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GENETIC LCAT DEFICIENCY AND ATHEROSCLEROSIS:
STUDIES ON ENDOTHELIAL CELLS AND MONOCYTES

BIO/14

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Abstract (English version)

Background

Lecithin:cholesterol acyltransferase (LCAT) is the unique enzyme in human able to esterify free cholesterol in plasma. Genetic lack of LCAT affects high-density lipoprotein (HDL) metabolism, leading to very low plasma concentrations of these lipoproteins. LCAT was believed to be an important driving force behind macrophage reverse cholesterol transport (RCT) and therefore playing a role in atheroprotection. Despite the low plasma HDL-C, LCAT deficiency does not remarkably increase preclinical atherosclerosis or could even be protective. We hypothesized that this discrepancy can be explained by an increased functionality of HDL particles, that are more effective in protecting against endothelium dysfunction.

Methods

HDLs were isolated from LCAT-deficient carriers and tested *in vitro* for their capacity to promote NO production and to inhibit vascular cell adhesion molecules in endothelial cells. Measurement of flow-mediated vasodilation (FMD) was used as marker of endothelial dysfunction *in vivo*. Reconstituted HDL containing only apoA-I (LpA-I) or apoA-I and apoA-II (LpA-I:A-II) were used to prove the structural responsible for increased HDL functionality. *Ex vivo* analysis of monocytes in carriers of LCAT deficiency, to assessed phenotype and function, was performed with fluorescence-activated cell sorter analysis, inflammatory stimulation and transendothelial migration assays. Comparison with other genetic hypoalphalipoproteinemia was conducted for the monocyte study.

Results

Genetic LCAT deficiency is characterized by selective depletion of LpA-I:A-II and high presence of immature preβ-HDL. HDLs isolated from carriers were more effective in promoting in *vitro* eNOS activation by phosphorylation and consequently NO production in endothelial cells, compared to controls, with a gene-dose dependent effect. Moreover, HDLs from carriers have an increased capability in inhibiting VCAM-1 expression. The effect was likely dependent on the selective depletion in LpA-I:A-II particles. In addition carriers' monocyte displayed a less inflammatory phenotype compared to controls, as evidenced by a reduced expression of integrin CD11c, a decreased capacity to transmigrate and a lower production of cytokines upon stimulation. Conversely, no changes in monocyte immunophenotype was found in other genetic HDL deficiency.

As *in vivo* confirmation, no difference in FMD was found between carriers of LCAT deficiency and controls.

Conclusions

Despite the low plasma HDL-C concentration, LCAT deficiency is not associated with increased endothelial dysfunction due to the enhance efficacy of HDL particles in stimulating endothelial NO production and in inhibiting adhesion molecules expression, as well to the reduce pro-inflammatory potential of carriers' monocytes.

Riassunto (Versione italiana)

Introduzione

La lecitin:cholesterol aciltransferasi (LCAT) è l'unico enzima nell'uomo in grado di esterificare il colesterolo nel plasma. Il deficit genetico di LCAT influenza il metabolismo delle lipoproteine ad alta densità (HDL), portando a concentrazioni plasmatiche ridotte di queste lipoproteine. Si ritiene che LCAT sia un'importante driving force per il trasporto inverso del colesterolo (RCT) nei macrofagi e che pertanto giochi un ruolo importante nell'ateroprotezione.

Nonostante i bassi livelli plasmatici di HDL-C, il grado di aterosclerosi preclinica non è risulta aumentato nel deficit di LCAT che addirittura sembra essere protettivo. Abbiamo quindi ipotizzato che questa discrepanza potesse essere spiegata da un aumento della funzionalità delle particelle HDL che sono più efficienti nel proteggere contro la disfunzione endoteliale.

Metodi

Le HDL sono state isolate da soggetti portatori di deficit di LCAT e testate *in vitro* per la loro capacità di stimolare la produzione di NO e di inibire l'espressione di molecole di adesione in cellule endoteliali. La misura della vasodilatazione flusso-mediata (FMD) è stata utilizzata come indice *in vivo* di disfunzione endoteliale. HDL ricostituite, contenenti solo apoA-I (LpA-I) o apoA-I e apoA-II (LpA-I:A-II) sono state utilizzate per dimostrare come quali modifiche strutturali siano responsabili dell'aumentata funzionalità. Analisi *ex vivo* del fenotipo e della funzionalità di monociti isolati da portatori di deficit di LCAT sono stati condotti tramite citometria a flusso, stimolazione e saggi di transmigrazione endoteliale. Portatori di altri deficit genetici di HDL sono stati utilizzati come confronto.

Risultati

Il deficit di LCAT genetico è caratterizzato da una deplezione selettiva di LpA-I: A-II e da alte concentrazioni di preβ-HDL immature. Le HDL isolate dai portatori si sono dimostrate più efficaci nel promuovere l'attivazione di eNOS *in vitro* mediante fosforilazione e, di conseguenza, la produzione di NO nelle cellule endoteliali, rispetto ai controlli, con un effetto gene-dose. Inoltre, le HDL dei portatori hanno una maggiore capacità di inibire l'espressione di VCAM-1. L'effetto è probabilmente dipendente dalla deplezione selettiva in lipoproteine LpA-I:A-II. Inoltre, i monociti dei portatori presentavano un fenotipo meno infiammatorio rispetto ai controlli, come evidenziato dalla ridotta espressione dell'integrina CD11c, dalla ridotta capacità di trasmigrazione e dalla minore produzione di citochine

dopo stimolazione. Al contrario, nessuna variazione è stata individuata nell'immunofenotipo di monociti isolati in soggetti con altri deficit genetici di HDL.

A conferma *in vivo* di quanto individuato precedentemente non è stata trovata alcuna differenza nella FMD tra portatori di deficit genetico di LCAT e controlli.

Conclusioni

Nonostante le ridotte concentrazioni plasmatiche di HDL-C, il deficit di LCAT non è associato a una maggiore disfunzione endoteliale dovuta all'aumentata efficacia delle particelle HDL nella stimolazione della produzione di NO endoteliale e nell'inibizione dell'espressione delle molecole di adesione, nonché al ridotto potenziale pro-infiammatorio dei monociti dei portatori.

Abbreviations

ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette sub-family G1
ApoA-I	apolipoprotein A-I
ApoA-II	apolipoprotein A-II
ApoB	apolipoprotein B
ATF3	activating transcription factor 3
BMI	body mass index
CAD	coronary artery disease
CCR2	C-C chemokine receptor type 2
CCR5	C-C chemokine receptor type 5
CCR7	C-C chemokine receptor type 7
CD11b	cluster of differentiation 11b
CD11c	cluster of differentiation 11c
CD29	cluster of differentiation 29
CD36	cluster of differentiation 36
CETP	cholesteryl ester transfer protein
CVD	cardiovascular disease
eNOS	endothelial nitric oxide synthase
FC	free cholesterol
FED	fish-eye disease
FLD	familial LCAT deficiency
FMD	flow-mediated vasodilation
HAEC	human aortic endothelial cells
HDL-C	high-density lipoprotein cholesterol
hsCRP	high sensitivity C-reactive protein
HUVEC	human umbilical vein endothelial cells

ICAM-1	intercellular Adhesion Molecule 1
IL-6	interleukin 6
IL-8	interleukin 8
IL-1β	interleukin 1β
IL-1Ra	interleukin 1 receptor antagonist
IL-10	interleukin 10
IMT	intima-media thickness
LCAT	lecithin:cholesterol acyltransferase
LDL-C	low-density liporprotein cholesterol
LpA-I:A-II	lipoprotein containing apoA-I and apoA-II
LPS	lipopolysaccharide
LpX	lipoprotein X
MCP-1	monocyte chemoattractant protein 1
MFI	mean fluorescence intensity
NLRP3	nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3
NO	nitric oxide
PAF-AH	platelet activating factor-acetylhydrolase
PON1	paraoxonase 1
POPC	phosphatidylcholine
RCT	reverse cholesterol transport
rHDL	reconstituted HDL
RPMI	Roswell Park Memorial Institute medium
rhLCAT	recombinant human LCAT
S1P	sphingosine-1-phosphate
SD	standard deviation
SEM	standard error of measurement
SR-A	scavenger receptor A

SR-BI	scavenger receptor class B type 1
TC	total cholesterol
TEM	transendothelial migration
TG	triglycerides
TLR	toll-like receptor
TNF-α	tumor necrosis factor α
VCAM-1	vascular cell adhesion protein 1

INTRODUCTION

1. Genetic high density lipoprotein disorders

Hypoalphalipoproteinemia is characterized by plasma high-density lipoprotein cholesterol (HDL-C) levels below the 5th percentile for the general population. Marked HDL deficiency (HDL-C < 20 mg/dL), without secondary causes (hypertriglyceridemia, diabetes mellitus, liver disease, etc.) is very rare (<1% of the population). Homozygous, compound heterozygous or heterozygous mutations in *APOA1, ABCA1* or *LCAT* genes account for the 10-16% of cases (1).

ApoA-I has a structural role in HDL particles that contain 2-5 molecules of apoA-I (2) and contributes to their anti-atherosclerotic properties, since lipid-free apoA-I mediates cholesterol efflux through the binding with the ATP binding cassette transporter, sub-family A, member 1 (ABCA1) (3).

Genetic apolipoprotein A-I (apoA-I) deficiency was first reported by Norum and colleagues in two sisters with very low HDL-C and premature cardiovascular disease (CVD) (4). Since apoA-I is essential for HDL biogenesis, mutations of two alleles in *APOA1* gene may lead to nearly complete absence of apoA-I and HDL-C in plasma. Among clinical signs, apoA-I deficiency may be accompanied by corneal opacities or xanthomas (5), as well as an increased risk for premature CVD (6).

At least 70 mutations have been identified since the discovery of ApoA-I$_{Milano}$ and only half of them are causing hypoalphalipoproteinemia (7). Furthermore, not all mutations have been associated with increased CVD risk (8). As the case of carriers of the variant R197C (ApoA-I$_{Milano}$) that have extremely low HDL-C levels, but seem to be protected from premature CVD (9).

After being released in the plasma apoA-I particles acquire lipids in a process mediated by ABCA1 transporter. Homozygous *ABCA1* mutations results in Tangier Disease (TD) (10) an autosomal dominant disorder characterized by absence of mature lipid-rich HDL (11), reduced LDL-C and enlarged yellow-orange lobulated tonsils, peripheral neuropathy (12), hepatosplenomegaly, corneal opacity, thrombocytopenia (13, 14). Heterozygous mutations produce a milder phenotype. TD is associated to increase cardiovascular risk, although not as great as expected, despite the severely low HDL-C (15). Thus, studies on the association of heterozygous mutations and

cardiovascular disease are not conclusive (16). More than 100 variants in *ABCA1* have been described (7) associated with a wide range of phenotypes (17).

After lipidation of lipid-free apoA-I, HDL undergo maturation by the action of lecithin:cholesterol acyltransferase (LCAT) that esterifies the free cholesterol on nascent HDL. Mutations in *LCAT* gene lead to very low HDL-C, with alteration in HDL structure and distribution (18). LCAT deficiency includes two rare disease: familial LCAT deficiency (FLD) and fish-eye disease (FED) and will be discussed later in details.

2. HDL and cardiovascular disease

Observational studies have established that HDL-C is inversely associated with cardiovascular disease and mortality (19, 20). This strong inverse association was first reported by the Framingham Heart Study, where risk of coronary artery (CAD) increased sharply as HDL levels fall progressively below 40 mg/dL (21). This observation arose the concept of “reverse cholesterol transport” as the mechanisms by which HDL protect against atherosclerosis (22). The hypothesis was supported by animal studies showing that HDL infusion or apoA-I overexpression inhibit or prevent atherosclerosis (23, 24). The association was further confirmed by prospective studies worldwide that included different racial and ethnic groups (25) (26) and patients in secondary prevention setting (27). In the PROCAM study men with HDL-C < 0.90 mmol/L (35 mg/dL) had a fourfold increased risk of coronary heart disease compared to men with higher HDL-C, independently of serum triglycerides levels (28). A reduction in HDL-C was also noticed in states that predispose to early atherosclerosis such as type 2 diabetes (29, 30). However, human genetics studies and randomized controlled trials (RCT) with HDL-C raising drugs however have failed in demonstrating benefit in reducing CV risk (31). Despite HDL-C concentrations, none of the three Mendelian disorders causing primary hypoalphalipoproteinemia (mutations in *ABCA1*, *APOA1, LCAT* gene) is unequivocally associated with premature coronary heart disease (32-35). Low-frequency and common genetic variants associated with raised HDL-C

concentrations, e.g. *LIPG*, have not proven any association with protection from coronary heart disease (36). One of the major issue concern the relation of HDL-C to other lipoproteins, thus the direct effect of HDL on atherosclerosis cannot be unravelled. Some reports on *CETP* gene variants showed higher HDL-C concentrations and reduced cardiovascular risk, however other atherogenic lipoproteins were lower in carriers compared to controls, thus affecting the interpretation of results (37). Moreover, some genetic variants associated with increased HDL-C have been paradoxically linked with higher risk (38) (39).

In addition, data from clinical trials did not show a beneficial effect on cardiovascular risk despite the increase in HDL-C concentrations. Outcome trials with niacin (AIM-HIGH and HPS2-THRIVE), failed to meet the primary endpoint (40, 41). More dramatically, trials with CETP inhibitors were stopped for futility (42) or even for the increased coronary events and mortality (43), despite the efficacy in arising HDL-C levels was clear. Very recently outcome data on anacetrapib showed that this CETP inhibitor was able to reduce by 9% the occurrence of major cardiovascular events, however the results have been considered non satisfactory and LDL-C reduction was advocate as the main responsible of this positive effect (44).

Very recently Madsen and colleagues have demonstrated on a large number of subjects (more than 100 000) a U-shaped association between HDL-C and cardiovascular mortality in both men and women (Figure 1). The same was found when single cardiovascular endpoints were considered (ischemic heart disease, myocardial infarction and ischemic stroke) (45).

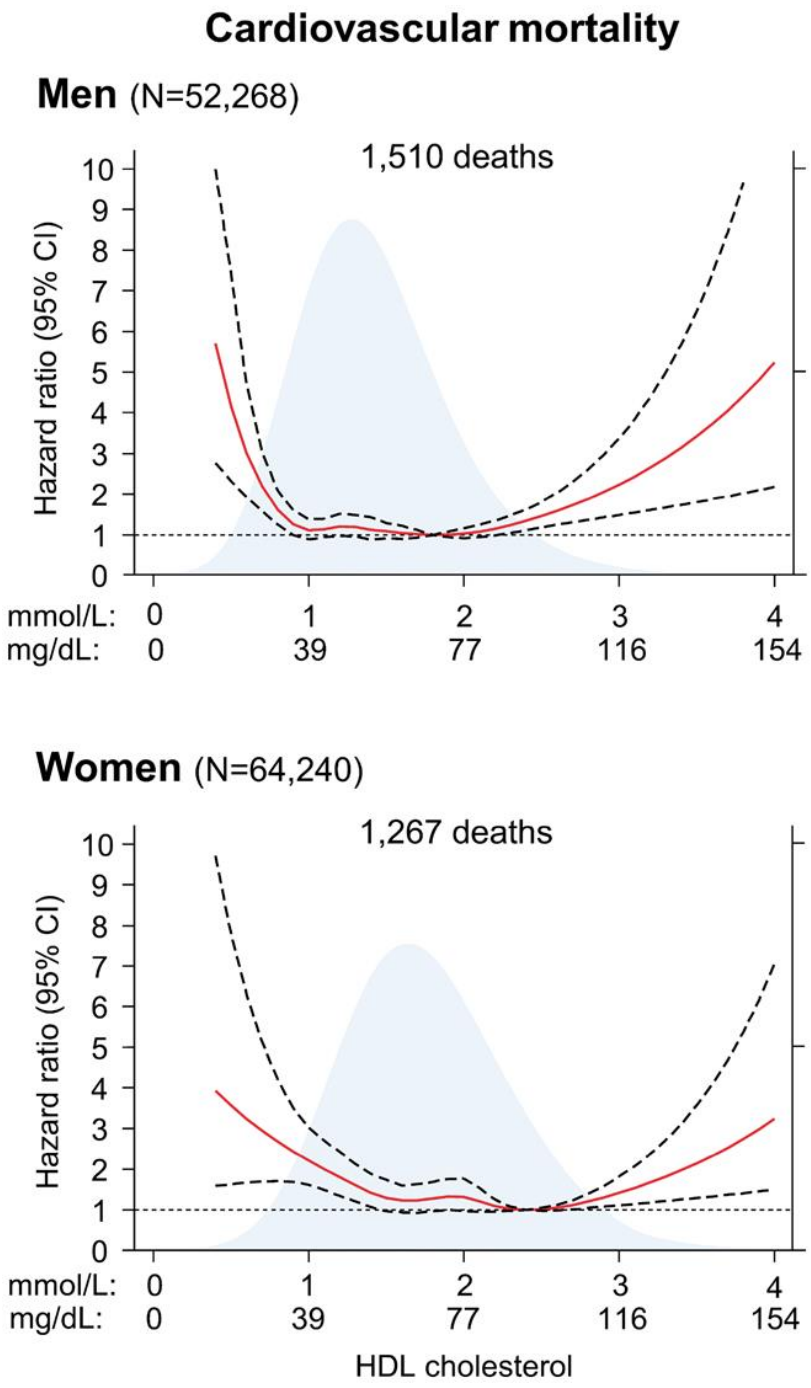

Figure 1. HDL cholesterol on a continuous scale and risk of cardiovascular, cancer, and other mortality in the general population. Solid line represents hazard ratio and dashed lines represent 95% confidence interval. Adapted from (45).

