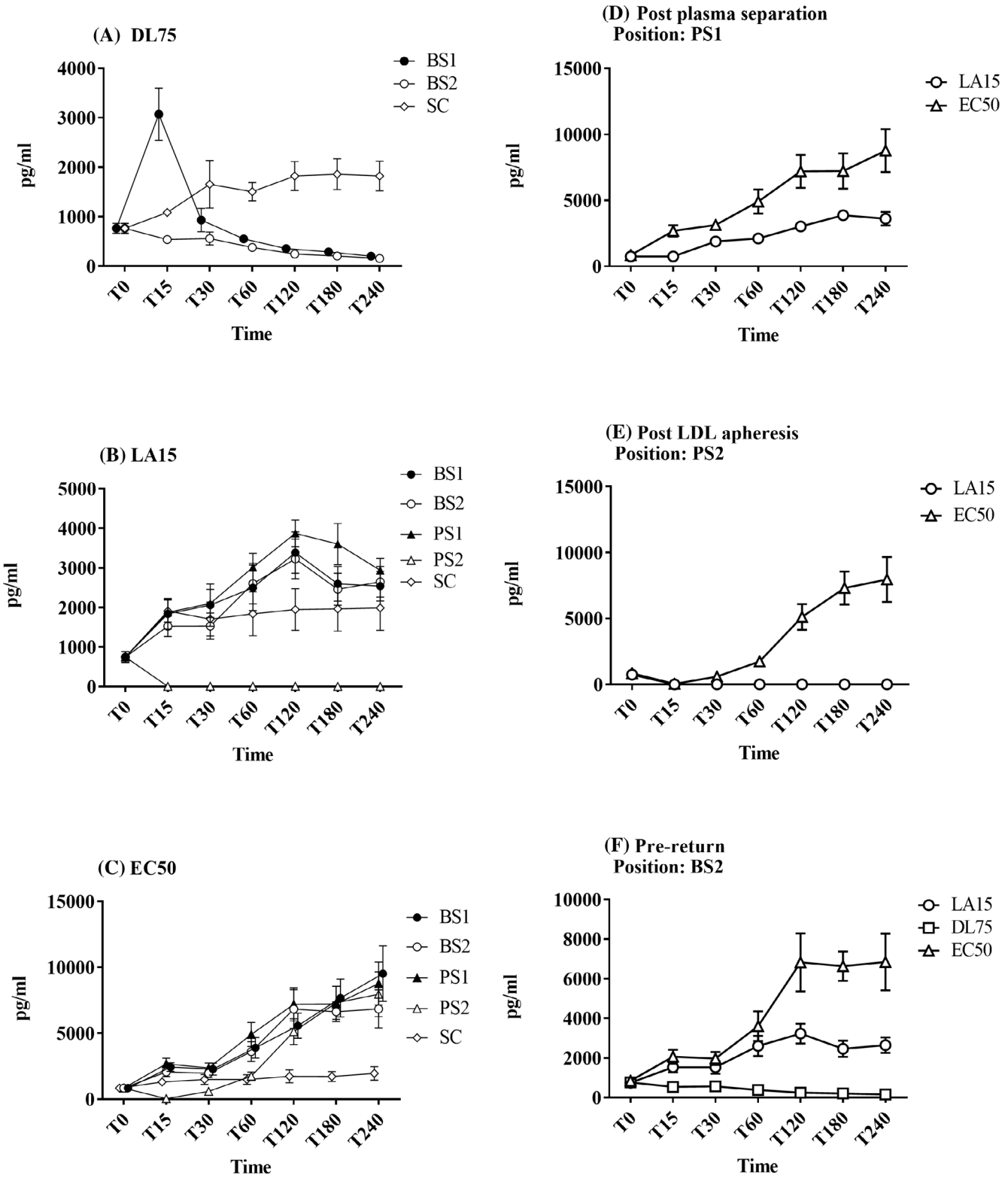

FIGURE 5 Regulated on activation T cell expressed and secreted (RANTES). Panels A-C: Change in RANTES concentration (pg/mL ± SEM) in position BS1, BS2, PS1, PS2, and sample control (SC) for systems LA15, EC50, and DL75 during 240 minutes. Panels D-F: Change in RANTES concentration (pg/mL) at selected positions in the LA15, EC50, and DL75 systems during 240 minutes

in position BS2 (Figure 5B,E); however, in position PS2, there was a reduction from 748.8 pg/mL ± 342.5 at T0 to 8.1 pg/mL ± 1.0 at T15 (Figure 5B,E). The concentration was at this level until T240 indicating effective reduction of RANTES in the LA15 column. In the EC50 system, position BS1, BS2, PS1, and PS2, there was an increase in

