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Lipoprotein apheresis affects lipoprotein particle subclasses more efficiently compared to the PCSK9 inhibitor evolocumab, a pilot study

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ABSTRACT

Lipoprotein apheresis and proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors are last therapeutic resorts in patients with familial hypercholesterolemia (FH). We explored changes in lipoprotein subclasses and high-density lipoprotein (HDL) function when changing treatment from lipoprotein apheresis to PCSK9 inhibition.

We measured the levels of low-density lipoprotein (LDL) and HDL particle subclasses, serum amyloid A1 (SAA1), paraoxonase-1 (PON1) activity and cholesterol efflux capacity (CEC) in three heterozygous FH patients. Concentrations of all LDL particle subclasses were reduced during apheresis (large 68.0 ± 17.5 to 16.3 ± 2.1 mg/dL, ($p=0.03$), intermediate 38.3 ± 0.6 to 5.0 ± 3.5 mg/dL ($p=0.004$) and small 5.0 ± 2.6 to 0.2 ± 0.1 mg/dL ($p=0.08$)). There were non-significant reductions in the LDL subclasses during evolocumab treatment. There were non-significant reductions in subclasses of HDL particles during apheresis, and no changes during evolocumab treatment. CEC was unchanged throughout the study, while the SAA1/PON1 ratio was unchanged during apheresis but decreased during evolocumab treatment.

In conclusion, there were significant reductions in large and intermediate size LDL particles during apheresis, and a non-significant reduction in small LDL particles. There were only non-significant reductions in the LDL subclasses during evolocumab treatment.

Keywords: LDL-cholesterol; lipoprotein particles, lipoprotein apheresis, PCSK9-inhibition.

1. Introduction

Lipoprotein apheresis has been considered the last treatment option when conventional cholesterol lowering treatment is not tolerated or when treatment targets are not met, particularly in patients with familial hypercholesterolemia (FH) [1,2]. The proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors are effective in reducing LDL cholesterol in heterozygous FH patients [3], and they also reduce coronary atheroma size [4]. The first clinical endpoint study with evolocumab was published in March 2017 and showed significant reductions in hard clinical endpoints [5]. Our group has recently demonstrated that when switching from lipoprotein apheresis to PCSK9 inhibition the LDL reduction is partly maintained, while avoiding reduction in high-density lipoprotein (HDL) cholesterol [6], furthermore PCSK9 inhibition elicits less inflammatory response than lipoprotein apheresis [7].

In addition to focus on LDL and HDL cholesterol there has been a growing interest in subclasses of lipoprotein particles, and traditionally small dense LDL particles have been considered particularly atherogenic [8-10]. The clinical effect of subclassing HDL particle size is perhaps more uncertain and the results have been more conflicting [11]. However, HDL composition and function have in recent times been assigned greater importance in lipid metabolism [12-14]. Paraoxonase-1 (PON1) is a HDL associated protein with anti-atherosclerotic properties by preventing oxidation of LDL and cell membranes [15]. Another crucial component of HDL is the acute phase protein serum amyloid A (SAA). Increased expression of SAA, as a result of infection or inflammation, alters the HDL composition and reduces the anti-inflammatory effects of HDL [16]. Regarding HDL functionality, research in reverse cholesterol transport including efflux capacity, has increased in recent years, and indeed efflux capacity is inversely associated with cardiovascular endpoints [17]. Contrary to previous research, small

HDL particles seem to be associated with more effective cholesterol efflux [18], underlining the difficulties in interpreting cholesterol efflux data.

In the present study, we explored subclasses of LDL and HDL particles, cholesterol efflux capacity, PON1 activity and SAA1, when switching from lipoprotein apheresis to PCSK9 inhibition with evolocumab.