In summary, although in epidemiological studies the inverse relation between HDL-C and cardiovascular risk is clear and strong, RCT and human genetics are not supporting the observation.

In the last years it has become more evident that the HDL hypothesis needed a recasting to a different concept that is the HDL function hypothesis (46): it is not HDL-C itself that has a causal relation with atheroprotection, but rather HDL function.

3. HDL and endothelial protection

Human HDLs are an heterogeneous class of lipoproteins. Each subclass express a different activity and the variety of effect displayed by HDL reflects the numerous amount of protein surrounding the particles, that can act synergistically (47, 48). Most of them contain apoA-I as the mayor component, but several other proteins are associated with HDL and confer physiological functions. Among these, apoA-II, apoCs, apoE, LCAT, PON, PAF-AH are the most important. Moreover, lipids and proteins composition contributes in defining different properties.

HDL has been shown to have various properties that can contribute to confer cardiovascular protection [44], dependent on their protective effect on vascular endothelium, which dysfunction represents a key early step of atherosclerosis development and is implicated in plaque progression and destabilization (Figure 2).

Endothelial dysfunction has been reported in primary hypoalphalipoproteinemia (49, 50). The effect of HDL on vascular endothelium translated *in vivo* into HDL-mediated endothelial protection and may be relevant in the development and prevention of vascular disease.

To this regards, maintenance of vascular endothelium homeostasis is crucial and HDL can contribute positively affecting several endothelial functions in the regulation of vascular tone, inflammation and hemostasis.

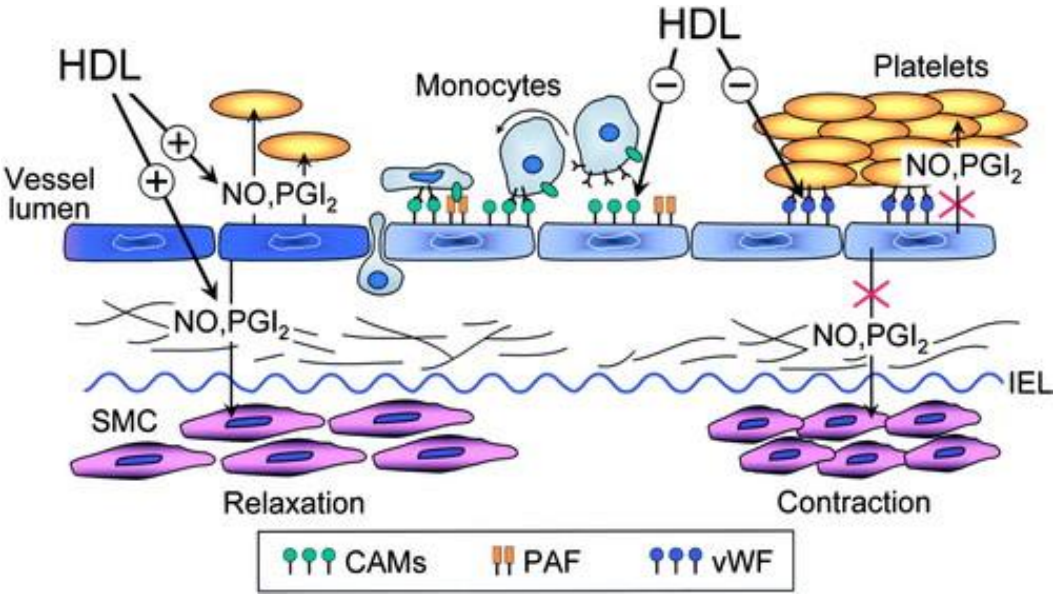

Figure 2. Multiple biological actions of HDL on vascular endothelium (51).

Cholesterol efflux

The best known HDL property resides in its ability to promote cholesterol efflux from peripheral cells, in particular from macrophages, and the related physiological process of reverse cholesterol transport (52). Lipid-poor apoA-I is an efficient acceptor of cholesterol and phospholipids via the ABCA1 transporter, that promote a fast, saturable and unidirectional efflux. This efflux permits the composition of small discoidal preβ-HDL, which can remove further cholesterol by the same pathway (53). Mature HDL can efflux cholesterol by ATP-binding cassette transporter G1 (ABCG1) and scavenger receptor class B type 1 (SR-BI) (54), mediating cholesterol efflux to large spherical HDL_2 and HDL_3 after their growth permit by the action ABCA1 and LCAT on small preβ-HDL. ABCG1 deletion in macrophages does not aggravate atherosclerosis demonstrating that ABCG1-mediated pathway of cholesterol efflux from macrophages appears to be quantitatively less important compared with that of ABCA1. But ABCA1 and ABCG1 may act synergistically in enhancing cellular cholesterol efflux (55, 56).

SR-BI mediates a slow, unsaturable and bi-directional efflux, that occur by passive diffusion. It acts on large, mature phospholipid-rich-HDL. The phospholipid content represent the principal characteristic that allow SR-BI binding. It acts primarily in hepatocytes but also in macrophages, adipocytes

and other cell types (57). Cholesterol transferred to HDL is then esterified under the action of LCAT and move to the lipid core of HDL helping to maintain a free-cholesterol gradient from cells to HDL. Recent data suggest that RCT can occur in the absence of functional LCAT (58, 59). The major of cholesteryl esters are transferred to the apoB containing particles thanks to CETP and metabolized into the liver. Alternatively HDL are taken-up by the liver through SR-BI, or SR-BI can also take-up unesterified cholesterol into the liver.

Cholesterol efflux power differs between HDL subpopulations. It depends on the transporter or receptor that HDL can activate: small discoidal preβ-HDL activate ABCA1, large spherical HDL activate ABCG1 and SR-BI. It depends on size and density: smaller particles are more potently inducers of cholesterol efflux. Finally it depends on apolipoprotein composition: LpA-I are more active than LpA-I:A-II. Modification of apoA-I by myeloperoxidase affects its ability to promote cholesterol efflux and apoA-I modified by myeloperoxidase is abundant in human atheroma (60). The correlation between HDL-C and efflux capacity is weak, thus suggesting that HDL-C does not predict the ability of HDL to promote reverse cholesterol transport.

Then, it has been suggested that cholesterol efflux capacity is more precise and predictive marker of atherosclerosis progression, compared to HDL-C (52).

Antioxidant effects

Oxidative stress in the arterial wall modified LDL to oxidized-LDL that are associated with the development of atherosclerotic plaque. The atherogenic property of oxidized-LDL is to accumulate in arterial wall cells, principally in macrophages that after phagocytising oxidized-LDL turn to foam cell, main representatives of the plaque.

Removal of oxidized lipids from LDL or cells represents the first step of HDL-mediated protection from oxidative damage induced by free radicals: phospholipid hydroperoxides are rapidly transferred from oxidized LDL to HDL; the transfer can occur directly or can be mediated by transfer protein CETP and then are removed from HDL by hepatocytes SR-BI uptaking. The HDL capacity to remove oxidized phospholipids depends on the surface lipid

monolayer fluidity; also this capacity is not specific for LDL but was detected also for erythrocytes and astrocytes (61, 62). The accumulation of oxidized lipids in HDL likely results not only from their transfer from LDL but also from triglyceride-rich remnant particles and endothelial cells (63).

HDLs are major carriers of plasma lipid hydroperoxides in animal model of atherosclerosis (64) and in humans (65). HDLs are also carriers of enzymes involved in lipid hydroperoxides destruction (66). The most important is paraoxonase-1 (PON-1) (67). A number of medical conditions including diabetes mellitus, chronic kidney disease, familial hypercholesterolaemia and autoimmune disease are associated with both decreased serum PON1 activity and increased CVD risk (68). ApoA-I contributes to the antioxidant capacity. Its structure in fact creates a perfect site for redox reaction, in particularly are involved Met112, Met 148 and Tyr115 residues (69).

Among other enzymes transported by HDLs, platelet-activating factor acetyl hydrolase (70) and LCAT (71) demonstrated anti-inflammatory capacity (71).

Vascular tone

Endothelial cells produce and release several vasoactive molecules that constrict and release the vessel. Among these molecules, nitric oxide (NO) is generated by endothelial NO synthase (eNOS), which transcription can be reinforce by endothelial insults and biochemical species (e.g. oxidized LDL). activates guanylate cyclase in vascular smooth muscles cells and induce relaxation. Decrease in NO bioavailability is a key feature in endothelial dysfunction. *In vivo* studies by coronary angiography (72) and intravascular ultrasound analysis (73) showed a strong correlation between plasma HDL and NO-dependent coronary vasodilation. Moreover, plasma HDL-C concentration has been shown to be an independent predictor of NO-dependent peripheral vasodilation in healthy subjects (74), as well as in hyperlipidemic and diabetic patients (75, 76). Strongly supporting these observations, intravenous infusion of sHDL in hypercholesterolemic subjects restores the endothelial dysfunction by increasing NO bioavailability (77). *In vitro* studies have shown that incubation of endothelial cells with HDL activates eNOS and this is mediated by the interaction of apoA-I with SR-BI (78). Subsequent intracellular events culminating in the activation of NO

production are mediated by PI3K/Akt signalling pathway and involve intracellular calcium mobilization, an increase in intracellular ceramide levels and eNOS phosphorylation (78, 79). ApoA-I is necessary, but not sufficient for eNOS stimulation, since no activation is observed with lipid free apoA-I.

Another vasoactive molecule that acts synergistically with NO is prostacyclin (PGI_2). It is generated from arachidonate derived from phospholipids of cell membrane or from exogenous substrate, by action of cycloxygenase (Cox). Oxidative stress and other factors can reduce the production of PGI_2 causing endothelial dysfunction and progression of atherosclerosis. Incubation of endothelial cells with HDL causes a dose-dependent increase of PGI_2 (80, 81). HDL cholesteryl esters are the most efficient substrate for PGI_2 production, HDL may act, as for NO release, by interacting with SR-BI (82). HDL can also activate a calcium-sensitive membrane bound phospholipase, which make endogenous arachidonate available for PGI_2 synthesis (83). Finally HDL increase cytokine induced expression of Cox-2 enzyme that occurs through an NF-kB independent pathway.

Anti-inflammatory effect

Atherosclerosis is an inflammatory disease and inflammation participates in all stages of atherosclerosis. Normal endothelium does not support leukocyte recruitment, however injury to vascular endothelium induces the expression of selective cell adhesion molecules (CAMs), that recall white blood cells to the atherosclerotic lesion (84).

Vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1, E-selectin and P-selectin are the main mediators attracting monocytes and other white blood cells to adhere to the endothelial surface and transmigrate into the intimal tissue. CAMs are produced in endothelial cells by different stimuli, including pro-inflammatory cytokines, as tumor necrosis factor-α (TNF- α). HDLs have shown ability in reducing leukocyte adhesion (85) through the inhibition of the expression of adhesion molecules, as demonstrated in studies in human umbilical vein endothelial cells (HUVEC) (86) with a dose dependent effect, that has its maximum at physiological HDL concentrations. Interestingly the HDL_3 subfraction is more effective than the larger HDL_2 (86), possibly because of a greater S1P

content (87). The inhibitory capacity is largely affected by apolipoprotein and phospholipid composition of HDL determining HDL particles shape: spherical HDL are more efficient than discoidal particles (86). Lipid composition is also important, in particular the length and degree of unsaturation of fatty acid residues (86). The anti-inflammatory pathway activated by HDL is not completely understood, but there are evidence of signalling perturbation at post receptor sites, probably inhibiting NF-κB translocation and DNA binding (88, 89). HDL interact with SR-BI through apoA-I and with S1P receptor through S1P, then activating an inhibitory PI3K/NOS pathway that eventually reduces translocation of NF- κB. Several studies in atherosclerosis-prone animal models proved that apoA-I overexpression significantly inhibits CAM expression on vascular endothelium and monocyte recruitment (90). The infusion of sHDL reduced VCAM-1 and E-Selectin in porcine model of acute inflammation (91). In human, low HDL concentrations are associated with increased plasma levels of soluble CAMs (49).

Antithrombotic activity

Platelets are involved in the progression of atherosclerosis because they can modify the proprieties of endothelial wall cells secreting growth factors and chemokines, and recruiting inflammatory cells (92). Platelets represent the key element for the formation of a thrombus formed following the rupture of the atherosclerotic plaque. Endothelium-derived NO and PGI_2 have a antithrombotic effect, inhibiting platelet aggregation, increasing cellular content of cGMP and cAMP, respectively. Thus, HDL stimulation of these two pathway reduces coagulatory ability. In addition, HDLs are effective in limiting, in a dose dependent manner, agonist-induced production of platelet-activating factor (PAF), a molecule that stimulate platelet aggregation and smooth muscle contraction. Moreover, circulating levels of Von Willebrand factor (vWF), expressed by endothelial cells and essential for platelet adhesion and aggregation is inversely correlated with plasma HDL (93). Interestingly, high HDL-C level in humans is associated with a reduced ex-vivo thrombogenic potential (94) and injection of sHDL in animal model of thrombosis reduces thrombus formation (95).

HDL decreases platelet aggregation mediated by glycoprotein IIb/IIIa in response to thrombin, collagen, ADP and adrenaline (92, 96, 97).
It has been recently demonstrated that plasma-derived, as well as synthetic HDL are able to inhibit tissue factor (TF) expression in endothelial cells and monocytes. Only complete HDL are effective, while lipid-free apoA-I does not retain this property, since the TF inhibition is dependent on modulation of cellular cholesterol content through the interaction with SR-BI. This interaction inhibits the activation of p38 MAPK and the repression of the PI3K pathway responsible for TF expression (98).

4. Lecithin:cholesterol acyltransferase

HDL inherited disorders also represent a unique tool to evaluate how specific HDL subpopulations are involved in the atheroprotective activity mediated by HDL. Genetic LCAT deficiency permits to evaluate a specific HDL subpopulation and to assess the relevance of LCAT enzyme in atherosclerosis. Carriers of two mutant *LCAT* alleles have severely low plasma HDL-C, apoA-I, and apoA-II levels, associated with alterations in HDL structure and particle distribution, with a selective depletion of large particles and predominance of small preβ-HDL (18, 99)

4.1. LCAT: from the gene to the enzyme

Human LCAT gene was firstly cloned by McLean in 1986: it was made by 6 exons and it is ~ 4.4 kB long (100). *LCAT* is located as a single gene on locus 22 of the long arm of chromosome 16 (16q22) (101) and encodes for a 416-aminoacid glycoprotein preceded by an hydrophobic sequence of 24 aminoacids, with a molecular mass of ~ 4.9 kDa (101). The gene is expressed mainly in the liver and to a lesser extent in brain and testis (102). Very low concentrations are in the cerebrospinal fluid, although its role in this compartment is not yet clear (103). The synthetized protein is secreted in the plasma and circulates in a glycosylated form (N-linked and O-linked) for a total molecular mass of approximately 67kDa (103). The LCAT protein structure is not completely known, although a partial model has been proposed, based on its homology with other lipases (104). This model

assigned LCAT to the α/β hydrolase fold family, with a central domain consisting of seven β-strands, linked by four α-helices and separated by loops. The catalytic triad includes Ser-181, Asp-345 and His-377 (Figure 3), together with Phe-103 and Leu-377 that form the oxyanion hole (104). The disulfide bridge between Cys50 and Cys74 was proposed as “lid” domain, typical of lipases, and it is involved in the substrate recognition and it is essential for the binding to lipoprotein surface (103, 105).