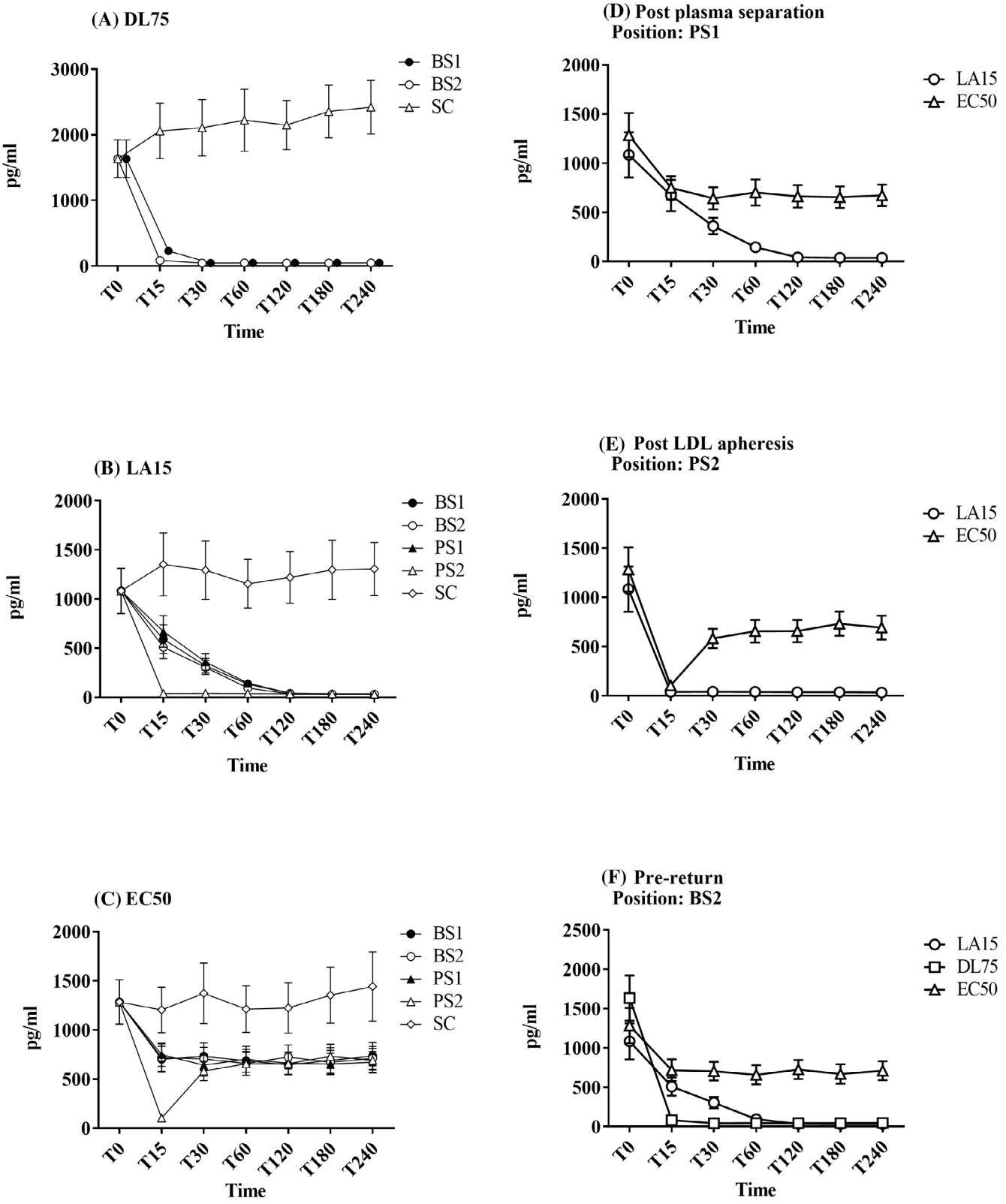

FIGURE 6 IP-10. Panels A-C: Change in IP-10 concentration (pg/mL ± SEM) in position BS1, BS2, PS1, PS2, and sample control (SC) for systems LA15, EC50, and DL75 during 240 minutes. Panels D-F: Change in IP-10 concentration (pg/mL) at selected positions in the LA15, EC50, and DL75 systems during 240 minutes

concentration of RANTES from T0 until T240 (Figure 5C-F).

3.10 | IP-10

IP-10 concentration was unchanged from baseline to T240 in SC (Figure 6A-C). In the DL75 system, position BS2, the IP-10 concentration was markedly reduced from T0 to T15 (Figure 6A,F), and remained on a low level until T240. In the LA15 system, position PS1, there was a gradual fall in concentration from T0 to T240 (Figure 6B,D). In position PS2, there was an initial fall from 1048.5 pg/mL ± 230.1 at T0 to 39.1 pg/mL ± 0.9 at T15 and then continuously low concentration until T240. In the position BS2, there also was a gradual concentration reduction from T0 until T240 (Figure 6B, F). This indicates effective reduction of IP-10 in the DL75 and LA15 columns (Figure 6B,E). In the EC50 column, position PS1, there was a fall from T0 to T15 after which the concentration remained stable until T240 (Figure 6C,D). In position PS2, there was a reduction from 1284.6 pg/mL ± 224.9 at T0 to 104.0 pg/mL ± 24.5 at T15, before an increase in concentration to 582.4 pg/mL ± 98.0 at T30, indicating a first pass reduction and a subsequent increase. From T30 until T240, the concentration was stable (Figure 6C,E). In position BS2, there was an initial reduction from T0 to T15 and thereafter a stable concentration until T240 (Figure 6C,F).

4 | DISCUSSION

Several studies presenting changes in inflammatory biomarkers, hemorheological parameters, and oxidative stress during in vivo lipoprotein apheresis with different columns have been published.[21,23,26-28] To our knowledge, this is the first study presenting a systematic comparison of three lipoprotein apheresis systems' impact on cytokines, also including temporal changes, in an ex vivo model. Fourteen of the 27 cytokines were out of range in the laboratory test kit used in this study and not usable for further analysis. It is known that the hemorheology is influenced by the level of cholesterol[29] and this might impact on the inflammation responses and the results from the test kit used in this study as the blood donors were healthy volunteers.

Uniform concentration changes in the SC indicate robust and stable testing conditions. The rate of increase in cytokine concentrations in SC were slow compared to changes in the apheresis systems as seen for RANTES and PDGF. Other biomarkers as VEGF, GM-CSF, and IP-10 showed only a small or no increase in SC indicating only minor activation of cells producing these cytokines in the environment of the SC. The temporal and relative changes in cytokine concentrations in the SC bag are due to known bioincompatibility mechanisms taking place.[30,31]

The chemical, electrical, and three-dimensional properties of the column membrane or adsorbing beads is the basis for removal of LDL cholesterol[32-34] and also affect biocompatibility properties and hence concentration change of cytokines. The LA15 and EC50 columns process plasma as compared to the DL75 column, which processes whole blood. Adding a second column, as in the plasma separation lipoprotein apheresis systems, might contribute to bioincompatibility. The choice of anticoagulation impacts the result as there is a crosstalk between coagulation and inflammation, termed thromboinflammation, and manipulation of coagulation may influence the inflammatory response when foreign surfaces are exposed to blood.[31,35] Unlike heparin, the thrombin inhibitor lepirudin, used as anticoagulation in this study, does not affect the complement system, an important biological factor for cytokine induction in bioincompatibility.[36] This is of crucial importance when comparing our results with previous ex vivo as well as in vivo studies where heparin, citrate or ethylene diamine tetraacetic acid were used as anticoagulation. Our results show that the adsorption lipoprotein apheresis systems, DL75 and LA15, are more effective in reducing the presented biomarkers compared to the filtration lipoprotein apheresis system EC50.

The temporal concentration change of eotaxin showed the difference between the whole blood and the plasma separation systems. In the LA15 and EC50 systems, eotaxin was filtered into plasma only to a small extent per time unit, as the concentration in post plasmapheresis position PS1 fell immediately after the treatment started, indicating that the plasma separation column can be a barrier to removal of eotaxin. This might be explained by the chemical structure of eotaxin having a disordered N-terminus as compared to, for example, RANTES.[37,38] No further removal was seen in the EC50 system. This indicates coating of the lipoprotein column membrane until saturation as the cause of the initial fall in concentration. Coating of foreign surfaces by plasma proteins has previously been described as the first step of the bioincompatibility process in contact between blood and foreign materials.[39]

Increase in concentration of VEGF was shown for all the apheresis systems tested compared to the SC indicating that tubing, columns, and shear flow and shear stress had an impact on production of VEGF.[40] Our results regarding VEGF are in contrast to former in vivo studies

which displayed a decrease in VEGF concentration in all systems used.[23,25] VEGF is known to bind heparin[41] which is used for anticoagulation in clinical settings of lipoprotein apheresis, and this might explain the difference as heparin binding can enhance capture of VEGF in the lipoprotein apheresis columns in an in vivo setting.

Platelets are activated and PDGF-BB released in the bioincompatibility process,[42] and the immediate increase in PDGF-BB in the DL75 system at T15 is a characteristic first pass induction effect seen in this system. This could probably be due to this column circulating whole blood and thus activating cytokine producing cells to a higher extent than the other lipoprotein apheresis columns. The direction of concentration change in PDGF-BB seen was the same as in an in vivo study, hence supporting the lipoprotein apheresis systems influence on PDGF-BB.[23]

The temporal concentration change of RANTES also demonstrated a difference between the whole blood and the plasma separation systems. The DL75 column, after a first pass induction, adsorbed RANTES leaving the final concentration below baseline. In the LA15 system, the concentration at position BS2 was at the level of SC or slightly above at T240. It is tempting to assume that the plasma separation column participates in the induction of RANTES as the concentration in position PS1, after the plasma separation column, in the LA15 system increased during time. Hirata et al demonstrated that the plasma separation column activated the complement system but not cell components of the blood.[43] A study on CD11b expression being complement factor 5 (C5) dependent, using the same plasma separation column, showed a clear C5 independent decrease in circulation platelets.[44] These findings put together indicate that the plasma separation column does activate the platelets hence increasing the production of RANTES. An in vivo study found that the DL75 and LA15 systems reduced RANTES concentration, supporting our findings for the DL75 column but being contradictory with regard to the LA15 column.[23] Stefanutti et al found, in an in vivo study using the DALI whole blood lipoprotein apheresis system, increase in RANTES, also contradictory to the findings in this study for the DL75 whole blood column, indicating a possible difference between whole blood lipoprotein apheresis systems with regard to concentration changes of RANTES.[45]