2. Material and Methods

The study design and results regarding lipid and inflammatory parameters have recently been published [6,7]. In brief, it was an observational study with three FH patients established in long-term lipoprotein apheresis. They were all heterozygous for the C210G missense mutation in the LDL receptor gene [19]. Treatment was converted to a PCSK9 inhibitor (evolocumab), and the patients were examined immediately before and after their last apheresis treatment (week 0), after one week (immediately before the first evolocumab injection (week 1)), then biweekly before administration of evolocumab (weeks 3, 5 and 7)

2.1 Patients and ethics

There were two women (52 and 53 years) and one man (49 years) with genetically confirmed FH, and they all had angiographically verified coronary artery disease. They were intolerant to statins due to myalgia, and they did not take any type of lipid lowering medication. The patients had been in lipoprotein apheresis on average for 11 years (11-13 years). All patients signed informed consent and the regional ethics committee approved the study. These were the only heterozygous FH patients in long term lipoprotein apheresis in Northern Norway.

2.2 Lipoprotein apheresis

The patients were established in weekly filtration lipoprotein apheresis, and the last lipoprotein apheresis in week 0 was performed with the semi-selective LDL filtration column Cascadeflo-EC-50W (Asahi Kasei Medical Europe) after previous plasma separation with Plasmaflo OP-50 (Asahi Kasei Medical Europe), using the Infomed HF440 apheresis machine (Infomed SA, Geneva, Switzerland), for all three patients a plasma volume of 4000 mL was treated. Anticoagulation was obtained by unfractionated heparin (7500 units was given for priming the unit, then an individualized infusion was administered to each patient in order to avoid clotting (range 150 to 750 units per hour, average treatment times 2 hours and 15 minutes).

2.3 Evolocumab treatment

Evolocumab was administered according to the manufacturers' instructions in week one, three, five and seven with the recommended dose of 140 mg subcutaneously (autoinjector). The injections were performed by the patients in the hospital, supervised by experienced nurses, after demonstrations with dummy autoinjectors.

2.4 Blood samples and analyses

Fasting blood samples were obtained by standard venipuncture (or from the AV-fistulas during apheresis). Blood samples for plasma preparation were placed on ice before centrifugation for 20 min, 3000xg at 4°C. Serum, EDTA plasma and citrate plasma were frozen in aliquots at -80°C and analyzed in batch at the end of the study.

LDL and HDL subfractions were determined electrophoretically by the use of lipid stained serum (Sudan Black), high-resolution 3% polyacrylamide gels and the Lipoprint® system (Quantimetrix Corporation, Redondo Beach, CA, USA, [20]). The different subfractions were identified by their migration distance - on basis of size - in the gel and the concentration of each subfraction was calculated using the Lipoprint analysis software. The Lipoprint LDL system can identify seven subfractions of LDL, (1= larger, buoyant particles, 2= intermediate and 3-7= small, dense particles). The diameter of the LDL particles at the cut-off point separating subfractions 1-2 from 3-7 was 251 Å. The Lipoprint HDL system separates ten various HDL subfractions (1-3= large HDL particles, 4-7= intermediate HDL particles, 8-10= small HDL particles). Hence the different subfractions of LDL and HDL were divided into three subclasses; large, intermediate and small.

Cholesterol efflux was measured with a commercial kit from Sigma-Aldrich (MAK192) according to the description. Briefly, a human monocyte cell line, THP-1, was differentiated into macrophages with 10 ng/ml phorbol myristate acetate (PMA) for 24 hours at 37° and 5% CO₂ in a 96-well plate. The PMA containing medium was replaced with complete cultivation medium (RPMI1640 including 10% foetal bovine serum, 2 mM Glutamine) and incubated for another 30 hours. The serum containing medium was removed and then washed with serum free medium. A reaction mix, containing equilibration buffer and fluorescence labelled LDL, was added to the cells and incubated for 16 hours. The reaction mix was removed and wells washed with serum free medium. Patient serum samples were precipitated with a reaction mix from the kit and the clear supernatant was added to the plate and incubated for 5 hours. After the incubation, supernatants were transferred to a new plate and the

fluorescence measured (482 ex/515 em). The cell layer was solubilized with a cell lysis buffer, incubated for 30 minutes on a shaker. The cell lysate was then transferred to the plate with supernatants and the fluorescence of the mixture was measured. Percent efflux was calculated as follows: $100 \times \text{fluorescence intensity of the medium} / \text{fluorescence intensity of the medium and cell lysate}$.