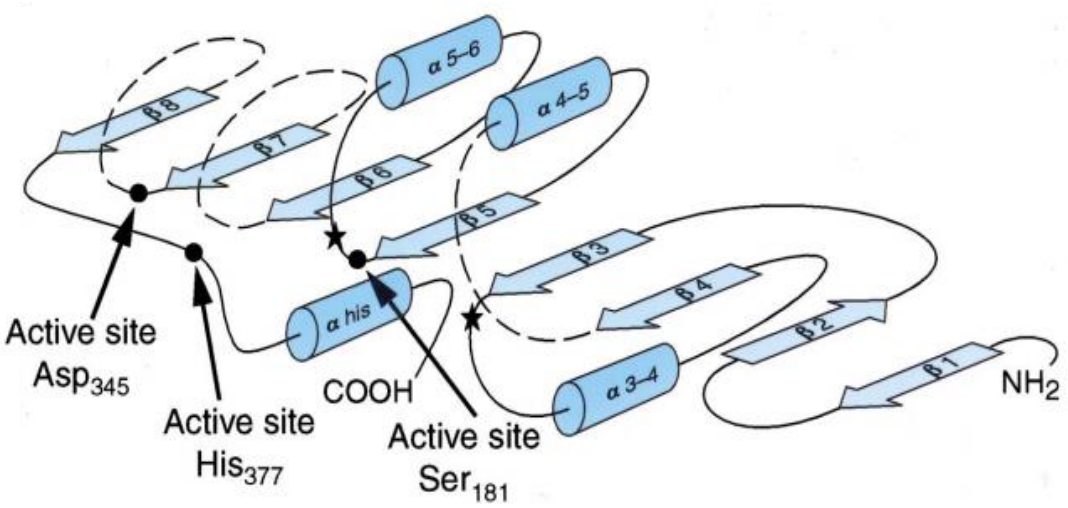

Figure 3. Structure of lecithin:cholesterol acyltransferase

Average plasma concentration of LCAT is 5 mg/L and is subjected to little variation with age, sex, lifestyle and smoking. LCAT mass correlates with enzyme activity either when measured on endogenous substrate (cholesterol esterification rate) either on standard exogenous substrate (LCAT activity) (106).

4.2. LCAT reaction and substrates

LCAT was demonstrated to own three different catalytic reaction: acyltransferase activity, phospholipase A2 and lysolecithin acil transferase (LAT) activity.

The most important physiologic reaction of LCAT is the conversion of cholesterol and phosphatidylcholine (PC) (lecithin) to cholesteryl ester and lysophosphatidylcholine (lysoPC). As depicted in

Figure **4**, it is a multistep reaction starting with the cleavage of the fatty acid in *sn-2* position of lecithin and the transfer to Ser181; then the fatty acid is transferred to the free 3-β hydroxyl group of cholesterol (transesterification), generating cholesteryl ester (CE) (103). LCAT is the unique enzyme in plasma able to esterify cholesterol.

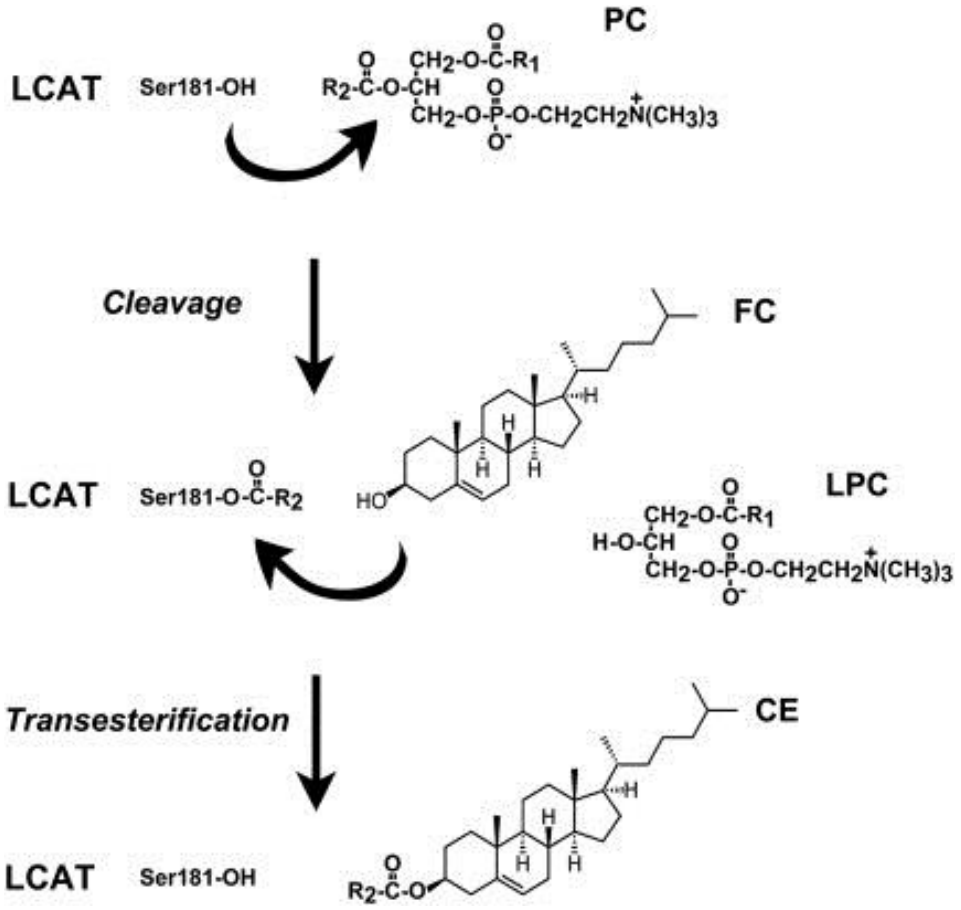

Figure 4. LCAT reaction. Firstly LCAT acts as a phospholipase A2, cleaving the sn-2 fatty acid of the phoshatidylcholine (PC) with formation of lysophosphatidilcholine (LPC). Then, the fatty acid, bound to Ser181 of LCAT is transferred to the free hydroxyl group

The preferential substrate of LCAT are small, discoidal and pre-β-migrating HDL (α-LCAT activity) (107), although it binds with lesser affinity also apoB-containing lipoproteins (β-LCAT activity) (108). Measurement of affinity of human LCAT for different substrate showed that it was 2.3 to 4-fold higher for HDL compared to LDL, with relative reactivity (appV_{max}/app Km) of 6.5%, 1.3% and 16% for LDL, HDL_2 and HDL_3 compared to rHDL (109). Although LCAT can directly esterify the free cholesterol on apoB-containing lipoproteins, these latter mainly acquire CE by CETP, which exchanges CE (made by LCAT) for TG between HDL and LDL. Either way, LCAT is the - main source of CE on apoB-containing lipoproteins. Other CE are produced by one intracellular enzyme Acyl-CoA:Cholesterol Acyltransferase (ACAT) in the liver and intestine when chylomicrons and VLDL are secreted (110).

Besides PC, LCAT can use other phospholipids containing 18:1 or 18:2 fatty acids (111), such as phosphatidylethanolamine (PE), but it has no activity on other phospholipids or acyl donors, such as phosphatidylserine or diacylglycerol (112). The initial binding of LCAT occurs to the lipid surface, followed by a specific activation by apoA-I. Other apolipoproteins (apoE, apoA-IV and apoC-1) can activate LCAT, but less effectively than apoA-I (113). Among these, apoE represents the cofactor for LCAT activity on apoB-containing lipoprotein (114). The effect of aminoacid substitution on apoA-I demonstrate that three arginine in 149, 153 and 160 are crucial for LCAT binding (115). LCAT activation by apolipoproteins and peptides requires lipid binding and amphipathic α-helical structures with nonpolar face of approximately 100°, a central region of acidic residues opposite to the nonpolar face and basic residues distributed at the polar-nonpolar interface. Alteration of residues from 143 to 187 makes apoA-I unable to activate the enzyme (103). ApoA-II is not able to activate LCAT and may impair the activation by apoA-I likely hiding active its sites. The presence of LCAT enhance fusion of lipidated apoA-I-only lipoproteins (LpA-I) and apoA-II-only particles (LpA-II) (116). In addition the two apolipoproteins may compete for binding sites on the PC-cholesterol vesicles (117). Studies on mice have shown that cholesterol transferring between preβ-HDL and α-HDL is slower in human apoA-I/apoA-II compared to apoA-I transgenic mice and is correlating with a more efficient cholesterol efflux in the latter (118).

The LAT activity of LCAT allows the reverse reaction of LCAT, which is the reacylation of lysophosphatidylcholine into phosphatidylcholine with LDL as a substrate (119).

In addition LCAT can also transesterify and hydrolyze PAF and oxidized PCs with shortened chains in the sn-2 position; through these reactions, LCAT contributes to the metabolism of oxidized phospholipids in plasma, especially those generated during LDL oxidation (120, 121).

Interestingly, LCAT is also produced in the brain by astrocytes and it is responsible of the esterification of FC on the apoA-containing lipoproteins derived from glia. This regulates the cerebral spinal fluid (CSF) concentration of apoE and apoA-I and affects the maturation of nascent lipoproteins derived from the glia (122).

Several reports have demonstrated that sphingomyelin is an inhibitor of LCAT activity. Other inhibitors include: hydroperoxides of PC, generated during oxidation of lipoproteins, PC with trans-fatty acids and chains of C18, likely changing the physical properties of the lipid e.i. altering apoA-I conformation or charge or competing with PC (103, 123, 124). Mild oxidation leads to a reduction in plasma PC and increase in short-chain polar PC and inhibition of the transfer of long-chain acyl groups to cholesterol (LCAT activity) or to lysoPC (LAT activity), suggesting a possible role of LCAT in metabolize oxidized PC (125).

4.3. LCAT is involved in HDL metabolism and reverse cholesterol transport

The interaction between lipid-free or lipid-poor apoA-I and the ABCA1 transporter on the cell membrane originates a small, discoidal pre-β-HDL, the preferred substrate for LCAT, which represents the first step for the efflux of cholesterol efflux and phospholipids (107).

LCAT esterify the FC. CE is more hydrophobic thus it migrates into the hydrophobic core of the lipoproteins, leading to the maturation of the HDL which becomes spherical and α-migrating (126). The action of CETP and various lipases leads to loss of CE from the HDL in exchange of TG. In this way α-HDL is converted back into a pre-β-HDL. While pre-β-HDL have a

short plasma half-life, since apoA-I is rapidly removed by the kidney, α-HDL have a slower turn over (126).

Consequently LCAT is involved in HDL metabolism, thus regulating their plasma levels.

LCAT has also long been believed to regulate macrophage reverse cholesterol transport (RCT) as proposed by Glomset et al LCAT contributes to RCT by maintain cholesterol gradient between the cell membrane and the extracellular compartment, promoting passive diffusion of cholesterol (127) or via ABCG1 transporter (128). Esterification of cholesterol is also required for cholesterol uptake and elimination from the hepatocytes, either through SR-BI or indirectly through CETP.

Nevertheless the role of LCAT in RCT is controversial, since there are evidences that the conversion of pre-β-HDL into α-HDL depletes the best acceptor for cholesterol efflux via ABCA1 transporter, the most important actor in cholesterol removal from macrophages (129). Supporting this observation, LCAT overexpressing mice show increased HDL-C, but do not show increased macrophage RCT (129). Conversely in LCAT-deficient mice, RCT occurs despite the lack of LCAT, mostly due to higher levels of pre-β-HDL and enhanced cholesterol removal by ABCA1 (58).

4.4. Genetic LCAT deficiency

In 1967 Norum and Gjone described an error in lipid metabolism in a Norwegian 33-year-old woman with corneal opacities, proteinuria and normochromic anemia (130). Although the renal function was normal, kidney biopsy revealed foam cells in the glomerular tufts. Total serum cholesterol was high and almost all was unesterified, accompanied by decrease serum lysolecithin and complete absence of HDL on agarose gel electrophoresis. Similar phenotype was reported in the proband's two sisters (131). Lack of LCAT was hypothesized and subsequently confirmed by measurement of LCAT activity in plasma (131).

Carriers of LCAT gene mutations showed a wide variety of phenotypic presentation. Most homozygous carriers develop corneal opacities and hemolytic anemia; chronic and progressing renal insufficiency, often resulting

in end-stage renal disease. Heterozygous subjects show a mild phenotype, supporting the evidence that LCAT deficiency is an autosomal recessive disorder. Loss-of-function mutations in both alleles, depending on the type, can lead to two distinct syndromes: familial LCAT deficiency (FLD) and fish eye disease (FED). A summary of the clinical and biochemical characteristics of these disorders is listed in Table 1 (132). Both are characterized by low HDL-c in plasma, but other clinical and biochemical features can be widely different (132). While in FLD the lack of LCAT impair the ability of the enzyme to esterify cholesterol on both HDL and LDL, in FED the enzymatic activity on LDL and VLDL is preserved. The direct consequence is that FLD have a very little amount of CE with high FC in all lipoproteins fractions, while FED have a subnormal CE/FC with concentration of CE in apoB-containing lipoproteins, that are present in normal plasmatic levels (133).

Among other characteristics, corneal opacity is common in both FLD and FED, whereas anaemia, proteinuria and renal disease are clinical features of FLD (35).

The differential diagnosis of FLD and FED in homozygous or compound heterozygous can only be made by the measurement of the ability of LCAT to esterify the FC on endogenous lipoproteins (α-LCAT plus β-LCAT activity) and on synthetic HDL (α-LCAT activity) (99). The classification of heterozygous subjects can be made by transient expression of LCAT mutants in cultured cells and measurement of LCAT concentration and activities in cell media (134). The prevalence of the disease is below 1:1,000,000 and more than 80 mutations in LCAT gene have been identified (132).

Table 1. Clinical and biochemical features of FLD and FED. Adapted from (132)

	FLD	FED
Clinical findings		
Corneal opacity	++	+
Anemia	+	-
Nephropathy	+	-
Routine laboratory		
Triglycerides	↑	→↑
Total cholesterol	↓	→
LDL-cholesterol	↓	→
HDL-cholesterol	↓↓	↓↓
VLDL-cholesterol	↓	→
Hemoglobin	↓	→
Proteinuria	+ (in most cases)	-
Special laboratory		
Unesterified cholesterol	↑	→
LCAT activity	Absent	↓
LCAT mass	↓	→↓
Renal biopsy	Abnormal (in most cases)	→
Cholesterol esterification rate	↓↓	→
Apolipoprotein A-I	↓↓	↓↓
Apolipoprotein A-II	↓	↓
Apolipoprotein E	↑	↑

Lipoprotein profile

Lack of LCAT leads to serum lipoproteins abnormalities, mainly characterized by very low levels of HDL-C (< 10 md/dL, < 0.26 mmol/L) and reduced apoA-I and apoA-II (< 30 mg/dL). Increased percentage of FC is one of the main features suggestive of genetic LCAT deficiency. The analysis of HDL subclasses in subjects with LCAT deficiency, by bidimensional electrophoresis, that separate HDL for surface charge and size, reveals a dramatic redistribution of apoA-I-containing-HDL. Homozygous carriers have small, lipid-poor, disc-shaped HDL particles (namely preβ-1 and α-4). Triglycerides levels are variable from normal to elevated. FLD subjects show a markedly reduced esterified cholesterol in both LDL and HDL fraction, while FED have a FC/TC ratio of about 50% compared to control, due to the retain of β-LCAT activity.

Differences between heterozygous carriers and controls are mild, since distribution of HDL subpopulations were comparable for size and electrophoretic mobility, with a shift toward the smaller HDL particles. Preβ were 2-fold increased. In addition heterozygous subjects show a 23% increase in α-4 and a 45% increase in preα-4 levels compared to non-carriers, whereas the intermediate-sized particle α-3 was similar to that of controls (18).