In the lipoprotein apheresis systems, reduction of cytokine concentration could be seen either immediately or after a period of time. For IP-10 in the DL75 system, a near complete removal from circulation was seen at T15, indicating an immediate adsorption. In the LA15 system, there was a gradual reduction in IP-10 concentration during 240 min. The difference is probably due to the DL75 column adsorbing cytokines direct form whole blood, as compared to the LA15 system, which adsorbs cytokines from plasma. Our results indicate that IP-10 is not easily filtered through the pores of the plasma separation column, and this is mandatory for IP-10 to be available for adsorption in the lipoprotein apheresis column LA15 and EC50. The LA15 column was effective in adsorbing IP-10 as the concentration in post plasma separation position PS2 was low from T15. We have previously demonstrated an in vivo increase in IP-10 with the same lipoprotein apheresis columns used in the present study.[23] This may indicate that lipoprotein apheresis affects expression of IP-10 differently in an in vivo and an ex vivo setting. Recently, Stefanutti et al demonstrated an impact on the messenger RNA of IL-1α, IL-6, and TNF in patients undergoing lipoprotein apheresis, indicating a possible regulatory effect on the expression of precursors in the chain of production of inflammatory mediators.[46,47] This could contribute to explaining the observed differences between in effect on IP-10 in in vivo and ex vivo studies.

5 | CONCLUSION

The results presented in this ex vivo study demonstrate differences between the whole blood adsorption, dextran sulfate plasma adsorption, and the double filtration plasmapheresis lipoprotein apheresis systems regarding their effects on cytokines, a fact that underlines the need to test every system independently, and not relying on results from similar or comparable systems. The results add to the current knowledge of effects of different lipoprotein apheresis systems on inflammatory mediators including temporal concentration changes, in ex vivo conditions. To answer the question whether differences in pattern changes of individual cytokines could play a role in therapeutic practice, further in vivo studies are needed, as this question is not answered in the present study. The results also underline the importance of studying biocompatibility processes not only in ex vivo but also in in vivo experiments close to the clinical setting in order to obtain a more complete understanding of the effects of contact between blood and the foreign material.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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Article IV

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Granulocyte and monocyte CD11b expression during plasma separation is dependent on complement factor 5 (C5) – an *ex vivo* study with blood from a C5-deficient individual

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Hardersen R, Enebakk T, Christiansen D, Bergseth G, Brekke O-L, Mollnes TE, Lappegård KT, Hovland A. Granulocyte and monocyte CD11b expression during plasma separation is dependent on complement factor 5 (C5) – an *ex vivo* study with blood from a C5-deficient individual. APMIS 2018; 126: 342–352.

The aim of the study was to investigate the role of complement factor 5 (C5) in reactions elicited by plasma separation using blood from a C5-deficient (C5D) individual, comparing it to C5-deficient blood reconstituted with C5 (C5DR) and blood from healthy donors. Blood was circulated through an *ex vivo* plasma separation model. Leukocyte CD11b expression and leukocyte–platelet conjugates were measured by flow cytometry during a 30-min period. Other markers were assessed during a 240-min period. Granulocyte and monocyte CD11b expression did not increase in C5D blood during plasma separation. In C5DR samples granulocytes CD11b expression, measured by mean fluorescence intensity (MFI), increased from 10481 ± 6022 (SD) to 62703 ± 4936, and monocytes CD11b expression changed from 13837 ± 7047 to 40063 ± 713. Granulocyte–platelet conjugates showed a 2.5-fold increase in the C5DR sample compared to the C5D sample. Monocyte–platelet conjugates increased independently of C5. In the C5D samples, platelet count decreased from 210 × 10^9/L (201–219) (median and range) to 51 × 10^9/L (50–51), and C3bc increased from 14 CAU/mL (21–7) to 198 CAU/mL (127–269), whereas TCC formation was blocked during plasma separation. In conclusion, up-regulation of granulocyte and monocyte CD11b during plasma separation was C5-dependent. The results also indicate C5 dependency in granulocyte–platelet conjugates formation.

Key words: C5 deficiency; bioincompatibility; plasma separation; CD11b/CD18; leukocyte–platelet conjugate.

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CD11b is part of the integrin heterodimer containing CD11b (integrin α_M) and CD18 (integrin β_2). CD11b/CD18 (CR3) has approximately 40 reported protein ligands. Biologic functions during the inflammatory response include adhesion of leucocytes, regulation of cytokine secretion, and there are indications of direct adhesion to platelets (1). In commonly accepted biocompatibility models activated leukocytes expressing CD11b/CD18 binds iC3b molecules included in the initial protein layer bound to the artificial surface, which is considered one of the first steps in the bioincompatibility reaction (2, 3).

Studies have shown that hemodialysis up-regulates the adhesion molecule CD11b on leukocytes, corresponding to elevated platelet–leukocyte

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aggregate counts, concluding that expression of CD11b is a reliable marker of leukocyte activation during hemodialysis (4). When plasma separation is used in treatment of, for example, vasculitis, the plasma fraction is disposed and substituted with either fresh frozen plasma or a Ringer/albumin solution before it is merged with the cellular component and returned to the patient. In plasma separation used in LDL apheresis systems, the cell fraction is merged with the plasma fraction before returned to the patient after LDL removal has taken place from the plasma fraction. If the plasma separation process in itself has adverse effects on the cellular components, either directly or by activation of the innate immune system, this may prove disadvantageous to the patient.

It has previously been demonstrated that plasma separation induces complement activation during *ex vivo* low-density lipoprotein (LDL) double filtration apheresis (5), and that CD11b up-regulation is dependent on the presence of complement factor 5 (C5) in an *ex vivo* model with polyvinylchloride tubing (PVC) (6). Studies have also shown up-regulation of CD11b on monocytes and granulocytes, and an increase in monocyte–platelet and granulocyte–platelet conjugates after circulating blood through PVC tubing. Furthermore, blocking of C5a receptor 1 (C5aR1, CD88) on granulocytes and monocytes largely counteracted the CD11b presentation. Thus, CD11b up-regulation by PVC is mediated through complement activation, mainly by C5a (7), and CD11b up-regulation on granulocytes is thus to some extent a proxy for complement activation. Crosstalk between parts of the innate immune systems (complement system, coagulation system, and contact activation system) in plasma separation procedures leads to activation of the cellular component of the immune system, and thus enhancement of the immune response. The result is an inflammatory response in the patient undergoing treatment including blood exposure to artificial surfaces, also involving the cellular component of the immune system (2). This treatment-induced systemic response may have unwanted consequences for the patient.

The aim of the present study was to investigate the role of complement in leukocyte activation during plasma separation, as measured by expression of CD11b and formation of leukocyte–platelet conjugates. We developed an *ex vivo* model of plasma separation and compared blood from an individual with C5 deficiency (C5D) with C5-deficient blood reconstituted with C5 (C5DR), and blood from healthy donors as control (CTR). C5 deficiency is extremely rare, with only a few dozen individuals reported worldwide (8), but as these individuals represent nature′s own knock-outs their blood is well-suited to study the role of complement in general and the role of C5 in particular in various models of inflammation.

MATERIALS AND METHODS

Ethics

The regional ethics committee approved the study and all blood donors signed an informed consent.