PON1 arylesterase activity was measured in citrate plasma. Briefly, plasma was diluted 1:80 with a salt buffer (20mM Tris–HCl and 1.0 mM CaCl_2). A triplicate of 20 μl diluted plasma were added to the wells in an UV-transparent 96-well plate (Sigma-Aldrich). 200 μl of phenyl acetate solution, containing 3.26 mM phenyl acetate in salt buffer, was added to each well and the absorbance of produced phenol was measured at 270 nm with 250 nm as background in a SpectraMax 190 plate reader (Molecular Devices, Sunnyvale, CA, USA). The initial period when the reaction was linear was used for calculation of activity, expressed as U/ml, using an extinction coefficient of phenol of $1310 \text{ M}^{-1} \text{ cm}^{-1}$.

To investigate the acute phase response by SAA, plasma SAA1 levels were measured by an ELISA (DY3019-05, R&D systems, Minneapolis, MN, USA) according to the manufacturers' instructions. In short, citrate plasma was added to the plate and incubated for 2 hours at room temperature. Following wash, a detection antibody was added and incubated for 2 hours. The plate was washed and streptavidin-horseradish peroxidase was added followed by incubation for 20 min. The plate was then washed a final time before a substrate solution was added before 20 min incubation. At the end of the incubation, stop solution was added and

absorbance was measured at 450 nm with correction at 570 nm using a Spectramax 190 plate reader (Molecular devices, Sunnyvale, CA, USA).

The measures of SAA and PON1 were integrated as SAA1/PON1 ratio, which has been proposed as a possible biomarker for dysfunctional HDL [21].

2.5 Statistics

Numerical data are presented with mean and standard deviation (SD). Age and duration of treatment are presented as mean and range. A repeated measures one-way analysis of variance (RM one-way ANOVA) was used to calculate the longitudinal effect of the evolocumab treatment (week one to week seven). Levels before and after lipoprotein apheresis treatment (week 0) and before apheresis vs. after the last evolocumab injection were compared by paired t-tests. All tests were two-tailed and results with a $p < 0.05$ were considered statistically significant. Analyses were performed using PRISM 6 (Graph Pad Software Inc, La Jolla, CA, USA).

3. Results

3.1 LDL particle subclasses (Fig. 1)

All subclasses of LDL particles were reduced during lipoprotein apheresis (Fig. 1).

The concentration of large LDL particles was reduced from 68.0 ± 17.5 mg/dL to 16.3 ± 2.1 mg/dL during apheresis ($p=0.03$) (Fig. 1a), intermediate LDL particles were reduced from 38.3 ± 0.6 mg/dL to 5.0 ± 3.5 mg/dL ($p=0.004$) (Fig. 1b) and small LDL particles were reduced from 5.0 ± 2.6 mg/dL to 0.2 ± 0.1 mg/dL ($p=0.08$) (Fig. 1c). During evolocumab treatment there were no significant changes in the large, intermediate or the small LDL particles, although there seemed to be a slight reduction over time for both large and intermediate particles. Average LDL particle diameter was unchanged during the course of study (data not shown).

3.2 HDL particle subclasses (Fig. 2)

All subclasses of HDL particles were reduced during lipoprotein apheresis (Fig. 2), however non-significantly. Large particles were reduced from 5.7 ± 1.5 mg/dL to 3.7 ± 1.2 mg/dL, (Fig 2a), intermediate particles were reduced from 15.0 ± 2.0 mg/dL to 9.3 ± 1.2 mg/dL, (Fig. 2b), and small HDL particles were reduced from 12.0 ± 4.6 mg/dL to 5.0 ± 1.7 mg/dL, (Fig. 2c). All types of HDL particles increased during the week after lipoprotein apheresis, and were unchanged during treatment with evolocumab.