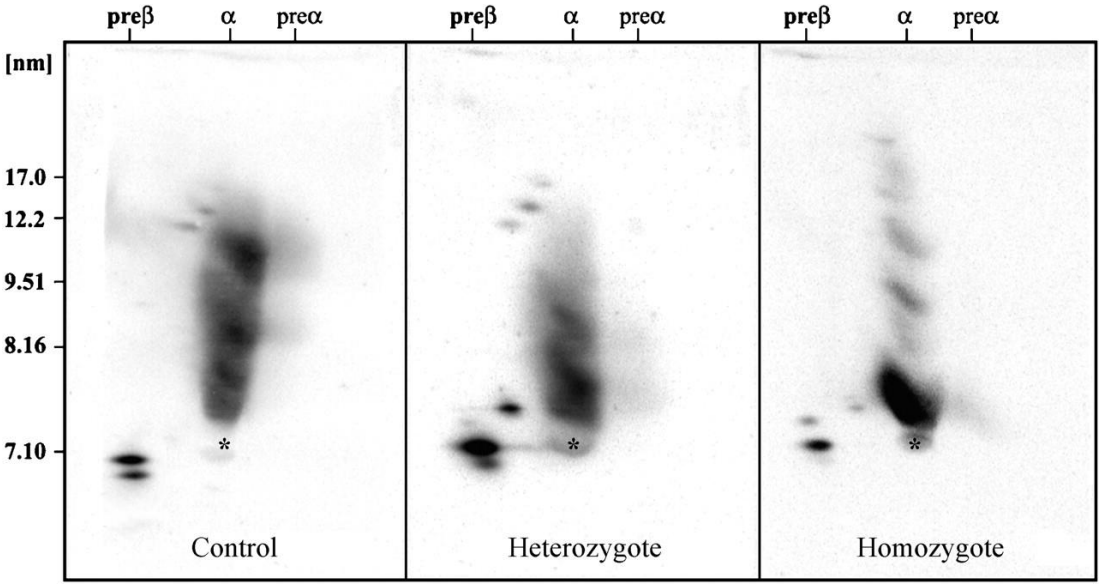

Figure 5. Apolipoprotein A-I (apoA-I)-containing HDL subpopulations of representative control, heterozygous, and homozygous LCAT-deficient subjects separated by two-dimensional, nondenaturing agarose-PAGE (18).

Corneal opacity

The most peculiar characteristics in FLD and FED patients is the presence of corneal opacity that is defined “fish-eye” (Figure 6). It is a gradual process, usually appearing in childhood and worsening over years (135). Despite the cloudy appearance of the cornea usually patients do not have impaired vision, but sometimes they may need corneal transplantation. Analyses of LCAT deficient subjects after transplantation revealed that opacity is due to FC and PL accumulation in form of 5-200 nm vacuoles, in particular in the corneal stroma, with more evident opacity near the limbus, often arranged in diffuse, grayish, circular bands (132, 133, 136, 137).

Linoleic acid (18:2) in predominant in esterify cholesterol fraction in the cornea, with a ratio C18:1/18:2 of 1:6.5 (normal ratio in the cornea is 1.4-4.6:1) (137).

Other HDL disorders are often associated with morphological and lipid composition of the cornea, such as Tangier disease (138), apoA-I deficiency (139) and apoA-I/C-III deficiency (4).

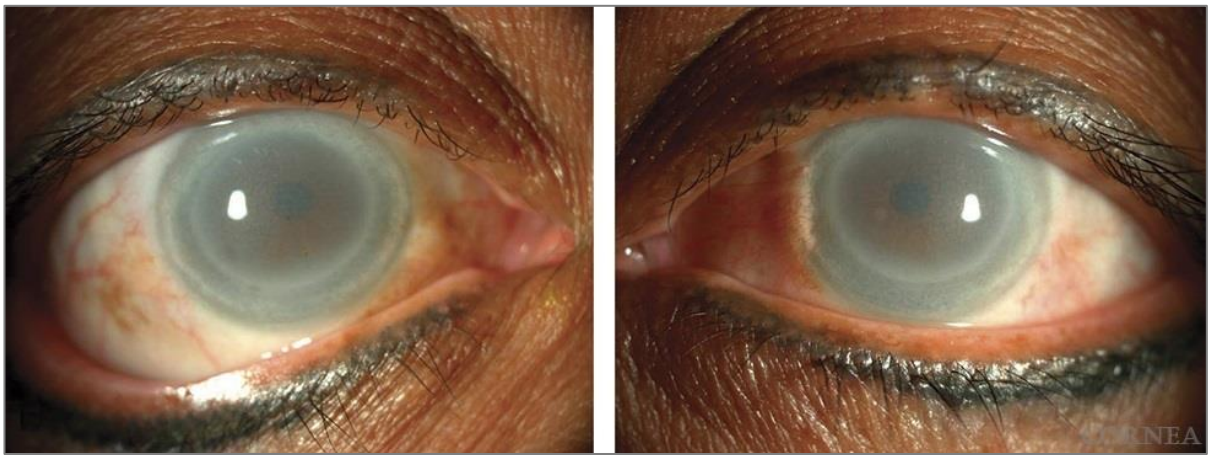

Figure 6. Slit-lamp photo of patient, showing diffuse bilateral opacity of the corneal stroma extending to the limbus, with a peripheral lipoid arc OU (140)

Anemia

Most FLD patients show mild normochromic anemia associated with reticulocytosis. Although anemia has not been described extensively, hematological exams showed that red blood cells are characterized by increased mean corpuscular volume, heterogeneous shape and peripheral

blood smear about 20% of cells are so-called Target cells and/or Knizocytes (erythrocyte with aberrant shape characterized by two concavities crossed by a thin strip of hemoglobin) (141).

Analysis of membrane lipids showed changes in composition, with increased free cholesterol and phosphatidylcholine with decreased phosphatidylethanolamine. Thus altering membrane properties and stability, such as fluidity and deformability, which are increased and decreases respectively (141). Radioisotypic studies highlighted reduced erythrocytes mean half-life (16-17 days vs 23-35 days of normal subjects (142).

Renal disease

Glomerulosclerosis represents the most severe consequence of LCAT deficiency and is the main cause of morbidity and mortality. Homozygosity seems to be a necessary condition for the development of the disease, while heterozygous subjects are not affected. Proteinuria is often diagnosed in the second or third decade and worsens in the fourth-fifth decade (133, 143) although the progression of renal disease is unpredictable, with subjects reaching renal failure in their early 20s.

Glomerulosclerosis is both focal and segmental that becomes global with the progression of the disease (144).

Renal function, measured by serum creatinine or creatinine clearance usually remain normal, with a sudden deterioration of renal function with progression of renal insufficiency and increase of proteinuria. FLD patients shows varying phenotype, depending on diet, abnormal lipoproteins and previous kidney disease (144, 145).

Renal biopsies reveal focal segmental glomerular sclerosis with mesangial expansion, mild increase in mesangial cellularity and thickening of the glomerular capillary walls (145). Lipid staining by Oil Red O shows lipid deposition with vacuolization of the glomerular basement membrane and foamy appearance. Increased free cholesterol and phospholipid are found in the glomerula (145).

The pathogenesis of renal disease is not completely understood. In some FLD patients oxidized phospholipids accumulation in the glomerula has been described (146).

It has been proposed that presence of an abnormal lipoprotein, called lipoprotein X (LpX) and/or possibly large molecular weight LDL, which accumulates in plasma, are involved in glomerulosclerosis development (147). The LpX is associated to the fraction of LDL (density 1.019-1.063 g/mL), that resembles a bilayer vesicle with a diameter of 30-70 nm. It contains a high content of free cholesterol (30%) and phospholipids (60%), mainly phosphatidylcholine. The core components are cholesteryl esters (2%) and triglycerides (2%). The major proteins are albumin, apoC-I, C-II, C-III and E that are on the surface. LpX is characterized by an opposite electrophoretic migration on agarose gel, since it migrates to the cathode and not to the anode as the other lipoproteins (148). The residual LCAT activity in FED presumably prevent the formation of a significant amount of LpX. These particles remain trapped in capillary loops of the glomerulus and induce endothelial and vascular injury (147). However, LpX is not always detected in FLD patients with renal disease (143).

4.5. Therapies for LCAT deficiency

Specific treatment for genetic LCAT deficiency is not currently available, probably due to the rareness of the disease. LCAT-deficient carriers are candidates for cornea and kidney transplantation, but the disease can reoccur in transplanted tissue (149). Therapy is mainly aimed at correcting the dyslipidemia and at delaying the evolution of chronic nephropathy, with changes in lifestyle an diet, anti-hypertensive treatment to avoid proteinuria (145). Combined treatment with nicotinic acid and fenofibrate have demonstrated amelioration of lipid profile, a reduction of LpX and proteinuria in one FLD carrier (150). Therapy with angiotensin II receptor blockers and lipid-lowering drugs showed in a case report study benefit in blood pressure, lipid abnormalities, proteinuria and also kidney function, probably delaying progression to renal failure (151).

Successful studies with infusion of normal plasma to correct the biochemical LCAT deficient phenotype (152) pave the way for the development of enzyme replacement therapy as a therapeutic strategy. Since LCAT has a relatively long half-life and does not require any specific tissue delivery,

enzyme replacement results feasible. *In vitro* studies with recombinant human LCAT proved efficacy in correct the abnormal lipoproteins observed in FLD plasma (153) and in promoting the maturation of immature HDL (154). Experiment in mouse model of LCAT deficiency and the first-in-human treatment provided encouraging results (153, 155) (156).

4.6. LCAT deficiency and atherosclerosis

Without LCAT, HDL-C, apoA-I and apoA-II levels are dramatically reduced, by lacking of mature HDL and the rapid catabolism of discoidal HDL by the kidney (157). Thus, theoretically, cases with LCAT deficiency should be at increased risk of cardiovascular disease because of HDL deficiency. More than one study supported the evidence that lack of LCAT is not associated with increased cardiovascular risk. In one study, two Canadian homozygotes and seven heterozygotes with FLD have been followed for 25 years and no vascular events were recorded in any carriers aged up to 70 years (158). Supporting observations came from another study on a larger number of carriers from 13 Italian families, that clearly showed that functional LCAT is not required for efficient atheroprotection (25). In this study carriers of LCAT deficiency had a 0.07 mm smaller average carotid intima media thickness (IMT) and 0.21 mm smaller maximum carotid IMT than controls with a gene-dose dependent effect (25) (Figure 7).

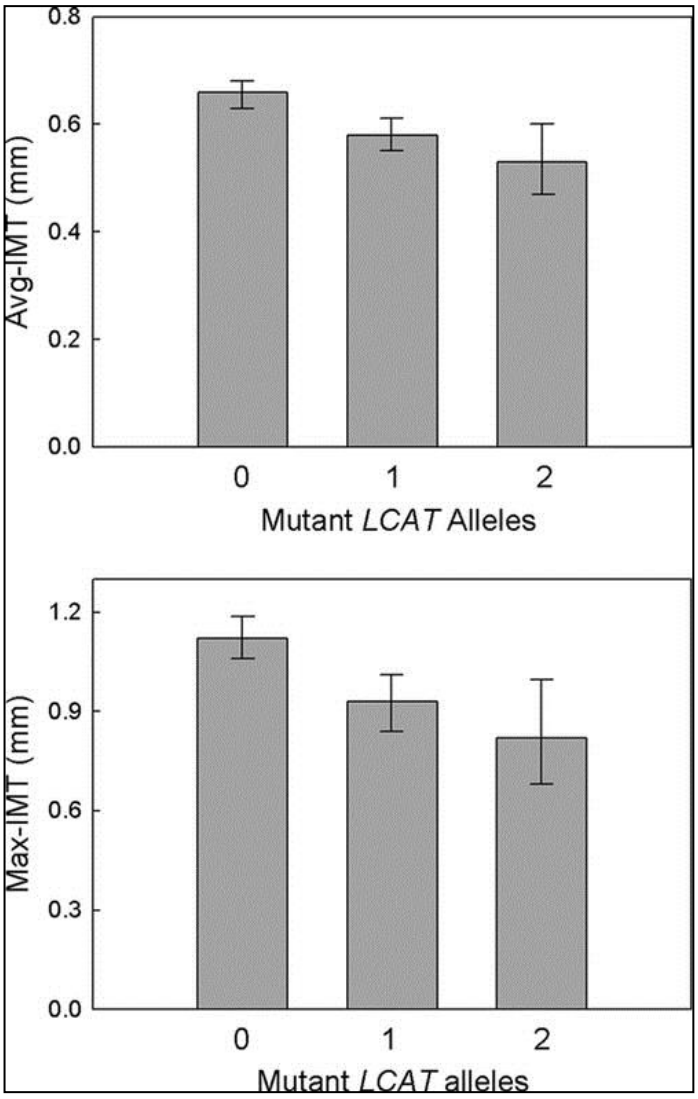

Figure 7. Average IMT (Avg-IMT) and maximum IMT (Max-IMT) values in carriers of 1 (n=28) or 2 (n=12) mutant LCAT alleles and in control subjects (n=80). IMT measurements were adjusted for age, sex, BMI, smoking status, hypertension, family history of cardiovascular disease, ultrasound device, and total cholesterol, HDL-C, and triglyceride levels. Data are expressed as geometric mean and 95% CIs. P=0.0003 for average IMT and P=0.001 for maximum IMT when tested for trend (25).

Contrasting results came from other groups, showing increased preclinical atherosclerosis in carriers compared to control, although only 2 out of 47 heterozygous carriers of the study had a premature cardiovascular event (159). Supporting the evidence that low LCAT levels are associated with increased carotid IMT, carotid magnetic resonance imaging studies were conducted in two homozygous and 38 heterozygous carriers. Results clearly

showed that LCAT deficient carriers had a greater increase in the thickness of carotid artery walls and 32% increase of plaque component compared with family controls (160). In the same population, arterial stiffness, an independent predictor of cardiovascular events, was also significantly increased (161).

Studies in animal models led to contrasting results, often dependent on the species utilized. LCAT overexpression (162-164) or downregulation (165, 166) in mice failed to demonstrated association with atherosclerosis. The increased atherosclerosis found in mice overexpressing LCAT was probably due to the accumulation in plasma of dysfunctional large apoE-rich HDL, that are defective in the delivery of cholesterol to the liver via SR-BI (164). Mice, which do not express CETP, are not protected against diet-induced atherosclerosis, while rabbits, which express CETP, show less atherosclerosis than control animals. Interestingly double transgenic mice for LCAT and CETP develop less atherosclerosis than LCAT-transgenic mice, although more than wild-type mice (167). Studies in rabbit gave opposite results (168, 169). Recent study in CRISPR/CAS9 mutant LCAT hamsters demonstrated that LCAT deficiency in hamsters develops pro-atherogenic dyslipidemia and promotes atherosclerotic lesion formation (170). Controversial results were also found in humans, summarized in Table 2. In general population studies, as the prospective Epic-Norfolk, the correlation between plasma LCAT and the development of coronary artery disease was investigated, however no association was found (171). The Copenhagen City Heart Study, together with The Copenhagen General Population Study, enrolled more than 60,000 subjects found an association of the *LCAT* variants S208T with reduction in HDL-C and apoA-I levels, but not with increased risk of major coronary events (172). Consistently, no association of low plasma LCAT levels with higher carotid intima media thickness in secondary prevention subjects (173). In a small study on 74 subjects LCAT activity was found to be directly associated with preclinical atherosclerosis in metabolic syndrome carriers, as well as in controls (174). Increased LCAT activity was associated with formation of small LDL particles, that are more atherogenic than large particles, but no data on subclinical atherosclerosis was analysed (175). Other studies demonstrated the opposite association in

high risk patients (176) as well a positive correlation of LCAT activity with the degree of coronary atherosclerosis (177). A very recent report on 267 patients with angiographically confirmed CAD showed that plasma LCAT mass concentration is upregulated and inversely related to plaque volume (178).

Table 2. Studies on LCAT and atherosclerosis. Adapted from (179).