Donors

Blood from a previously described C5-deficient individual (9) and blood from three healthy donors were used. Blood was drawn four times from the C5-deficient donor, and twice from each healthy donor. The individuals donated 450 mL of blood on each occasion. Time duration between blood donations was approximately 6 months.

Blood sampling and plasma separation

Lepirudin (Refludan®, Celgene, Marburg, Germany), 25 mg in 50 mL of 0.9% NaCl, was added to a 600 mL filterless Blood Pack Unit (Fenwal, Lake Zürich, USA, made from polyvinylchloride copolymer plasticized with di-2-ethylhexyl phthalate, without other additives, before blood donation, giving a final concentration of 0.05 mg lepirudin/mL blood and a final volume of 500 mL in the blood pack unit. Lepirudin in this concentration gives efficient anticoagulation without affecting complement activation (10).

Fifty milliliters of blood were then transferred to an empty blood pack to serve as a control for time-dependent, contact-induced activation (no-plasma separation blood reservoir, NPS). The remaining 450 mL served as the reservoir for blood circulating in the plasma separation model (plasma separation blood reservoir, PS) (Fig. 1). Both blood packs were placed in a temperature-controlled heater (Binder, Binder GmBH, Tuttlingen, Germany) set at 37 °C, with constant movement by means of a modified test tube rotator (Rock ‘n Roller, Labinco BV, Breda, The Netherlands). The blood reservoir was attached to the plasma separation system which consisted of an Octo-Nova (MeSys Gmbh, Hannover, Germany) machine with a PlasmaFlo OP-05W column (Asahi Kasei Medical Europe) plasma separation column and PVC tubing. The flow rates were 100 mL/min for the blood pump and 20 mL/min for the plasma pump. Blood samples for flow cytometry were obtained at 0, 5, 15, and 30 min (T0–T30), whereas the other markers were obtained at 0, 5, 30, 120, and 240 min (T0–T240).

All blood samples were drawn simultaneously (within a time frame of 1 minute) for each sample time and location. Blood samples were drawn into polystyrene tubes containing EDTA (to block any further complement activation) to a final concentration of 10 mM and then placed on ice before centrifugation for 15 min at 3220 g at 4 °C. The plasma was frozen in aliquots at −80 °C until analysis in batches. Blood smears were made immediately after blood sampling, at T0 and at T240.

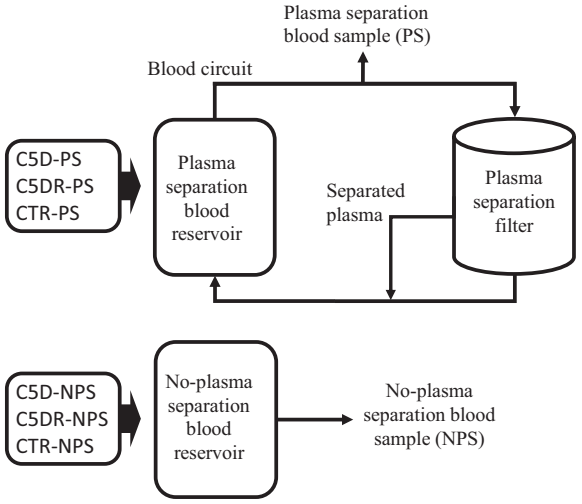

Fig. 1. Schematic drawing of the *ex vivo* model. The plasma separation blood bag served as the reservoir for the plasma separation circuit. Plasma separation blood samples were obtained from the tubing blood sample outlet after the plasma separation blood reservoir. The arrows show the direction of blood flow and plasma flow in the system. The no-plasma separation blood reservoir was kept at 37 °C on the test tube rotator next to the plasma separation blood reservoir. No-plasma separation blood samples were drawn directly from the no-plasma separation blood reservoir.

Flow cytometric studies of CD11b

Flow cytometric studies were performed with an LSRII (Becton Dickinson (BD), San Jose, CA, USA). At each point of time, blood (100 μL) was fixed with 100 μL 0.5% paraformaldehyde for 4 min at 37 °C, and 25 μL fixed blood was then incubated for 15 min at room temperature in the dark with anti-CD11b-PE (BD) or the isotype control IgG2a-PE (BD). For threshold, the nuclear stain LDS-751 (Molecular Probes, Invitrogen™ Thermo Fisher Scientific, Waltham, MA, USA) was added. In addition, anti-CD14 FITC (BD) was used for gating purpose. One mL PBS was added and samples were acquired after 15 min. Granulocytes and monocytes were gated in an SSC/anti-CD14-dotplot, and the mean fluorescent intensity values for CD11b were calculated (Fig. 2). The antibody used in our study (mouse anti-human-CD11b-Phycoerythrin, clone D12, Becton Dickinson, San Jose, CA, USA) is specific for the 165-kilodalton (kd) α-subunit of the CD11b/CD18 antigen heterodimer, and is as such unable to disclose if the CD11/CD18 integrin is conformationally changed into its active form. However, the same anti-human CD11b antibody is used by our and other groups in studies exploring CD11b up-regulation in inflammation, and taken into account for the conformational change of the heterodimer into its active form and activation of leukocytes (11–14).

Leukocyte–platelet conjugates

Fixed blood cells were stained with anti-CD14 PE (BD), anti-CD61 FITC (BD), LDS-751 and re-suspended as described above. Granulocytes and monocytes were gated in an SSC/anti-CD14-dotplot, and the mean fluorescent intensity values for CD61 were calculated.

Routine biochemistry

Hemoglobin, leukocytes, and platelets were analyzed using a Siemens ADVIA® 2120 Hematology System (Siemens Healthcare Diagnostics Ltd., Camberly, UK). Total protein, albumin, C4, IgG, IgM, and IgA were analyzed in an ADVIA®1800 system (Siemens Medical Solutions Diagnostics, Japan) with reagents from Siemens Healthcare Diagnostics Ltd.

Complement components and functional activity assays

Purified human complement protein C5 was obtained from Quidel (Quidel Corporation, San Diego, CA, USA). Purified C5 was added to C5-deficient blood to give a final plasma concentration of 80 μg/mL, corresponding to the concentration of C5 in normal individuals (15). The complement activation products C3bc and the terminal complement complex (TCC) were measured using enzyme immunoassays based on capture antibodies reacting with neoepitopes exposed selectively in the activation product and not in the native component as described in detail previously (16).

Correction for dilution

A small amount of priming solution (isotonic saline) was used to prepare the tubing and columns before the *ex vivo* loop was started. Hematocrit was used to correct the concentration for the plasma parameters, according to a standardized formula (17).

Statistics

Formation of leukocyte–platelet conjugates and CD11b expression was measured as mean fluorescent intensity ± standard deviation (SD), all other measurements are median ± range. The rarity of the C5 deficiency precluded the use of many repeated samples in this study. Due to the few numbers of observations, we have presented the data without further tests of statistical significance. All calculations presented were performed with Prism 5.0 for Windows, Graphpad software (San Diego, CA, USA).