3.3 CEC, PON1 activity and SAA1 (Fig. 3)

There was no significant change in cholesterol efflux capacity during apheresis ($42.6 \pm 3.8\%$ to $44.8 \pm 8.8\%$, and no significant changes at week 1 ($50.3 \pm 7.1\%$), (Fig. 3a). Likewise, during evolocumab treatment there were no significant changes in efflux capacity.

There was a significant decrease in PON1 activity during apheresis from 87.5 ± 8.3 U/ml to 53.5 ± 3.6 U/ml ($p=0.03$) (Fig. 3b). One week after apheresis PON1 activity had increased to levels comparable to pre-apheresis, and was unchanged during evolocumab treatment.

There was a non- significant reduction in SAA1 during apheresis (1.9 ± 1.1 μ g/ml to 1.3 ± 0.6 μ g/ml ($p=0.18$), and SAA1 was unchanged during evolocumab treatment (Fig. 3c).

The SAA1/PON1 ratio was unchanged during apheresis but appeared to decrease during evolocumab treatment (Fig. 3d).

4. Discussion

We observed reductions in all subclasses of LDL particles after semi-selective lipoprotein apheresis. Although the reduction in small LDL particles did not reach statistical significance it seems numerically convincing, as there were virtually immeasurable amounts of small LDL particles present after apheresis. Subclasses of HDL particles were statistically unaffected by apheresis and evolocumab treatment, even though we previously have demonstrated significant reduction in total HDL cholesterol during apheresis [6]. This could be a result of the low number of participants. There was no significant change in cholesterol efflux capacity one week after apheresis compared to baseline before apheresis, and efflux was also unaffected by evolocumab.

The novel PCSK9 inhibitors have been shown in several clinical trials to lower LDL and coronary atherosclerosis significantly [4,22,23]. Clinical endpoints were reduced in a recent evolocumab study, however total mortality was not reduced [5]. Further long-term studies are expected to be reported in 2018. Lipoprotein apheresis, which - although time-consuming and expensive – also has a proven clinical effect [24]. Furthermore, whether the intermittent, very low values of LDL seen after apheresis with a rebound before next treatment is preferential to a more constant, moderate reduction seen with PCSK9 inhibition is also a matter of debate. A recent double-blind study has demonstrated that the rate of apheresis treatment can be reduced with PCSK9 inhibition [25]. However, it seems premature to recommend that all patients established in apheresis should be switched to PCSK9 inhibition.

4.1 Lipoprotein particle subclasses

Over the last decades, small LDL particles have been considered especially atherogenic [26], even if the clinical use of fractioning or subclassing of lipoprotein particles has been debated [27,28].

Otto et al have previously demonstrated reduction in large, intermediate and small subfractions of LDL (measured with ultracentrifugation) during one session of lipoprotein apheresis [29], this finding was later confirmed by Geiss et al [30]. The PCSK9 inhibitor alirocumab lowered large, intermediate and small LDL particles (measured by mass spectroscopy) when compared to placebo [31,32]. We demonstrate reductions in large, intermediate and small LDL particles during apheresis, and even if the latter was not statistically significant, virtually all of the small LDL particles were removed. This finding could be of particular importance for FH patients with high risk of atherosclerotic complications [33]. When changing the treatment from lipoprotein apheresis to evolocumab the findings are less clear; there was a trend for reductions in both large and intermediate LDL particles, while the findings regarding small LDL particles were less consistent and not in line with the findings cited above. This could be due to a number of factors including small sample size and short observation period.