Human studies	*LCAT mass/activity*	*CAD*	*Reference*
General population	=	Yes	(171)
	↓	No	(172)
Subjects at high cardiovascular risk	↓	Yes	(176, 180, 181)
	↑	Yes	(174, 175, 177, 182)
	↓	No	(173)

A possible explanation for the contrasting findings about LCAT and atherosclerosis have been recently proposed by a study of Our group. The development of preclinical atherosclerosis follows a different development in partial LCAT deficiency compare with complete LCAT deficiency, since in FLD the lack of LCAT affects the ability to esterify free cholesterol on both HDL and LDL, reducing the cholesteryl esters in plasma, while in FED, LCAT maintain the capacity to generate esters on apolipoprotein B-containing lipoproteins (183).

AIM

The lecithin:cholesterol acyltransferase (LCAT) is the unique enzyme in human able to esterify the free cholesterol in plasma and play an important role in high-density lipoprotein (HDL) metabolism. Genetic LCAT deficiency leads to abnormal plasma lipoprotein profile, characterized by low HDL-C, mainly small discoidal and immature HDL (preβ-HDL).
Despite the severely low HDL-C concentrations, LCAT deficiency is not unequivocally associated with increased atherosclerosis (179) and it has been often associated with atheroprotection (134). Trying to explain this controversy, we hypothesized that HDL in carriers of LCAT deficiency were more effective in atheroprotection. For this purpose we defined two aims:

I) evaluate the ability of HDL in LCAT deficient patients to maintain endothelial cell homeostasis

II) assess the phenotype and function of monocytes in LCAT deficiency

For both aims, I took advantage of the large availability of plasma samples from LCAT deficient patients of Prof. Calabresi's laboratory at the University of Milan and of Prof. Stroes' laboratory at the Academic Medical Center in Amsterdam.

For aim (I) HDLs were isolated from plasma of homozygous and heterozygous LCAT deficient patients and from control subjects, and tested on endothelial cells (HUVEC) to prove their ability to promote nitric oxide production and to inhibit adhesion molecules expression. The project was completed with additional analysis to demonstrate the structural characteristic in HDL responsible for the increased efficiency of these particles.

For aim (II) the project was conducted in Amsterdam, in Prof. Stroes' laboratory where monocytes were freshly isolated from a small number of patients selected among carriers of LCAT deficiency. Immunophenotype, cytokine production upon stimulation, migratory capacity of monocytes were assessed. In addition these monocytes were compared with monocytes isolated from carriers of other genetic hypoalphaliproteinemias (*ABCA1*, *APOA1* mutations).

In addition, measurement of flow-mediated vasodilation (FMD) in carriers was used as *in vivo* measurement of endothelial functionality.

MATERIALS AND METHODS

1. SUBJECTS

11 homozygous, 9 compound heterozygous and 67 heterozygous carriers of LCAT deficiency were recruited for the study, for a total of 87 subjects. 41 family members accepted to participate as control group. All subjects were recruited among those belonging to the Italian LCAT-deficient families (99, 167). 11 homozygotes and 49 heterozygotes are FLD, remaining are FED.

The monocyte studies were conducted at the Academic Medisch Centrum of the University of Amsterdam on a representative subgroup of carriers of *LCAT* mutations. 12 carriers of genetic LCAT deficiency were selected (7 heterozygotes and 5 homozygotes) and 9 control subjects matched for age, gender and BMI. For the comparison study, carriers of 10 *ABCA1* and 9 *APOA1* mutations were recruited. Exclusion criteria were history of cardiovascular diseases, chronic kidney disease, diabetes or any condition associated with acute inflammation. Each subject provided written informed consent. The study was approved by the local Institutional Review Board and conducted according to the principles of the International Conference of Harmonization-Good Clinical Practice guidelines.

Blood samples for the study on endothelial cells and for lipid profile measurement were collected after an overnight fast, centrifuged at 4°C. Aliquots were immediately frozen and stored at -80°C until assayed.

2. STUDIES ON ENDOTHELIAL CELLS

2.1. Plasma lipids and apolipoproteins

Plasma total cholesterol, HDL-C, triglycerides and apolipoproteins levels were measured by automated analyser Roche Diagnostics c311. LDL-C was calculated using Friedewald's formula. The plasma concentrations of HDL containing only apoA-I and particles containing both apoA-I and apoA-II were determined by electroimmunodiffusion in agarose gel in a slightly alkaline (pH 7.6) buffered solution (Sebia Italia).

2.2. HDL purification

HDLs (d = 1.063-1.21 g/mL) were purified by sequential ultracentrifugation from plasma of six homozygotes carrying different *LCAT* mutations (Thr274-Ile, Arg147-Trp, Lys218-Asn,

and Leu372-Arg), 10 heterozygotes carrying six different *LCAT* mutations (Thr274-Ile, Arg147-Trp, Lys218-Asn, delG stop 16, Val309-Met, and delG Thr13-Met), and 10 family controls. Since homozygotes have very low plasma HDL concentrations, plasma samples were pooled in two preparations of three subjects each before ultracentrifugation, to allow enough HDL for the *in vitro* experiments. KBr salt was used to modify density at 1.080 g/mL, then stratified with a KBr solution at the same density and ultracentrifuged overnight to obtain separation of HDL from other lipoproteins with smaller density. Then density was corrected with KBr salt to 1.21 g/mL and stratified with a same density KBr solution, then ultracentrifugated overnight again. Thus, HDL were separated from other greater density plasmatic protein and dialyzed against sterilized saline immediately before use. HDL protein concentration was determined by Lowry colorimetric assay.

2.3. HDL subclasses analysis

HDLs were separated according to their size by non denaturating gradient gel electrophoresis of the d < 1.21 g/mL using precast 4-30% polyacrylamide gels (184).
Preβ-HDL content was measured after separation by 2D electrophoresis followed by immunodetection against human apoA-I and expressed as percentage of total apoA-I (184)**.**

2.4. Sphingosine-1-phosphate content

Shpingosine-1-phosphate (S1P) content on HDLs was assessed by a commercial competitive ELISA assay (Echelon Biosciences) and expressed as ρmol/mg of HDL protein. The S1P coated 96-well plate was blocked to reduce non-specific binding. The S1P standard curve and samples were mixed with the anti-S1P antibody before adding to the plate. The anti-S1P antibody bound to the S1P coated plate or the S1P in the sample. Following incubation and plate washing, streptavidin-horseradish peroxidase (HRP) was added to the plate and bound to all anti-S1P antibodies (labelled with biotin) bound to the plate. After an additional incubation and plate washing, tetramethylbenzidine (TMB) substrate was added and the colorimetric reaction stopped by the addition of 1N sulfuric acid. The absorbance at 450 nm was measured and the concentration of S1P in the samples was determined by comparison to the standard curve.

2.5. Measurement of plasma adhesion molecules

Soluble forms of intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin were measured with commercial ELISA kits (R&D Systems). A monoclonal antibody specific for the adhesion molecules were pre-coated onto a microplate. Standards, samples, controls were added and any sVCAM1 or sICAM-1 or sE-Selectin presents were bound by the immobilized antibody and the enzyme-linked monoclonal antibody specific for sVCAM1 or sICAM-1 or sE-Selectin. Unbound materials were washed away. HRP-conjugate antibody was added and binds to the captured analyte. By washing any unbound substances and/or antibody-enzyme reagent was removed, thus a substrate solution was added to the wells and blue color develops in proportion to the amount of protein bound. The color development was stopped turning the color in the wells to yellow and the intensity of the color was measured at 450 nm.

2.6. Reconstituted HDL preparation

ApoA-I and apoA-II were purified from human plasma, as previously described (185) and discoidal reconstituted HDLs (rHDLs) containing apoA-I and POPC (rLpA-I) were prepared by the cholate dialysis technique (186). rHDLs containing both apoA-I and apoA-II (rLpA-I:A-II) were obtained by incubating rLpA-I with lipid-free apoA-II (apoA-II:apoA-I = 1:2, w:w), as previously described (185). rHDLs were then isolated by ultracentrifugation (d < 1.21 g/ml) and the presence of apoA-I and apoA-II was verified by SDS-PAGE followed by Coomassie blue R250 staining. The size of the particles was estimated by gradient gel electrophoresis (186) using the Pharmacia Phast system. Phospholipid content of rHDL was determined by an enzymatic method (187), and proteins were measured by the method of Lowry et al. (188).

2.7. Endothelial cells

All experiments were performed on primary cultures of human umbilical vein endothelial cells (Clonetics, Lonza) in M199 with 0.75% BSA and 1% FCS. Plasma HDLs were used at protein concentration of 1.0 mg/mL and were sterilized with 0.22 μm filters before use. Cells were growth with complete growth media (M199 with 10% serum, 1% L-Glutamine,

1% antibiotics, EGF 0.1 ng/ml + FGF 1 ng/ml + ECGF 2 µl/ml) in 12-well plates until 90% confluence and starved 18 hours before experiment in M199 with 0.75% BSA, 1% FCS.

2.8. VCAM-1 expression in endothelial cells

Endothelial cells were incubated overnight with HDL, washed with PBS and then stimulated with TNF-α (10 ng/mL) for 8 hours. Then media was collected and cells were lysate with lysis buffer containing 20 mM Tris, pH 6.8, 1 mM EDTA, 4% SDS, 20% glycerol, 1 mM NaOv, 1 mM NaF, 1 ug/ml leupeptin, 1 mM benzamidin, 10 ug/ml soybean trypsin inhibitor, 0.5 mM DTT, 1 mM Pefabloc. VCAM-1 expression in medium was measured by commercial matched antibody pairs ELISA kit (BioSource) that employs a quantitative sandwich enzyme immunoassay technique and normalized by the protein concentration of the total cell lysate. Pre-coated microplate with specific antibody for VCAM-1 was used. Standards and samples are pipetted into the wells and any VCAM-1 present is bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for VCAM-1 was added to the wells. After washing, avidin conjugated- HRP was added. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution was added and color develops in proportion to the amount of VCAM-1 bound in the initial step. The color development is stopped and the intensity of the color is measured.

2.9. eNOS expression

Endothelial cells were incubated overnight with HDL (1.0 mg/mL), washed with PBS and lysated with lysis buffer. Expression of eNOS was assessed by SDS-PAGE and immunoblotting. Total protein concentration was assayed by micro-BCA method (ThermoScientific). 25 µg of total protein for each samples were loaded into 8% SDS-polyacrylamide gel. The separation takes place on the basis of the effects of molecular sieve due to the size of the meshes of the gel. While the smaller proteins can pass through easily, the larger particles are slow down by frictional forces. Electrophoresis started at 60V, so that samples can distributed in stacking gel and once entered into the running gel the electrophoresis was raised to 80V. At the end of the electrophoresis the proteins were transferred into a nitrocellulose membrane (Schleicher & Schuell) for 1 hour at 180 mA, in buffer containing 25 mM Tris, 192 mM glycin and 15% (v/v) methanol. Membranes were

immediately saturated with a TBS-T buffer (0,018 M Tris, 0,25 M NaCl, pH 7.4, and 0,1% (v/v) Tween 20) containing 5% (w/v) non-fat milk, overnight at 4°C. Membranes were developed against total eNOS antibody (BD Biosciences) diluted 1:1000 and incubated overnight at 4°C, washed 50 minutes in T-TBS solution, incubated with a secondary antibody (Dako) conjugated with HRP for 1 hour at 37°C and washed again 50 minutes in T-TBS solution at room temperature. Bands were visualized by enhanced chemiluminescence (GE Healthcare Biosciences) and analyzed with a GS-690 Imaging Densitometer and Multi-Analyst software (Bio-Rad Laboratories). The membranes were stripped with a buffer solution containing 0.2 M Glycin, pH 2.2, SDS 0.1% (w/v) SDS e 1% (v/v) Tween 20 and reprobed with an antibody against β-actin (Sigma-Aldrich Chemie) diluted 1:2000.

2.10. eNOS activation

Endothelial cells were incubated 20 minutes with HDL (1.0 mg/ml), washed with PBS and lysated with lysis buffer. eNOS activation by phosphorylation was evaluated by SDS-PAGE and immunoblotting using antibodies against phosphorylated eNOS (Ser1177, Cell Signalling Technology) diluted 1:1000 overnight 4°C, stripped and reprobed with an antibody against total eNOS with method described previously.

2.11. Nitric oxide production

Endothelial cells were incubated 30 minutes with HDL (1 mg/ml), washed with PBS and incubated 2 hours in dark conditions at room temperature with buffer containing diacetate 4,5-diaminofluorescein (DAF-2 DA, Sigma-Aldrich Chemie). DAF-2DA is a non-fluorescent cell permeable reagent that can measure free NO and nitric oxide synthase (NOS) activity in living cells under physiological conditions. Once inside the cell the diacetate groups on the DFA-2DA reagent are hydrolyzed by cytosolic esterases thus releasing FAD-2 and sequestering the reagent inside the cell. Production of nitric oxide converts the non-fluorescent dye, DAF-2, to its fluorescent triaole derivative, DAF-2T. Fluorescence intensity was detected (λ excitation= 485 nm, λ emission= 530 nm) with a Synergy Multi-Mode microplate reader equipped with the GEN5 software (BioTek). After detection, cells were lysated with lysis buffer and protein concentration was measured with micro-BCA assay. For each sample, fluorescence was normalized by the protein concentration of the total cell lysate.

2.12. Statistical analysis

Results are reported as mean ± SD, if not otherwise stated. Variables with non-Gaussian distribution are presented as median and interquartile ranges and were log-transformed before analysis. The association of biochemical with *LCAT* genotype was assessed as the linear trend versus the number of mutant *LCAT* alleles and adjusted for age, sex and family by covariance analysis. The association of HDL functions with LCAT genotype was assessed as the linear trend versus the number of mutant *LCAT* alleles. Independent predictors of HDL functionality were identified by multiple regression with stepwise selection of variables. All tests were two-sided and $P<0.05$ was considered as significant. All analyses were performed by using the SAS statistical package v.9.2 (SAS Institute Inc., Cary, NC).

3. STUDIES ON MONOCYTES

3.1. Plasma lipids and apolipoproteins

Lipid levels were measured using fast protein liquid chromatography (FPLC) (189).
The system contained a PU-980 ternary pump with an LG-980-02 linear degasser, FP-920 fluorescence and UV-975 UV/VIS detectors (Jasco, Tokyo, Japan). An extra PU-2080i Plus pump (Jasco, Tokyo Japan) was used for in-line cholesterol PAP or Triglyceride enzymatic reagent (Roche, Basel, Switzerland) addition at a flowrate of 0.1 ml/min. Plasma lipoproteins were separated with a Superose 6 HR 10/30 column (GE Healthcare Hoevelaken, The Netherlands) using TBS pH 7.4, as eluent at a flow rate of 0.31 ml/min. Quantitative analysis of the chromatograms was carried out with ChromNav chromatographic software, version 1.0 (Jasco, Tokyo, Japan). Commercially available lipid plasma standards (low, medium and high) were used for quantitative analysis (SKZL, Nijmegen, the Netherlands) of the separated lipoprotein fractions.
Apolipoproteins were measured by automated analyser Roche Diagnostics c311.

3.2. Flow cytometry

Blood samples were collected into EDTA-coated Vacutainer® tubes (BD, Plymouth, UK), after which 50 µL of whole blood was added to each flow cytometry tube plus the requested amounts of each antigen specific mAb were added, according by manufactures. The following fluorochrome labeled mAb were used: phycoerythrin-CyChrome7 (PECyTM7)-anti CD14, allophycocyanin (APC)-Cy7-anti CD16, peridinin-chlorophyll-protein (PerCP)-Cy5.5-anti HLA-DR, APC-anti CD192 (CCR2), fluorescein isothiocyanate (FITC)-anti CD195 (CCR5), PE-anti CD11b, APC-anti CD11c, , APC-anti CD36, APC-anti CD29, FITC-anti CD282 (TLR2), PE-anti CD284 (TLR4) [all from BD Biosciences, San Josè, CA, USA]; PE-anti CD197 (CCR7) [Biolegend, SanDiego, CA, USA]; PE-anti CD204 (SR-A) [R&D systems, Abingdon, UK]; Fluorescence was measured with BD Canto II and analysed with FlowJo software version 7.6.5 (FlowJo, LLC, Ashland, OR). The expression of surface markers was calculated as delta (Δ) median fluorescence intensity (MFI) (ΔMFI = MFI surface staining - MFI isotype control).