RESULTS

Expression of CD11b on leukocytes

Granulocytes (Fig. 3A): Blood from control individuals showed an increase in CD11b expression in the plasma separation sample from 2621 ± 498 (mean and SD) at T0 to 30727 ± 9165 at T30 (CTR-PS: Fig. 3A, left panel). In contrast, the C5-deficient blood (C5D) showed no increase in granulocyte CD11b expression in the plasma separation sample changing from 5351 ± 919 at T0 to

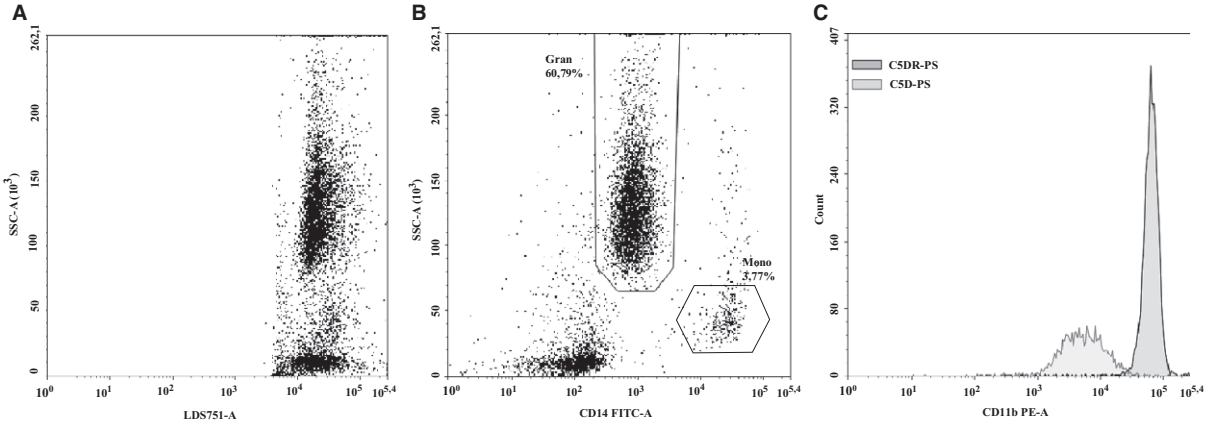

Fig. 2. Flow cytometric readouts at 30 min of granulocyte and monocyte CD11b. During acquisition, threshold was set on the nuclear stain LDS 751 (A). Granulocytes and monocytes were gated for in a CD14 FITC/SSC dotplot (B). Granulocyte CD11b expression in samples C5DR-PS and C5D-PS shown in a histogram (C).

7935 ± 1648 at T30 (C5D-PS: Fig. 3A, left panel). After reconstituting the C5-deficient blood with purified C5 (C5DR), granulocyte CD11b expression in the plasma separation sample increased from 10481 ± 6022 at T0 to 62703 ± 4936 at T30 (C5DR-PS: Fig. 3A, left panel).

In the time-dependent, spontaneous activation, no-plasma separation sample (NPS), there was a small increase in CD11b expression in blood from the control persons from 2443 ± 725 at T0 to 6419 ± 218 at T30 (CTR-NPS: Fig. 3A, right panel). Similarly, there was a small increase in C5-deficient blood from 5351 ± 919 at T0 to 10537 ± 890 at T30 (C5D-NPS; Fig. 3A right panel). After reconstitution, there was an increase in CD11b expression from 10481 ± 6022 at T0 to 47080 ± 17186 at T30 (C5DR-NPS: Fig. 3A, right panel).

Monocytes (Fig. 3B): In blood from control individuals, the plasma separation sample showed a marked increase in monocyte CD11b expression from 3894 ± 285 at T0 to 23575 ± 6765 at T30 (CTR-PS; Fig. 3B, left panel). In C5-deficient blood, there was no increase in CD11b expression as it changed from 9027 ± 456 at T0 to 11478 ± 1461 at T30 (C5D-PS: Fig. 3B, left panel)

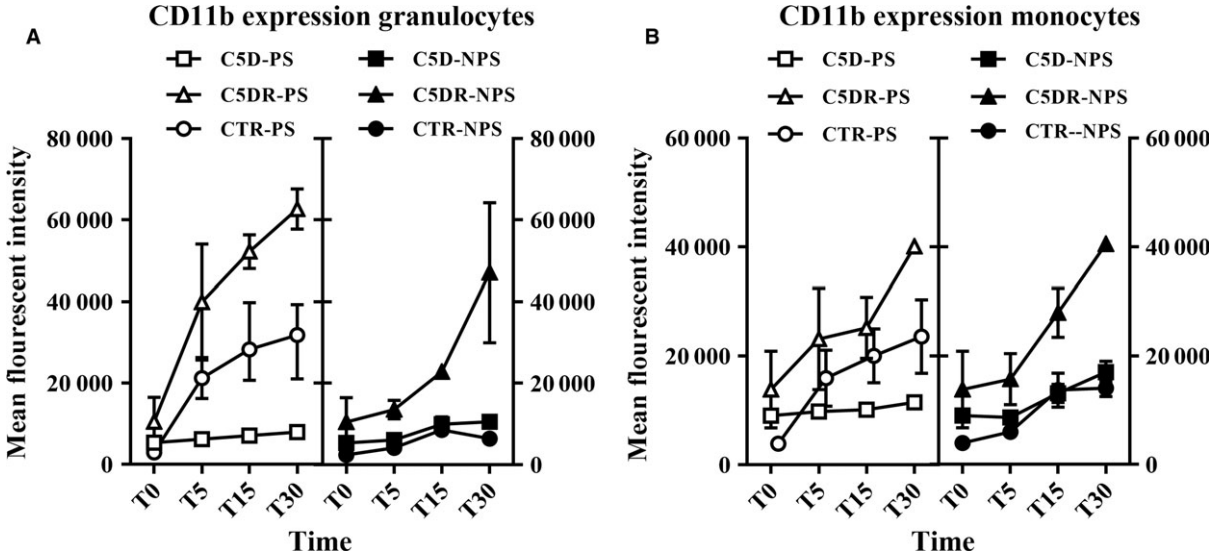

Fig. 3. CD11b expression. Expression of CD11b on granulocytes (A) and monocytes (B). CD11b expression from baseline (T0) through 30 min (T30) expressed as mean fluorescent intensity and standard deviation in the C5D, C5DR, and CTR samples in the plasma separation blood samples (PS) and in the no-plasma separation blood samples (NPS).

similar to that seen for the granulocytes. Upon reconstitution with C5 monocyte expression of CD11b in C5-deficient blood also increased, from 13837 ± 7047 at T0 to 40063 ± 713 at T30 (C5DR-PS: Fig. 3B, left panel).

In the no-plasma separation samples, there was a small and equal increase in CD11b expression on monocytes for controls and C5D (CTR-NPS and C5D-NPS: Fig. 3B, right panel). Similar to the granulocytes, there was an increase in CD11b expression in the monocyte C5DR-NPS samples from 13837 ± 7047 at T0 to 40532 ± 543 at T30 (Fig. 3B, right panel).

Formation of leukocyte–platelet conjugates

Granulocyte–platelet conjugates (Fig. 4A)*:* In blood from control individuals, the plasma separation sample showed an increase in granulocyte–platelet conjugate formation from 1325 ± 250 at T0 to 5633 ± 3199 at T30 (CTR-PS: Fig. 4A, left panel). In C5D blood, granulocyte–platelet conjugate formation during plasma separation increased from 1931 ± 337 at T0 to 3247 ± 1066 at T30 (C5D-PS: Fig. 4A, left panel). The reconstituted C5-deficient blood increased from 1851 ± 805 at T0 to 4743 ± 485 at T30 (C5DR-PS: Fig. 4A, left panel).

In the no-plasma separation samples (Fig. 4A, right panel), there was a small and equal increase during the observation time in the three groups.

Monocyte–platelet conjugates (Fig. 4B): There were increases in monocyte–platelet conjugate formation for all groups during plasma separation from T0 to T30 (Fig. 4B left panel). The increases seen in the no-plasma separation samples during the 30 min observation time were less than in plasma separation samples and also similar in all groups (Fig. 4B right panel).