Large HDL particles have previously been associated with better clinical outcome [34], however this notion has been challenged, and more recent studies indicate that smaller HDL particles may be more beneficial [35]. Small, dense HDL particles have also been identified as more efficient mediators of cholesterol efflux [18].

Orsoni et al have demonstrated that lipoprotein apheresis reduces all sizes of HDL particles (mass spectroscopy), however relatively more of the larger subtypes of HDL [36]. Koren et al noted a non-significant increase of all three particle sizes after 12 weeks of alirocumab compared to baseline [31]. We found non-significant reductions in all three subclasses of HDL cholesterol particles during apheresis, and no significant differences when changing the cholesterol lowering treatment from apheresis to PCSK9 inhibition.

4.2 CEC, PON1 activity and SAA1

Cholesterol efflux capacity is inversely related to atherosclerotic disease in FH patients [37]. Nenseter et al did not find any change in cholesterol acceptor capacity after lipoprotein apheresis in patients with homozygous FH [38]. Adorni et al demonstrated a reduction in cholesterol efflux capacity after lipoprotein apheresis in patients with hypercholesterolemia [39]. At present, there are few data on PCSK9 inhibition and cholesterol efflux capacity, however an increase in cholesterol efflux during PCSK9 inhibition has been hypothesized [40,41].

A number of previous studies have been performed in non-human cell-lines [39,42]. However, in order to avoid any possible species-related differences [43], we routinely use a human monocyte cell line. We demonstrate unchanged cholesterol efflux capacity moving from lipoprotein apheresis to PCSK9 inhibition, even if HDL cholesterol was reduced during lipoprotein apheresis and restored during PCSK9 inhibition. Our contradictory CEC results compared to Adorni et al [39], may be explained by the fact that human monocytes and not acetyl-coenzyme A

acetyltransferase inhibitor were used in the present study, but this has to be further investigated in a larger study.

PON1 activity is closely connected to HDL function, and could have an atheroprotective effect [44]. PON1 contributes to the antioxidative functions of HDL in the vascular wall but is also an important detoxifying agent [15], therefore techniques measuring enzyme activity may differ depending on the aim. Our finding of reduction of PON1 during apheresis could hence be of importance, also when taking into account the simultaneous reduction in HDL [6]. Furthermore, the return of PON1 to pre-apheresis levels at the start of PCSK9 inhibition at week 1 and during treatment of evolocumab is interesting and should be further explored in larger studies.

SAA is an acute phase protein, present in HDL in several isoforms⁴⁵ that has been associated with atherosclerotic disease [46], and from previous studies it is known that lipoprotein apheresis reduces serum amyloid A and serum amyloid P [47]. However, in the present study we only find a non-significant reduction in SAA1 during apheresis. Evolocumab treatment had no effect on SAA1 levels.

However, paired measurements of SAA and PON1 have been suggested as a possible marker for dysfunctional HDL since it combines the antioxidant capacity of PON1 with the marker of inflammatory responses SAA [21], and should therefore be investigated in a larger population before any conclusions could be drawn since we have previously shown that apheresis elicits an innate immune response [48,49], and we have recently reported that evolocumab seems inert in this respect [7]. In the present study, the ratio was unchanged after apheresis while treatment with

evolocumab caused a non-significant reduction. This finding possibly indicates a favorable reduction of SAA1/PON1 with evolocumab treatment but further studies in larger populations are needed.

A very recent paper establishes the need for more data on combination of apheresis and new LDL lowering therapy, and international collaboration is established [50].

4.3 Limitations

There are several limitations to this study. It has a low number of participants (n=3) and is merely observational. This limits generalizability. The apheresis method used is a semi-specific method, and may not reflect more specific lipoprotein apheresis systems. Furthermore, the effects seen when converting from lipoprotein apheresis to PCSK9 inhibition may not necessarily reflect the effects of PCSK9 inhibition in apheresis-naïve patients. Finally, some of the non-significant trends seen after seven weeks of treatment may reflect the relatively short observation time.