3.3. Monocyte isolation and stimulation

Buffy coats were obtained from study participants after written informed consent. Venous blood was drawn in EDTA-coated Vacutainer® tubes (BD, Plymouth, UK) to prevent coagulation. PBMC isolation was performed by diluting whole blood in sterile PBS, after density centrifugation over Lymphoprep™ (d=1.077) as described elsewhere (190). Shortly, blood was diluted in a 1:1 ratio with PBS plus 2 mM EDTA and therefore added to a layer of Lymphoprep (Axis-Shield, Dundee, Scotland). Cells were centrifuged for 20 min at 600 g at room temperature with slow acceleration and no brake. After washing twice with PBS/2 mM EDTA, cells were counted by a CASY counter (Roche Innovatis, Bielefield, Germany). After PBMC isolation, monocytes were selected using human CD14 magnetic beads and MACS® cell separation columns according to protocol (Miltenyi, Bergisch Gladbach, Germany). Briefly, cells were resuspended in MACS buffer (0.5% BSA in PBS/2 mM EDTA), CD14 MicroBeads (Miltenyi Biotec, Leiden, The Netherlands), incubated for 30 min at 4°C. Then cells were washed with MACS buffer and resuspended in MACS buffer for CD14 isolation using the MACS separation columns. Monocytes were resuspended in Dutch modified RPMI culture medium (Roswell Park Memorial Institute

medium; Invitrogen, CA, USA) supplemented with 10µg/ml gentamicin, 10mM Glutamax, and 10mM pyruvate) and counted by means of a CASY counter. All experiments were performed with freshly isolated circulating monocytes.

A total of $5*10^5$ monocytes were seeded per well on flat-bottom-96 well plates and stimulated for 24 hour with RPMI only as a negative control, 10 ng/mL LPS or 10 µg/mL Pam3Cys. Then, plates were centrifuged and supernatants were stored at -20°C until cytokine measurement.

3.4. Cytokines measurement

Cytokine production was determined in supernatants using commercial ELISA kits for IL1β, TNFα and IL-1Ra (R&D systems, MN, USA), IL-6, IL-8 and IL-10 (Sanquin, Amsterdam, The Netherlands) following the instructions of the manufacturer. MCP-1 ELISA was performed following the manufacturers' instructions using antibodies anti-Human CCL2 Purified (14-7099), antiCCL2 Biotin (13-7096) and Human CCL2 Recombinant Protein (14-8398), all obtained from eBioscience (CA, USA).

Plasma cytokines were determined using commercial high sensitive ELISA kits for TNF-α (eBioscience, CA, USA) and IL-6 (R&D systems, MN, USA) and MCP-1 as described above. hsCRP in plasma was determined using the high sensitive ELISA kit for CRP (R&D systems, MN, USA).

3.5. Monocyte transendothelial migration

To assess adhesive and migratory capacity of monocytes, transendothelial migration (TEM) was performed. Human aortic endothelial cells (HAECs) were cultured on a fibronectin (FN)-coated glass cover to confluency and stimulated overnight with TNF-α (10 ng/mL). Monocytes ($1*10^6$ cells/mL) were added to the HAECs and incubated for 30 min at 37°C and 5% CO_2, then fixed with 3.7% formaldehyde (Sigma-Aldrich, Zwijndrecht, the Netherlands). Cells were later imaged with a Zeiss Axiovert 200 microscope (Plan-apochromat 10x/0.45 M27 Zeiss-objective; Carl Zeiss Inc., Jena, Germany) and analysed using Image-J software. Transmigrated monocytes were distinguished from adhered monocytes by their transitions from bright to dark morphology and quantified using the cell counter plugin on a minimum of 4 acquired images.

3.6. RNA isolation and quantitative Real-Time PCR

Monocytes isolated form patients and controls were lysed in Trizol reagent and stored at -80°C. Total RNA purification was performed according to the manufacturer's instructions (PreAnalytiX GmbH, Switzerland). RNA concentrations were measured using NanoDrop software, and isolated RNA was reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-rad, Hercules, CA). qPCR was performed using the SYBR Green method (Applied Biosciences, Carlsbad, CA). Used primers are listed below in Table 3 (Biolegio, Malden, The Netherlands). Samples were analyzed using the ΔCt method, and HPRT was used as a housekeeping gene.

Table 3. Primer sequences used for qRT-PCR analysis (5'-3')

Gene	Forward primer	Reverse primer
HPRT	CCTGGCGTCGTGATTAGTGAT	AGACGTTCAGTCCTGTCCATAA
TNF-α	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
IL-6	AACCTGAACCTTCCAAAGATGG	TCTGGCTTGTTCCTCACTACT
IL-1β	CAGCTACGAATCTCCGACCAC	GGCAGGGAACCAGCATCTTC
MCP-1	CCAGTCACCTGCTGTTATAAC	TGGAATCCTGAACCCACTTCT
CD11c	CGTTCGACACATCCGTGTA	TTTGCCTCCTCCATCATTTC
IL-18	ATGCACTGGGAGACAATTCC	CTCCCTCCACCTTCTTCCTC
IL-10	GCCTAACATGCTTCGAGATC	TGATGTCTGGGTCTTGGTTC
CD36	CTTTGGCTTAATGAGACTGGGAC	GCAACAAACATCACCACACCA
SR-A	CCAGGTCCAATAGGTCCTCC	CTGGCCTTCCGGCATATCC
ABCA1	GGTGATGTTTCTGACCAATGTGA	TGTCCTCATACCAGTTGAGAGAC
ABCG1	CGGAGCCCAAGTCGGTGTG	TTTCAGATGTCCATTCAGCAGGTC
HMGCoAR	CAGGATGCAGCACAGAATGT	CTTTGCATGCTCCTTGAACA

3.7. Statistical analysis

To examine the difference in monocyte phenotype and function between subjects with genetic mutation affecting HDL-C concentration (LCAT deficiency) and controls, we performed the Student t test or Mann-Whitney U test for normal and non-normal distributed variables, respectively. Normality was examined by Shapiro-Wilk test. Variances in both groups were examined and tested for similarity with the Levene's test for variance.

Differences between the different genotypes (*ABCA1* and *APOA1*) were analysed with one-way ANOVA followed by Tukey's or Bonferroni's multiple comparisons test or Kruskal-Wallis followed by Dunn's multiple comparisons test. Correlations between HDL-C, apoA-I and mRNA expression of inflammatory cytokines were assessed by Spearman's rank correlation. A 2-sided value of $P<0.05$ was considered statistically significant. All data were analyzed with SPSS version 24.0 (SPSS Inc., Chicago, IL, USA Software, La Jolla, CA).

4. *In vivo* assessment of vascular structure and function

4.1. Flow-mediated vasodilation

Evaluations were performed at fasting, between 8.30 am and 10.30 am to exclude circadian variations, abstaining from physical activity and smoking since midnight. Ultrasonic scans were performed in a quiet temperature-controlled (22±2°C) room by using a B-mode ultrasound device (ESAOTE AU4) equipped with a 7.5–10.0 MHz linear array transducer, held throughout the scan at the same point of the brachial artery (BA) of the non-dominant arm using a stereotactic device. The ultrasonic device was gated to the peak R-wave on ECG and images were collected during the end-diastolic phase of each cardiac cycle and recorded on SVHS videotapes for off-line measurements. Images of the BA were continuously recorded: (i) for 1 min at rest, (ii) during 5 min of low-flow obtained by inflating a pneumatic tourniquet placed in the forearm to a pressure 30–50 mmHg above the individual systolic blood pressure and (iii) for 3 min after cuff deflation (i.e. during the reactive hyperemic phase). In each patient, the BA diameter (BAD) was measured using dedicated software, which allows the automatic and continuous detection of the distance between the media–adventitia interfaces of the near and far wall of the vessel. BAD at rest was the average of the sixty BAD values obtained throughout the pre-ischemic phase. BAD max was the highest BAD value in the hyperemic phase. FMD was calculated as the percentage change between BAD at rest and BAD max.

4.2. Carotid intima media thickness

The extent of preclinical atherosclerosis was determined in all available subjects by measuring carotid intima-media thickness (IMT) by non-invasive B-mode ultrasonography as previously described (25). High resolution B-mode ultrasonography of carotid IMT with a linear ultrasound probe (4.0 – 13.0 MHz frequency,14x48 mm footprint, 38 mm field of view; Vivid S5 GE Healthcare ®, Wauwatosa, WI, USA) was performed by a single sonographer blinded to subject's identity. The IMT was assessed at the far wall distance between the interface of the lumen and intima and the interface between the media and adventitia in a standardized number of points. The maximal IMT was recorded and averaged for the left and right sides of the common carotid artery (30 mm proximal to the carotid bulb). Presence of extra-cardiac atherosclerotic vascular involvement was determined with presence of focal plaques (> 1.3 mm in longitudinal resolution, lateral or medial angle) and/or diffusive mean IMT > 1.3 mm (> 1.3 mm in longitudinal resolution, lateral or medial angle).

RESULTS

1. SUBJECTS

In Table 4 are reported the clinical and biochemical characteristics of the Italian and Dutch subjects included in the study. As expected total cholesterol, HDL-C, apoA-I and apoA-II were lower in carriers of LCAT mutations compared to controls. The effect was gene-dose dependent, with heterozygous subjects showing an intermediate phenotype.

Table 4. Clinical and biochemical characteristics of carriers of *LCAT* gene mutations and controls

	Carriers of two mutant LCAT alleles	Carriers of one mutant LCAT allele	Controls	P_{Trend}*	P_{Anova}
n	20	67	41		
Age (years)	44.1 ± 4.1	48.4 ± 2.5	45.4 ± 3.2	0.687	0.611
Gender (male/female)	15/5	43/24	16/25	0.025	0.009
BMI (Kg/m^2)	24.1 ± 0.72	25.1 ± 4.7	24.4 ± 3.3	0.575	0.593
TC (mg/dL)	158.2 ± 18.0	168.9 ± 5.1	196.4 ± 6.0	0.019	0.001
LDL-C (mg/dL)	104.1 ± 14.4	101.3 ± 4.8	113.6 ± 5.0	0.746	0.145
HDL-C (mg/dL)	10.9 ± 1.6	41.6 ± 1.9	60.6 ± 2.3	<0.0001	<0.0001
Triglycerides (mg/dL)	176.7 (116-372)	112.0 (86-159)	91 (67-121)	<0.0001	<0.0001
ApoA-I (mg/dL)	44.6 ± 5.1	103.8 ± 3.2	127.8 ± 4.3	<0.0001	<0.0001
ApoA-II (mg/dL)	9.6 ± 1.5	29.0 ± 0.8	33.9 ± 1.3	<0.0001	<0.0001
ApoB (mg/dL)	81.3 ± 12.7	93.4 ± 3.2	89.8 ± 3.4	0.124	0.068
Glucose (mg/dL)	105.5 ± 10.5	101.8 ± 7.3	88.8 ± 2.4	0.359	0.437
Hypertension (n,%)	6 (30)	10 (14.9)	3 (7.3)	0.064	0.085
Diabetes (n,%)	2 (10)	2 (3)	2 (4.9)	0.251	0.593
Smoking (n,%)	7 (35)	13 (19.4)	7 (17.1)	0.756	0.359
Statin treatment (n,%)	6 (30)	4 (6.0)	1 (2.4)	0.001	0.003
Familial history of CVD (n, %)	12 (60)	31 (46.3)	16 (39.0)	0.494	0.996
Premature CVD (n,%)	0 (0)	1 (1.5)	1 (2.4)	0.895	0.770
Renal disease (n,%)	9 (45)	11 (16.4)	0 (0)	<0.0001	0.000

Data are expressed as mean ± SEM for all variables, except for triglycerides that are expressed as median and interquartile range. Categorical variables are expressed as number, percentage* *P* adjusted for age, gender, and family, except for age. Age was adjusted for gender and family; gender was adjusted for age and family.

Plasma apoB was also reduced compared to the unaffected family members, although not significantly. Risk factors for cardiovascular disease were comparable between carriers and controls, but the presence of renal disease was predictably higher when LCAT gene is mutated. Renal disease was diagnosed by kidney biopsy or proteinuria or eGFR calculation.

Besides the drastic reduction in HDL-C levels, also HDL subclass distribution appears highly modified in carriers of LCAT deficiency. Lipoproteins containing only apoA-I (LpA-I) and lipoproteins containing both apoA-I and apoA-II (LpA-I:A-II) concentrations were both reduced in carriers, with a gene dose effect, but notably the decrease in LpA-I:A-II particles was much greater (-72% and -30% in homozygotes and heterozygotes respectively) than the decrease in LpA-I (-50% and -18% in homozygotes and heterozygotes respectively). Homozygous carriers displayed a higher content of immature, discoidal HDL, namely preβ-HDL, versus controls (Figure 8). Furthermore, among spherical HDL, HDL_3 were smaller in homozygous carriers, who completely lack of the largest HDL subpopulations, i.e. HDL_2. Heterozygous carriers instead showed a normal HDL distribution, with HDL of normal size (Table 5).

Table 5. Plasma HDL subpopulations in carriers of LCAT gene mutations

	Carriers of two mutant *LCAT* alleles	Carriers of one mutant *LCAT* allele	Controls	P_{Trend}*	P_{Anova}
LpA-I (mg/dL)	28.0 ± 4.2	46.3 ± 1.7	56.4 ± 2.5	<0.0001	<0.0001
LpA-I:A-II (mg/dL)	22.5 ± 4.3	55.8 ± 2.4	79.8 ± 3.8	<0.0001	<0.0001
Preβ-HDL (% of apoA-I)	40.0 (23.0-49.0)	16.0 (11.8-22.0)	12.8 (10.9-14.1)	<0.0001	<0.0001
HDL_2 size (nm)	ND	11.2 ± 0.1	11.1 ± 0.1	0.15	0.35
HDL_3 size (nm)	7.4 (7.2-7.7)	9.0 (8.8-9.3)	9.0 (8.8-9.0)	<0.0001	<0.0001

Data are expressed as mean ± SEM for all variables, except for preβ HDL and HDL_3 size that are expressed as median and interquartile range. ND not detectable.* *P* adjusted for age, gender, and family.

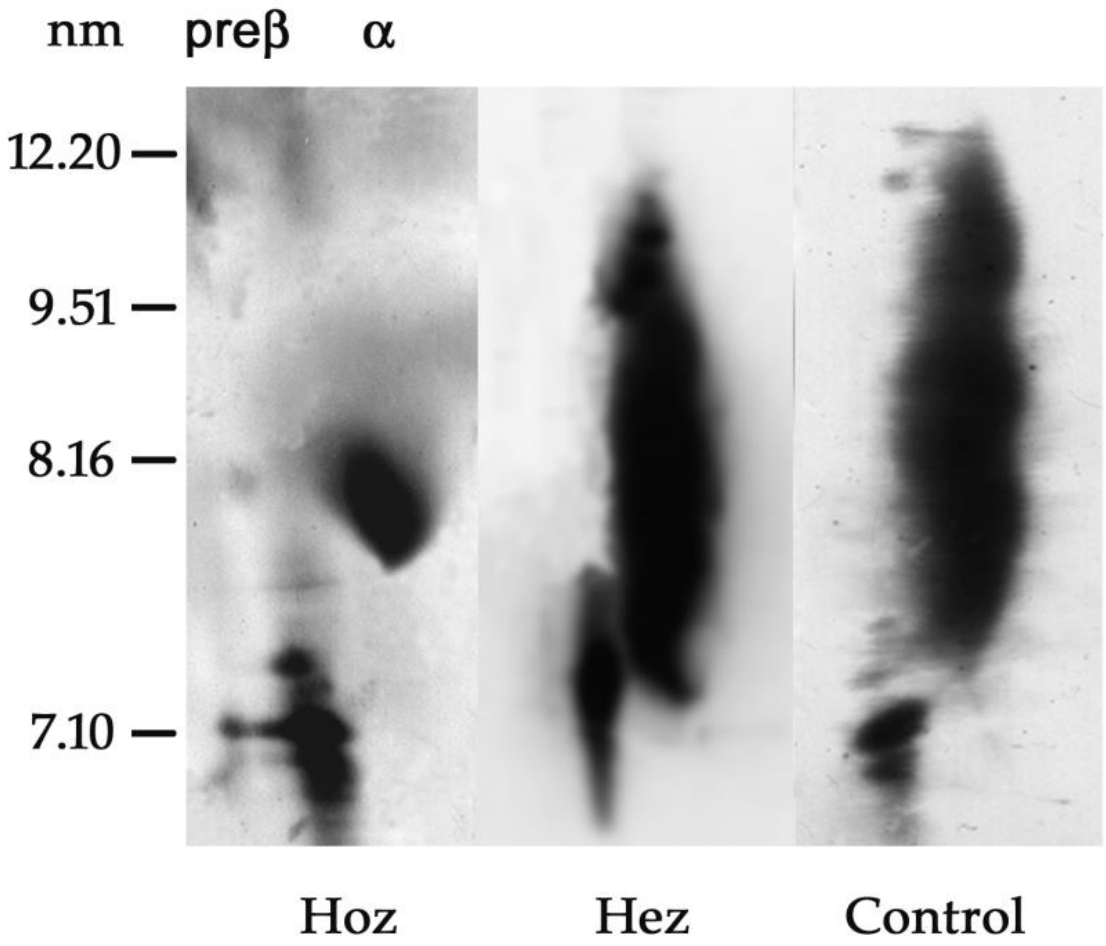

Figure 8. HDL subclasses displayed through 2-dimensional electrophoresis of homozygotes for LCAT deficiency, heterozygotes and controls

2. STUDIES ON ENDOTHELIAL CELLS

2.1. Characteristics of study subjects

Seventy-five carriers of *LCAT* gene mutations participated in the study on endothelial cells. Clinical and biochemical characteristics of this group essentially recapitulated the characteristics of the total populations, summarized in Table 6.