Platelet and leukocyte counts

Platelet count was reduced in all groups during plasma separation (T0–T240); CTR: 235 10^9/L (218–246) (median and range) to 67 × 10^9/L (26–68), C5D: 210 × 10^9/L (201–219) to 51 × 10^9/L (50–51), C5DR: 191 × 10^9/L (158–224) to 31 × 10^9/L (26–36) (Fig. 5A, left panel). Blood smears obtained from the plasma separation samples at the end of the apheresis session showed platelet agglutination (data not shown). None of the groups changed in platelet count in the no-plasma separation samples (Fig. 5A right panel). Furthermore, there were no significant changes in the leucocyte counts during plasma separation or in the no-plasma separation samples in the different groups (Fig. 5B).

Total protein and complement factor 4 (C4)

In the plasma separation control sample, there was a reduction in total protein from T0 53 g/L (45–54) (median and range) to 25 g/L (18–31) at T5 with no further reduction at T240 (CTR-PS: Fig. 6A, left panel). There were similar reductions in total

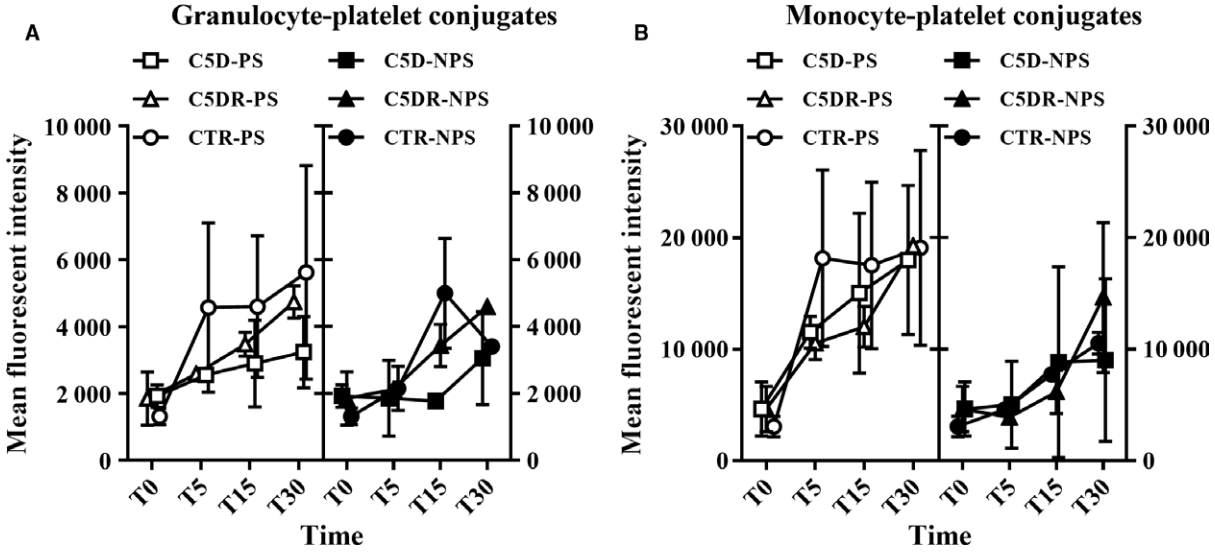

Fig. 4. Leukocyte–platelet conjugates. Granulocyte–platelet (A) and monocyte–platelet (B) conjugate formation from baseline (T0) through 30 min (T30) expressed as mean fluorescent intensity and standard deviation in the C5D, C5DR, and CTR samples in the plasma separation blood samples (PS) and in the no-plasma separation blood samples (NPS).

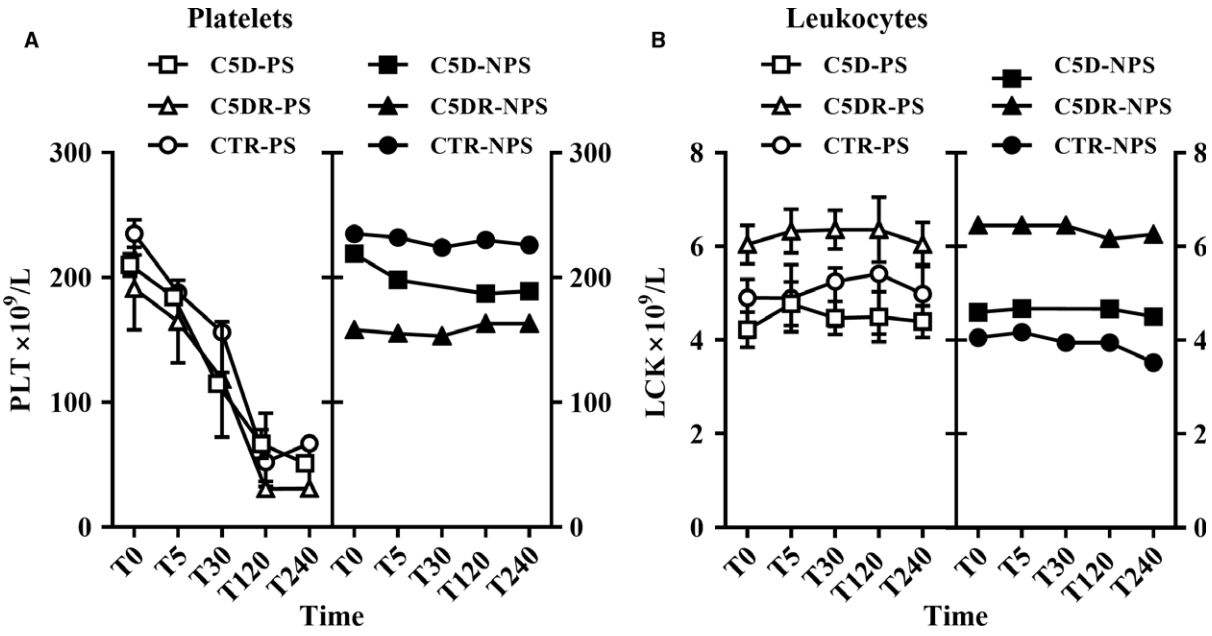

Fig. 5. Platelet and leukocyte counts. Platelet (A) and leukocyte (B) from baseline (T0) through 240 min (T240) expressed as median and range in the C5D, C5DR, and CTR samples in the plasma separation blood samples (PS) and in the no-plasma separation blood samples (NPS).