In conclusion, there were significant reductions in large and intermediate size LDL particles during lipoprotein apheresis, and a non-significant reduction in small LDL particles. There were non-significant reductions in all three subclasses of HDL particles during lipoprotein apheresis, while treatment with evolocumab did not significantly affect LDL or HDL particle subclasses. Cholesterol efflux capacity was not affected by lipoprotein apheresis or evolocumab treatment while the SAA1/PON1 ratio appeared to decrease slightly during evolocumab treatment.

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Figures.

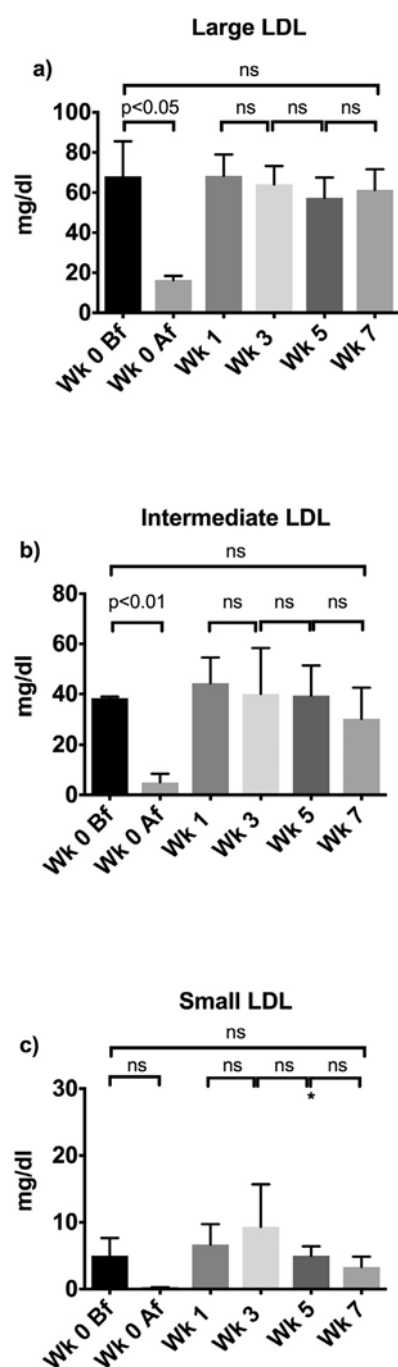


Figure 1. Concentrations of subclasses of LDL particles during lipoprotein apheresis and after starting evolocumab.

LDL: Low-density lipoprotein. Wk 0 bf: Week 0 before apheresis. Wk 0 af: Week 0 after apheresis. Wk 1-7: Week 1-7 (samples taken before evolocumab administration).