Table 6. Plasma lipid/lipoprotein levels in carriers of LCAT gene mutations included for the study on endothelial cells

	Carriers of two mutant LCAT alleles	Carriers of one mutant LCAT allele	Controls	P_{Trend}*	P_{Anova}
n	15	60	32		
Age (years)	44.1 ± 4.9	48.7 ± 2.7	46.6 ± 3.6	0.86	0.73
Gender(male/female)	14/1	39/21	11/21	<0.001	0.0003
TC (mg/dL)	159.6 ± 22.9	166.5 ± 5.5	193.0 ± 6.5	0.015	0.03
LDL-C (mg/dL)	98.5 ± 17.6	97.2 ± 5.0	111.4 ± 5.5	0.21	0.30
HDL-C (mg/dL)	12.1 ± 2.0	41.9 ± 2.1	59.8 ± 2.3	<0.0001	<0.0001
Triglycerides (mg/dL)	180 (114-387)	110.5 (81-155)	77.5 (62-113)	0.0002	<0.0001
ApoA-I (mg/dL)	50.5 ± 5.7	102.1 ± 3.0	136.3 ± 4.6	<0.0001	<0.0001
ApoA-II (mg/dL)	9.6 ± 1.5	29.0 ± 0.8	33.9 ± 1.3	<0.0001	<0.0001
ApoB (mg/dL)	67.8 ± 13.0	92.2 ± 3.5	88.6 ± 3.9	0.16	0.03

Data are expressed as mean ± SEM for all variables, except for triglycerides that are expressed as median and interquartile range. Categorical variables are expressed as number, percentage* *P* adjusted for age, gender, and family, except for age. Age was adjusted for gender and family; gender was adjusted for age and family.

2.2. HDLs ability to modulate NO production in endothelial cells

To assess if changes in HDL subclass distribution can affect HDL vasoprotective properties, anti-inflammatory and NO-promoting activities mediated by isolated HDL were tested in endothelial cells. The effect of HDL in modulation of endothelial production of nitric oxide (NO) was tested *in vitro* in HUVEC after incubation overnight with HDLs. As shown in Figure 9 (Panel A) HDLs isolated from carriers and controls displayed a comparable ability in inducing eNOS expression, with a small decrease in subjects with two mutated alleles (P_{Anova}= 0.115). However when tested for their ability to activate eNOS

by phosphorylation, HDLs of LCAT deficient subjects proved to be more effective with a gene dose dependent effect (Figure 9, Panel B). Consequently, the total NO production was higher when endothelial cells were incubated with HDL isolated from carriers compared to those of controls (P<0.001). As previously, the induction was gene dose dependent (Figure 10).

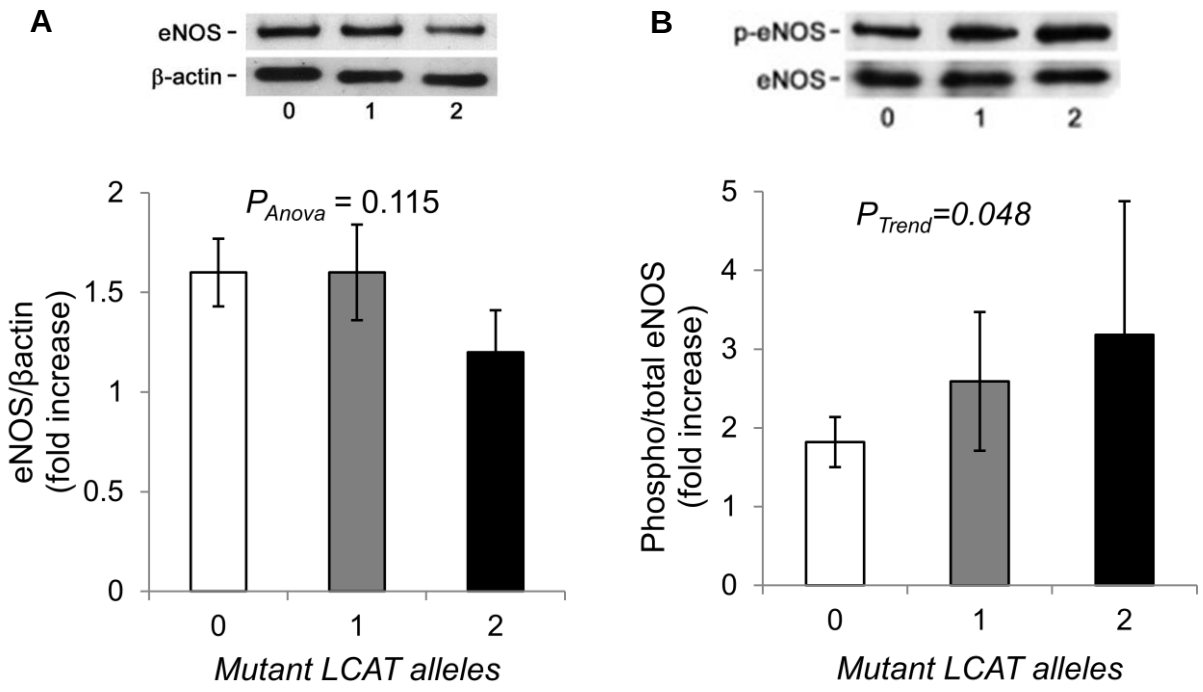

Figure 9. eNOS expression (Panel A) and activation (Panel B) induced by HDLs in endothelial cells. Data are expressed as fold of increase in HDL-treated vs untreated cells. Data are expressed as mean ± SD; n=10 controls, n=10 for heterozygotes and n=6 for homozygotes (pooled in two preparations).

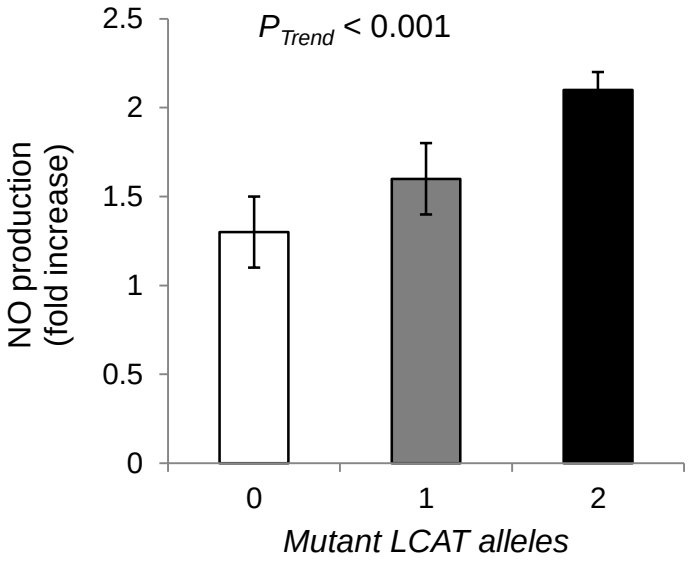

Figure 10. NO production by HDL. Data are expressed as fold of increase in HDL-treated vs untreated cells; n=10 controls, n=10 for heterozygotes and n=6 for homozygotes (pooled in two preparations).

Sphingosine-1-Phospate (S1P) is a bioactive lipid, playing a key role in vascular biology (191) that circulates bound to HDL via apoM. It is mainly associates with small, dense HDL consistently with the high content of apoM (47). S1P binds the S1P3 receptor, expressed in endothelial cells (79) and modulate the activity of eNOS through phosphorylation operated by Akt (192). S1P concentration in isolated HDL from LCAT deficient carriers and controls was measured to assess whether the ability of HDL in LCAT deficiency to activate eNOS was dependent on S1P content. As represented in Figure 11, S1P content in HDL in homozygous carriers was comparable to that of controls, while heterozygous subjects showed a slightly higher content of S1P in HDL.

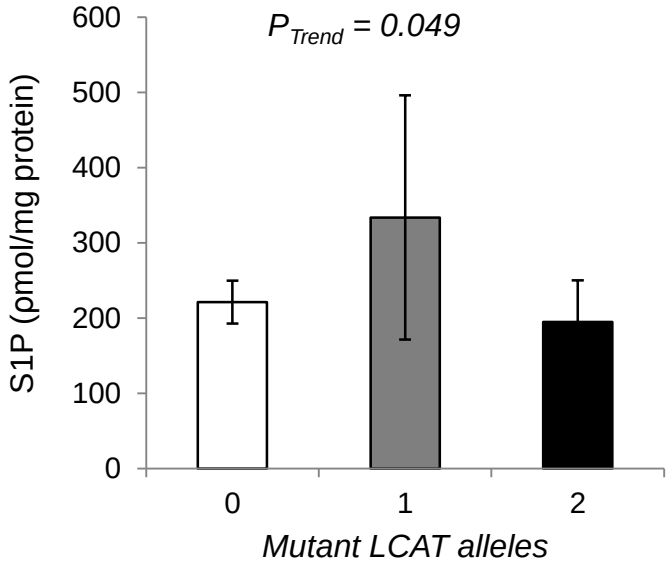

Figure 11. S1P content in HDL. Data are expressed as mean ± SD; n=9 controls, n=9 for heterozygotes and n=3 for homozygotes.

2.3. Inhibition of VCAM-1 expression by HDLs in endothelial cells

As the stimulatory effect of HDLs on endothelial NO production is critical for their anti-inflammatory activity on endothelium (193)**,** the *in vitro* effect of HDL isolated from carriers on the expression of endothelial adhesion molecules after stimulation by TNFα was assessed. As expected, HDLs from LCAT deficient patients were more effective in inhibiting VCAM-1 expression compared to HDLs isolated from controls. In particular HDL from heterozygotes reduced VCAM-1 by 53.1 ± 7.2%, from homozygotes by 65.0 ± 8.6% vs 44.4 ± 4.1% in controls (Figure 12).

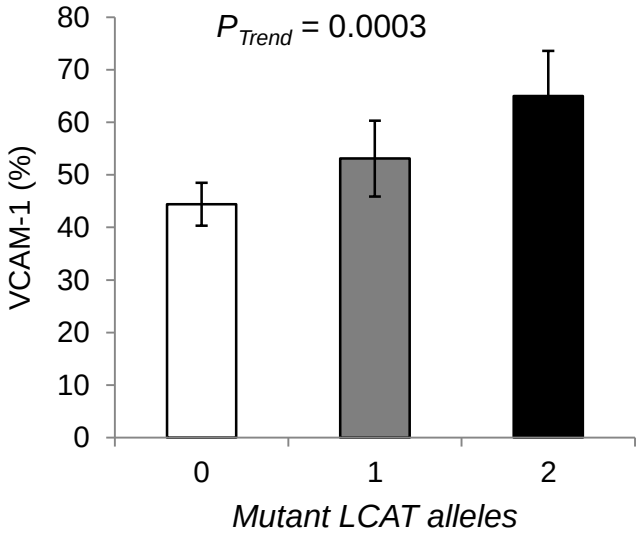

Figure 12. Inhibition of VCAM-1 expression by HDL. Data are expressed as percentage of VCAM-1 concentration in media after TNFα-stimulated cells ± SD; n=10 controls, n=10 heterozygotes and n=6 homozygotes (pooled in two preparations).

2.4. Circulating levels of sCAMs

As markers of systemic inflammation VCAM-1, ICAM-1 and E-Selectin were measured in plasma of LCAT deficient patients and controls. Consistently with the *in vitro* data, no differences were found between carriers and controls when values were tested for trend (Figure 13). Levels of sVCAM-1 were significantly higher in homozygotes compared to controls and this was possibly attributable to the presence of renal disease in 9 out of 15 carriers of two mutated alleles (943.5 ± 395.7 ng/mL vs 761.1 ± 394.5 ng/mL, *P*=0.397). Conversely, stratifying for the presence of renal disease, homozygotes did not show a significant increase of sICAM-1 levels and sE-selectin concentrations were even lower (*P*=0.026).

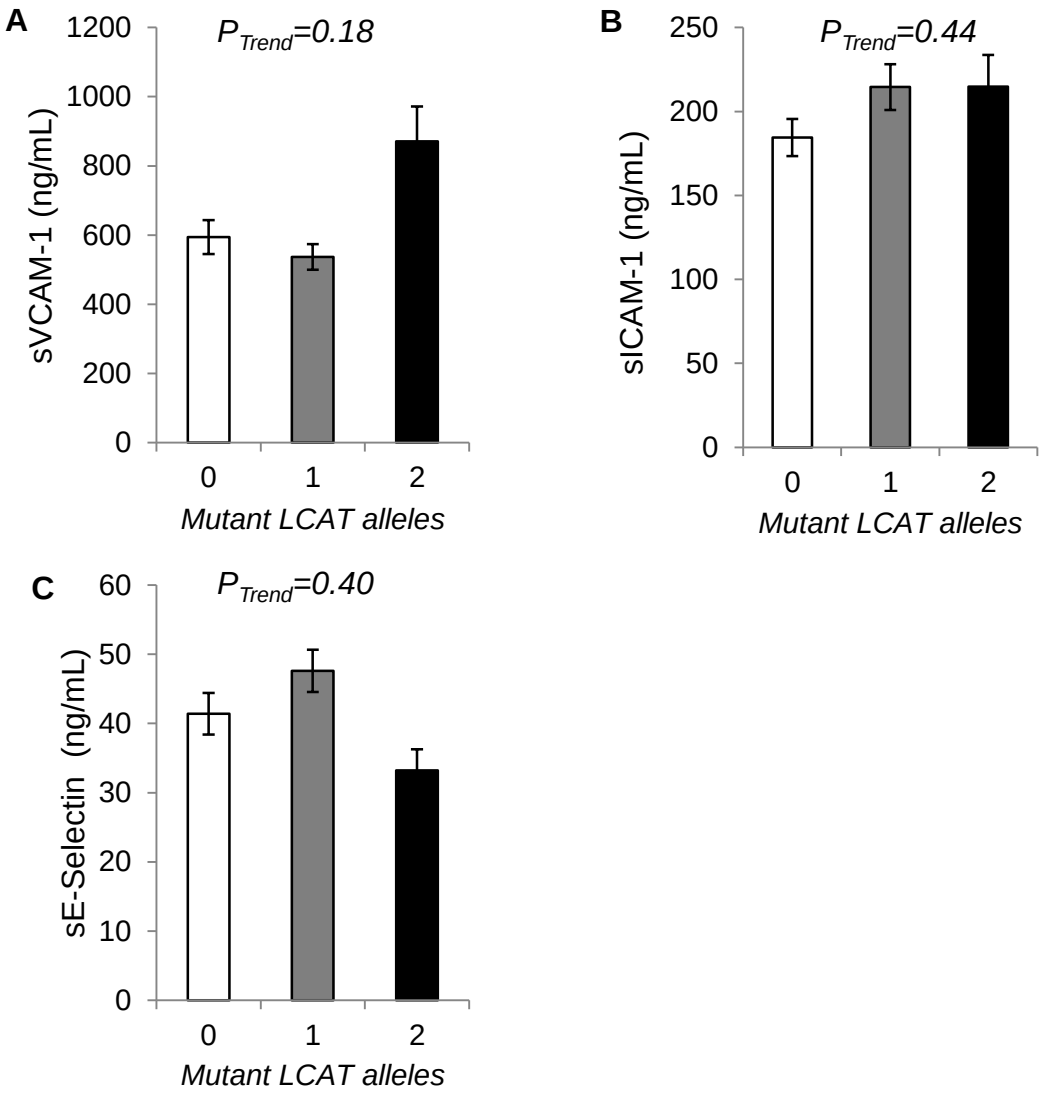

Figure 13. Plasma levels of soluble CAMs in carriers of two or one mutated *LCAT* alleles and controls. Data are expressed as mean ± SEM; n=32 controls, n=62 heterozygotes and n=15 homozygous. *P* for trend adjusted for age and gender are reported

2.5. Nitric oxide production induced by rLpA-I and rLpA-I:A-II in endothelial cells

Notably, among HDL-related parameters, only LpA-I:A-II levels were independently correlated of HDL-induced eNOS activation (β = -0.017 ± 0.006, *P*=0.007). Thus we tested the hypothesis that the selective depletion in LpA-I:A-II, typically observed in LCAT deficiency carriers, could account for the increased endothelial protection exerted by HDL. For this purpose rHDLs containing only apoA-I or rHDLs containing both apoA-I and apoA-II were prepared and characterized. Properties of rHDLs are summarized in Table 7 and Figure 14.

