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

Running head: Cytokine changes in lipoprotein apheresis

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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 IFN-γ, IL-8, IL-1ra, eotaxin, TNF, MCP-1, PDGF-BB, RANTES, MIP-1β and IP-10 ($p<0.05$). The dextran sulfate plasma adsorption system reduced IFN-γ, IL-8, IL-1ra, eotaxin, TNF, MCP-1, PDGF-BB, MIP-1β and IP10 ($p<0.05$). VEGF and GM-CSF were increased in the whole blood and dextran sulfate plasma adsorption systems ($p <0.05$). The double filtration plasmapheresis system reduced IFN-γ, IL-1ra, TNF, MIP-1β and IP-10 ($p<0.05$), while MCP-1,VEGF, GM-CSF and RANTES were increased ($p <0.05$). 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, lipoprotein apheresis, e*x vivo*.

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 post-implantation 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 proinflammatory 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

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 one month intervals.

Lipoprotein apheresis (Figure 1)

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 bag (SC) 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., Osaka, Japan) 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, Europe) 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).

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, 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 (FGF), 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.

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, CA). For calculation of differences in SC between baseline (T0) and after 240 minutes perfusion (T240) a two-tailed unpaired students t-test was used. Significance level was set at <0.05. For pairwise comparison of the lipoprotein apheresis systems a regular two-way ANCOVA model with Sidak`s multiple comparison modification was used. Significance level was set at <0.05.

3. Results

Overall concentration changes in the sample control bag and the lipoprotein apheresis systems (Fig. 2)

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 in Figure 2. Figure 2 is divided into A and B to discriminate between small (A) and large (B) concentration changes.

Sample control 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) ($p < 0.05$) (Fig. 2A and B white bars). The other biomarkers did not differ in concentration in SC at T240 compared to baseline.

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 ($p < 0.05$), IL-17 was unchanged and VEGF and GM-CSF were increased ($p < 0.05$) in position blood sample 2 (BS2), before the apheresis blood reservoir, compared to the SC at T240. (Fig. 2A and 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 ($p <$

0.05). The other biomarkers did not differ between the DL75 and EC50 systems at T240 (Table 1).

LA15 lipoprotein apheresis system

In the LA15 system IFN-γ, IL-8, IL-1ra, eotaxin, TNF, MCP-1, PDGF-BB, MIP-1β and IP10 were reduced ($p < 0.05$), IL17 and RANTES was unchanged and VEGF and GM-CSF were increased ($p < 0.05$) in position BS2 compared to the SC at T240. (Fig. 2A and B light grey 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 ($p < 0.05$). The other biomarkers did not differ between the LA15 and EC50 systems at T240 (Table 1).

EC50 lipoprotein apheresis system

In the EC50 system IFN-γ, IL-1ra, TNF, MIP-1β and IP-10 were reduced ($p<0.05$), IL-8, eotaxin, IL17 and PDGF-BB were unchanged and MCP-1,VEGF, GM-CSF and RANTES were increased ($p < 0.05$) in position BS2 compared to the SC at T240 (Fig. 2A and B dark grey bars).

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.

Eotaxin (Fig. 3)

Eotaxin concentration was unchanged from baseline to T240 in SC (Fig. 3A, B, C).
In the DL75 system, position BS2, eotaxin showed a marked reduction from T0 to T15 and remained on this level until T240. (Fig. 3A and 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 (Fig. 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 (Fig 3B, F). In the EC50 system, position PS1, a similar pattern was seen as for the LA15 system (Fig 3C, D), however in position PS2, eotaxin increased slightly from T15 until T240 (Fig. 3C, E).

PDGF-BB (Fig. 4)

PDGF-BB concentration increased 25-fold from baseline to T240 in SC (Fig. 5A, B, 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 (Fig. 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 (Fig. 5A, D). From T120 until T240 there was a decrease in concentration towards baseline (Fig 5B, F). In position PS2 the concentration stayed at baseline from T0 to T240 (Fig 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 (Fig 5C, D, E, F).

RANTES (Fig. 5)

RANTES concentration increased from baseline to T240 in SC (Fig. 5A, B, C). In the DL75 system, position BS1, RANTES increased 4-fold until T15 before a gradual reduction to below baseline at T240 (Fig 5A). In the LA15 system, position BS2, concentration increased form baseline to T240 (Fig 5B, F) indicating that the LA15 system as a whole increased the concentration of RANTES. In position PS1 the same pattern was seen as in position BS2 (Fig. 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 (Fig 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 concentration of RANTES from T0 until T240 (Fig 5C, D, E, F)

IP-10 (Fig. 6)

IP-10 concentration was unchanged from baseline to T240 in SC (Fig. 6A, B, C). In the DL75 system, position BS2, the IP-10 concentration was markedly reduced from T0 to T15 (Fig. 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 (Fig 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 (Fig 6B, F). This indicates effective reduction of IP-10 in the DL75 and LA15 columns (Fig 6B, E). In the EC50 column, position PS1, there was a fall from T0 to T15 after which the concentration remained stable until T240 (Fig 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 (Fig

6C, E). In position BS2 there was an initial reduction from T0 to T15 and thereafter a stable concentration until T240 (Fig. 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 cross-talk 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 (EDTA) 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 plasmaseparation 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 e.g. 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 minutes. 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 plasmaseparation 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. and others demonstrated an impact on the messenger RNA of IL-1α, IL-6, 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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Disclosure.

The authors have no conflicts of interest to disclose.

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