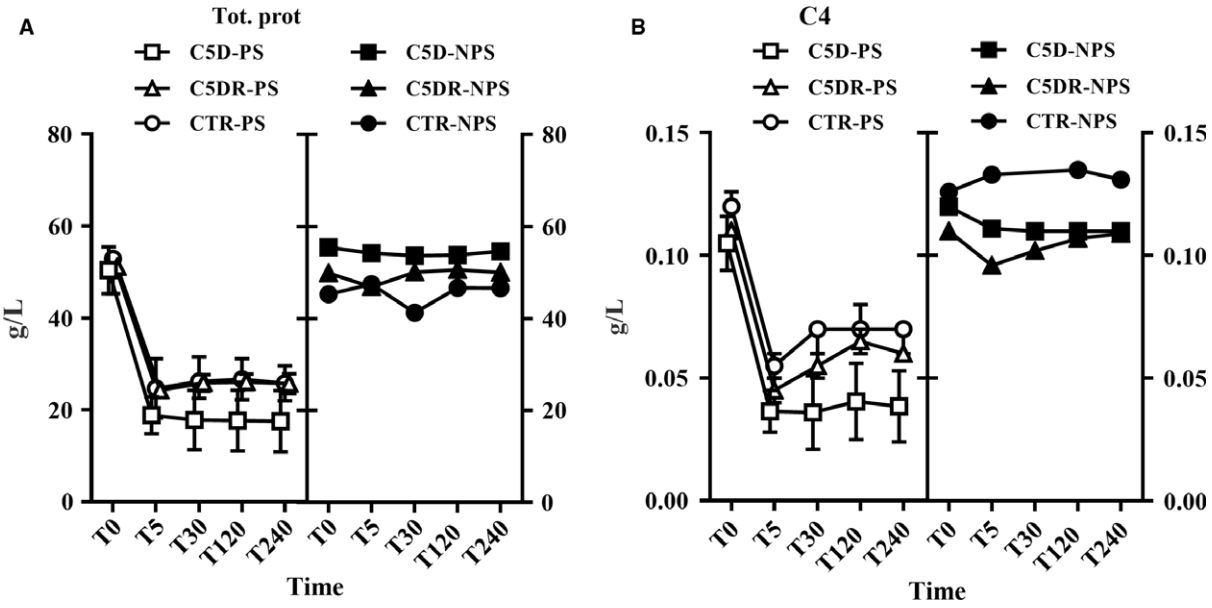

Fig. 6. Plasma protein concentrations. Total protein (A) and complement factor 4 (B) from baseline (T0) through 240 min (T240) expressed as median and range in the C5D, C5DR, and CTR samples in the plasma separation blood samples (PS) and in the no-plasma separation blood samples (NPS).

protein for all groups during plasma separation (C5D-PS and C5DR-PS: Fig. 6A, left panel). No such reduction was seen in the no-plasma separation samples (Fig. 6A, right panel). The same pattern was seen for albumin, IgG, IgM, and IgA (data not shown).

Resembling the pattern for total protein, there were reductions in C4 for all groups during plasma separation starting at T5, with no further reduction at T240 (Fig. 6B, left panel), whereas the levels in the no-plasma separation samples remained unchanged during time (Fig. 6B, right panel).

Complement activation

In all plasma separation samples, there were increases in the complement activation product C3bc, starting at 5 min with further increase after 30 min (Fig. 7A, left panel). In the no-plasma separation samples, the increase occurred later, starting at 30 min and continuing up to 240 min, however not reaching the same maximum as in the plasma separation samples (Fig. 7A, right panel).

The terminal C5b-9 complement complex (TCC) was measured to assess endpoint complement activation. In the controls and in the C5 reconstituted samples, there was a 15-fold increase in TCC during plasma separation (CTR-PS, C5DR-PS: Fig. 7B, left panel), and as expected there was no TCC formation in the C5D sample consistent with the lack of C5 (C5D-PS: Fig. 7B, left panel). Similarly, in the no-plasma separation samples, there was a 10-fold TCC increase in the CTR sample and a 20-fold increase in the C5DR sample, while there was no TCC formation in the C5D sample (Fig. 7B, right panel).

DISCUSSION

We have previously shown that individuals deficient of C5, nature's own knock-outs, can be used as a robust model for exploring the role of C5 in different experimental settings (14). In the present study, using C5D blood compared to controls in an *ex vivo* model of plasma separation, we demonstrate that CD11b expression was C5 dependent both for granulocytes and monocytes supporting previous published observations. Formation of granulocyte–platelet conjugates was to some extent C5-dependent. Monocyte–platelet conjugates, however, were C5-independent. These findings underscore the importance of the complement system in leukocyte activation during extra-corporeal treatments involving surface activation.

Expression of CD11b on leukocytes

Our finding that CD11b expression on granulocytes is C5-dependent in our model of *ex vivo* plasma separation is supported by previous studies. Rinder et al. showed that C5aR1 blockade significantly decreased CD11b up-regulation on granulocytes and that anti-human C5 antibody blocks CD11b up-regulation on granulocytes in an *ex vivo* model with simulated extracorporeal circulation (18, 19), and it has also been demonstrated that blocking the C5aR1 in a model with PVC tubing counteracted the CD11b expression on granulocytes (7). Our group has previously demonstrated that CD11b expression on granulocytes in a *Neisseria meningitides* model using blood from a C5-deficient donor only occurred after reconstitution of C5, also indicating the importance of C5 in CD11b expression on granulocytes (14) and Bergseth et al. showed

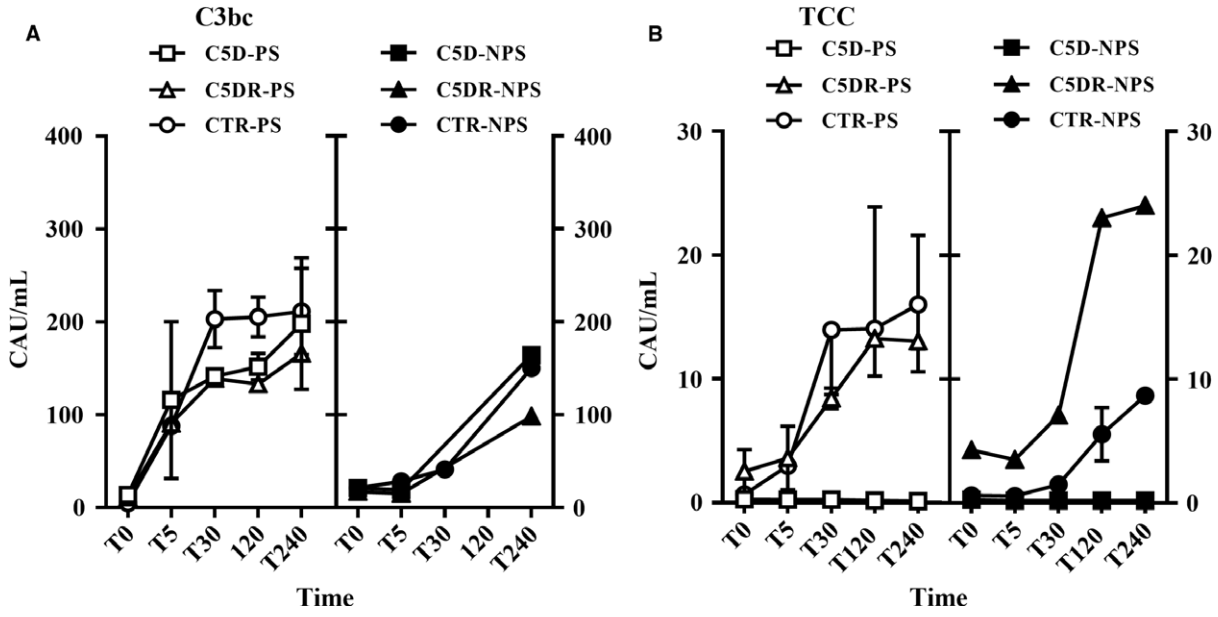

Fig. 7. Complement activation. Complement activation products C3bc (A) and TCC (B) from baseline (T0) through 240 min (T240) expressed as median and range in the C5D, C5DR, and CTR samples in the plasma separation blood samples (PS) and in the no-plasma separation blood samples (NPS).

that C5 deficiency decreased CD11b expression on granulocytes in a model with C5-deficient and C5 reconstituted blood in PVC tubes (6).