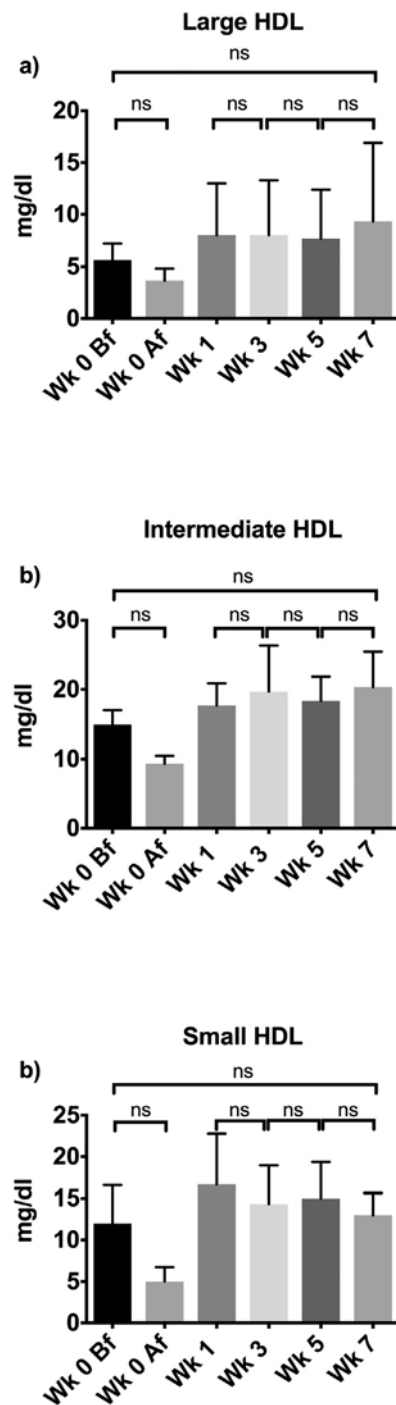


Figure 2. Concentrations of subclasses of HDL particles during lipoprotein apheresis and after starting evolocumab.

HDL: High-density lipoprotein. Wk 0 bf: Week 0 before apheresis. Wk 0 af: Week 0 after apheresis. Wk 1-7: Week 1-7 (samples taken before evolocumab administration).

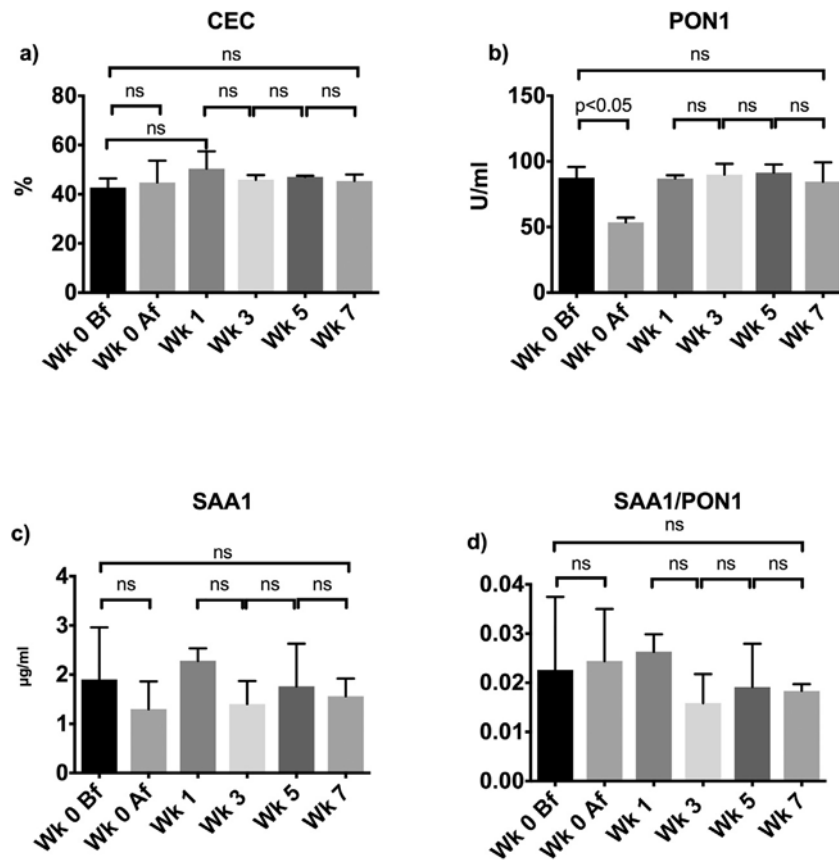


Figure 3. Cholesterol efflux capacity, PON1 activity, SAA1 concentration and PON1/SAA1 ratio during lipoprotein apheresis and after starting evolocumab.

CEC: Cholesterol efflux capacity. PON1: paraoxonase-1. SAA1: serum amyloid A1.

Wk 0 bf: Week 0 before apheresis. Wk 0 af: Week 0 after apheresis. Wk 1-7: Week 1-7 (samples taken before evolocumab administration).