Our findings also indicate that CD11b expression on monocytes in the plasma separation samples is C5-dependent. This is also partly in accordance with former studies. Rinder et al. found that anti-C5a only trended toward blocking CD11b expression on monocytes (19). We have previously shown that CD11b expression on monocytes to some extent was dependent on C5 in a model using C5aR1 antagonist for blocking C5 effect on monocytes in a PVC tubing model (7), and Bergseth et al. found that lack of C5 decreased monocyte CD11b expression in a PVC tubing model, using C5D-deficient blood reconstituted with C5 (6). Rinder et al. also demonstrated that CD11b expression on monocytes can be reduced by down-regulating both classical and alternative C3/C5 convertases using the complement activation blocker (CAB-2; CD46–CD55 conjugate). Thus, blocking formation of C3 cleavage products points to C3a and probably other C3 fragments as additional candidates possibly able to facilitate up-regulation of CD11b expression on monocytes (20). This indicates that biocompatibility between different materials cannot be readily compared, and that every material and model has to be evaluated separately. The main difference between our study and others is the presence of the plasma separation column and the blood and plasma flow rates. Since plasma separation involves shear stress and shear force, this could also affect CD11b expression.

Formation of leukocyte–platelet conjugates

Formation of leukocyte–platelet conjugates is recognized as a component of inflammation in many circumstances, and conjugate formation can be induced by artificial surfaces and thus seen as a marker of bioincompatibility (21, 22). In an *in vitro* model of artificial-surface-induced inflammation using monoclonal antibodies and small peptides as complement inhibitors, we have previously shown that conjugate formation is mediated by activation of complement and the formation of C5a, which also up-regulates CD11b on leukocytes (7).

In the present study, formation of granulocyte–platelet conjugates increased fairly equal in the C5DR-PS and C5D-PS samples until T15. From T15 to T30, there was a 2.5-fold increase in granulocyte–platelet conjugate formation in the C5DR-PS sample compared to the C5D-PS sample. In the CTR-PS sample, there was a 4-fold increase in granulocyte–platelet conjugate formation. Other studies have shown increase in formation of granulocyte–platelet conjugates in models including PVC tubing and a membrane oxygenation device and at the same time also shown that granulocyte–platelet conjugate formation can be reduced either by blocking C5aR1 or by blocking cleavage of C5 (7, 19).

We found an increase in monocyte–platelet conjugate formation in the plasma separation samples in our study, and this increase appeared to be C5-independent. Previous studies have indicated C5 dependence to a certain degree in the formation of monocyte–platelet conjugate formation (6, 7, 19).

The difference in result from other studies regarding formation of leukocyte–platelet conjugates indicates that the plasma separation column and the blood bag or the model as a whole can mediate conjugate formation also through mechanisms other than complement C5a generation and CD11b expression on leukocytes. Previous studies have pointed out the ability of shear stress and shear force in blood circulating circuits both *ex vivo* and *in vivo* to activate cellular components of the blood (23–25). Gutensohn et al. described, in a model of platelet apheresis, interaction between platelets and monocytes simultaneously as up-regulation of P-selectin and CD63 was observed on platelets (26). At the same time, activated platelets binding CD41a+ leukocytes forming conjugates was observed. Importantly, they noticed that most of the binding between the platelets and leukocytes happened during the first 5 min of circulation. This was also the case in our study with regard to the monocyte–platelet conjugate formation in the plasma separation samples.

The plasma separation column used in the current study has been described to mediate complement activation, but not to activate cellular components of the blood (27). By measuring C3, C3a and C5a in models with different types of lipoprotein apheresis and in lone plasmapheresis models, other studies have also concluded that the plasmapheresis column activates complement (5, 28). Taken together, these findings indicate that shear stress and shear force also mediate conjugate formation through mechanisms other than C5 activation.

Platelets and leukocytes

Activation, adherence, and clotting of platelets are recognized as bioincompatibility in artificial devices. Primary activation of platelets and secondary activation as a result of activation of the coagulation and immune systems have been discussed as possible mechanisms (2, 3). In our study, there was a marked reduction in platelets in the plasma

separation samples compared to the no-plasma separation samples, but the reduction was not C5-dependent. The observation of C5 independence is not in alignment with earlier studies stating that inhibiting C5 did preserve the platelet count in a cardiopulmonary bypass model (18). On the other hand, it is clearly demonstrated that platelets bind vigorously to PVC used in the tubing in our model (29, 30). Shear stress and shear forces can activate platelets enhancing adhesion of platelets to biomaterials (31, 32). Platelet agglutination was also clearly shown in blood smear taken from the plasma separation samples at T240. This is probably part of the explanation for the observed platelet reduction in the plasma separation samples in our study, despite the use of lepirudin as an anticoagulant. The plasma separation column used in our study is not yet known to interact with the platelets (27). The leukocyte count remained stable and unchanged in all samples in our study despite formation of conjugates and the change in platelet count in the plasma separation samples, indicating that leukocytes did not adhere to the plasma filtration column or the tubing.

Plasma proteins

We observed a reduction in circulating plasma proteins (albumin, immunoglobulins, and C4) in the plasma separation samples independent of C5. When blood interacts with foreign material, the first step in the bio-incompatibility cascade is that a layer of plasma proteins binds to the surface (33). These proteins, bound to the material, undergo conformational changes making the proteins able to activate inflammatory cascade and network systems (34). In the no-plasma separation samples, there were no or only marginal changes in protein concentration, consistent with the different biomaterials in the blood bag and in the plasma separation system and the different mode of contact between blood and plasma circulating in the plasma separation column causing shear stress with increased binding of protein to the biomaterial. Thus, testing of bio-incompatibility for any material should take place under the same conditions as the biomaterials are supposed to be used in clinical practice.

Complement activation

Generation of C3bc is formed by C3 cleavage irrespective of which initial pathway(s) that are activated (35). A model for activation of complement on artificial surfaces has been suggested by several authors (2, 36, 37). Put together, it is possible for both classical, lectin, and alternative pathway to activate the amplification loop of complement and secondly lead to the generation of the terminal complement complex. In accordance with this, we observed an increase in C3bc in our study, whereas the lack of C5 as expected prevented the generation of TCC in the C5-deficient samples. When C5D blood was reconstituted with purified C5, we observed an enhanced activation reflected by increased TCC formation compared to the controls also in the no-plasma separation control sample. The most likely explanation for this is the fact that purified proteins may undergo changes in configuration enhancing their biologic effects, in addition to the inherent risk of contamination. We have experienced this with the purified C5 in our laboratory (non-published observations) and it has been described that different forms of C5b have different potency in generating TCC (38). Our data indicate that the purified C5 might have increased capacity to generate TCC.

CONCLUSION

In an *ex vivo* model of plasma separation, the upregulation of leukocyte CD11b was C5 dependent both on granulocytes and monocytes. The results also indicate a possible C5 dependency of granulocyte–platelet conjugate formation. Platelet count was reduced during plasma separation, whereas the leukocyte count was unchanged. Further improving biocompatibility and reducing complement activation by materials used in routine plasma separation could prove to be of clinical benefit.

We acknowledge that the findings are not new as such and our results are based on a small sample size and conclusions should be drawn with care; however, previous studies in the field have used inhibitors of the complement system, such as purified or monoclonal antibodies or smaller peptides. Such experimental approaches always carry the risk of contamination and cross reaction. To our knowledge, our study is the first using blood from a C5-deficient individual to investigate complement activation in a model with plasma separation, and in our opinion this model adds important information even if confirming previous findings. Furthermore, although C5 deficiency is extremely rare, the increasing clinical use of inhibitors of C5 (e.g. eculizumab) emphasizes the importance of studying mechanisms related to complement activation in more depth.

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