Table 7. Properties of reconstituted HDL

Particle	Size	POPC:Protein	Molecules/particles	
			ApoA-I	ApoA-II
	nm	*w/w*		
rLpA-I	9.7	2.0:1	2	-
rLpA-I:A-II	9.8	1.7:1	1	2

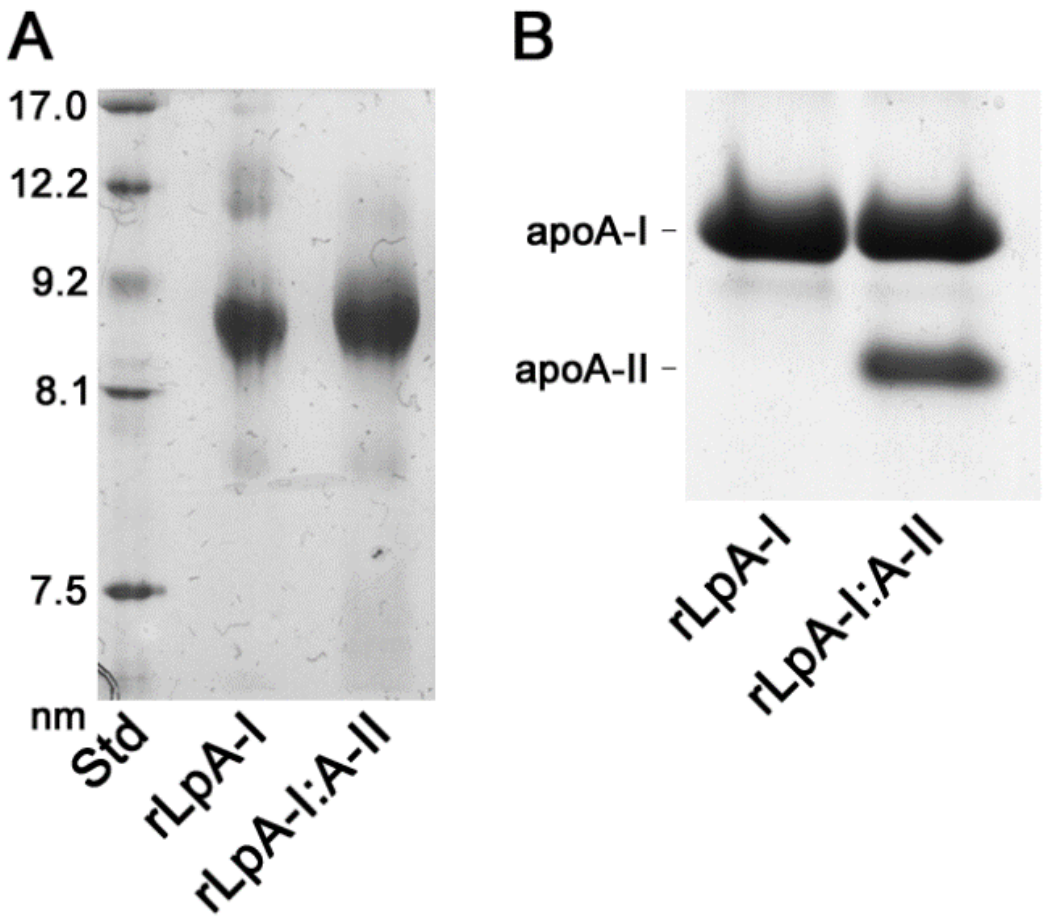

Figure 14. Characterization of rLpA-I and rLpA-I:A-II particles. Particle size by native GGE (Panel A) and protein composition by Coomassie-stained SDS-PAGE (Panel B) (194).

When endothelial cells were incubated with rLpA-I the NO production was higher than in cells incubated with rLpA-I:A-II at all concentrations tested, as presented in Figure 15.
LpA-I:A-II failed to stimulate NO production or even, at lower concentrations seems to have a detrimental effect, although not significant. Consistently with the previous results, eNOS activation by phosphorylation was more effective when only apoA-I was present (Figure 16, Panel A), although the total eNOS protein abundance was comparable (Figure 16, Panel B). In addition, inhibition of VCAM-1 expression in TNFα-stimulated cells were similarly effective (Figure 16, Panel C).

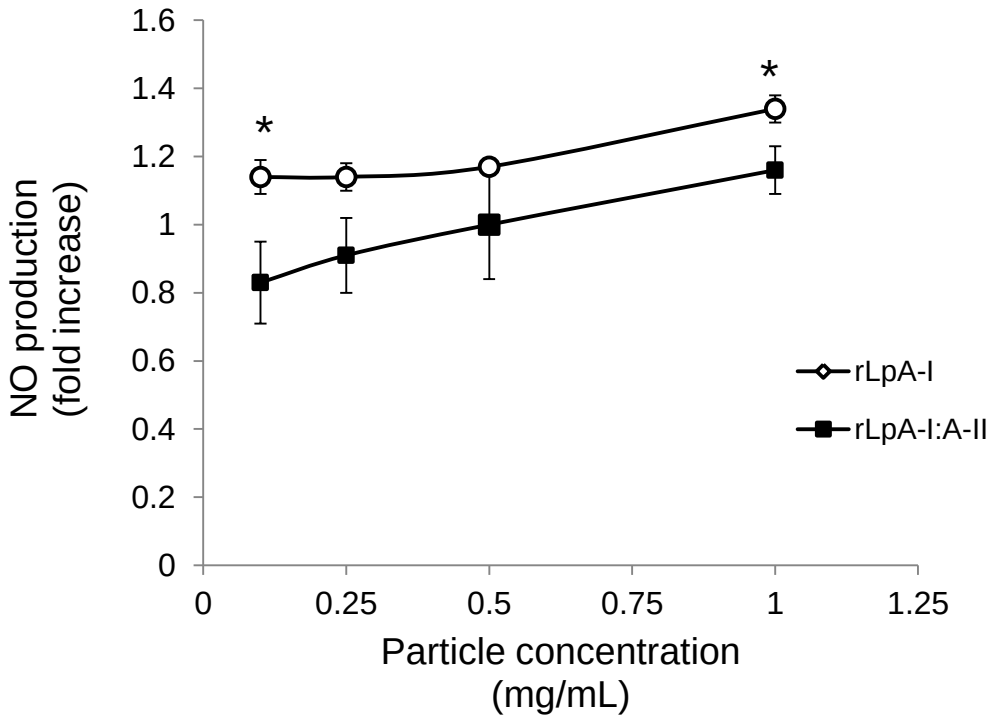

Figure 15. NO production in endothelial cells incubated with rLpA-I and rLpA-I:A-II. Data are expressed as fold of increase in treated versus untreated cells, n=3. *P < 0.05 versus rLpA-I:A-II at the same concentration

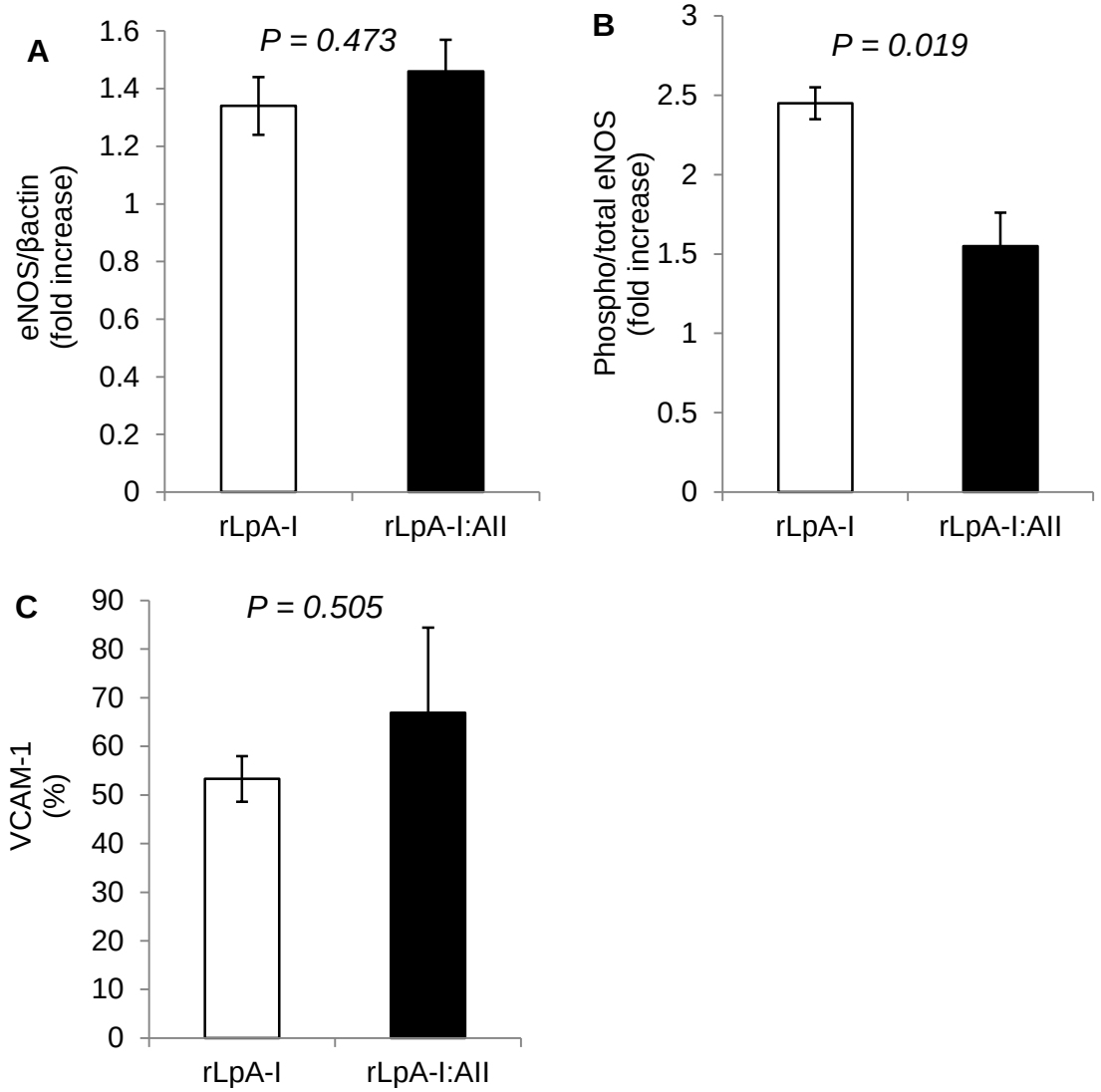

Figure 16. Effects of rLpA-I and rLpA-I:A-II on eNOS expression (Panel A), NO production (Panel B) and TNFα-induced VCAM-1 expression (Panel C) in endothelial cells. Values are expressed as fold of increase in treated versus untreated cells, n=3.

To confirm these findings, when rLpA-I:A-II were added to HDLs of carriers to restore the LpA-I/LpA-I:A-II ratio observed in controls (HDL/rLpA-I:LpA-I:A-II weight ratio 3:1), the ability to promote NO production by endothelial cells was impaired (Figure 17).

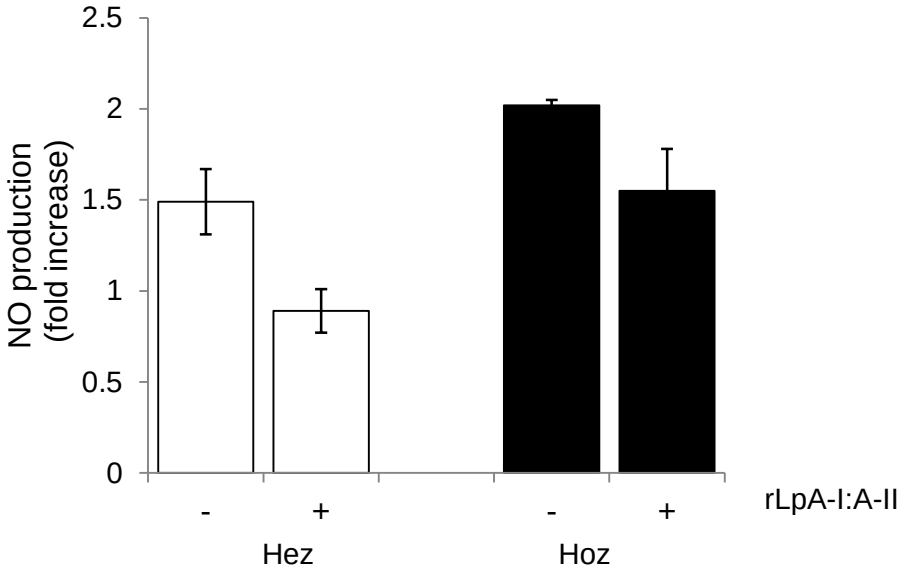

Figure 17. NO production in endothelial cells incubated with HDLs from heterozygous and homozygous patients before and after supplementation with rLpA-I:A-II. Data are expressed as fold of increase in HDL-treated versus untreated cells; n = 3 for heterozygotes

3. STUDIES ON MONOCYTES

3.1. Characteristics of study subjects

For the monocyte studies we included 12 genetic LCAT deficiency carriers (LCAT-def) were representative of the study population and 9 control subjects matched for age, gender and BMI. Inclusion criteria were absence of cardiovascular disease, renal disease and diabetes at the time of recruitment, as well as absence of any acute inflammatory condition. An overview of their clinical and biochemical characteristics are listed in Table 8. As expected LCAT-def had lower HDL-C and apoA-I levels (P<0.0001), while they showed comparable levels of total cholesterol, triglycerides and LDL-C. Circulating hsCRP and other cytokines were measured, but any significant difference was found in carriers compared to controls, as reported in Table 9. Hence, no clue of chronic/acute inflammatory was detected.

Table 8. Clinical characteristics of carriers of LCAT deficiency and control subjects

	LCAT-def	Controls	*P-value*
n	12	9	
Age (years)	45.5 ± 4.8	41.2 ± 4.0	0.519
Gender (male/female)	5/7	5/4	0.670
BMI (kg/m^2)	25.9 ± 1.1	24.5 ± 1.3	0.416
Smoking (n,%)	3 (25)	5 (44)	0.319
TC (mg/dL)	174.6 ± 12.7	208.5 ± 9.6	0.060
TG (mg/dL)	157 (102-213)	113 (93-128)	0.095
LDL-C (mg/dL)	128.7 ± 11.0	121.5 ± 7.7	0.621
HDL-C (mg/dL)	26.0 ± 5.0	63.4 ± 5.9	<0.0001
ApoA-I (mg/dL)	63.1 ± 9.8	121.1 ± 7.9	<0.0001
ApoB (mg/dL)	109.0 ± 9.7	93.9 ± 2.9	0.247
Glucose (mg/dL)	82.1 ± 4.3	87.1 ± 4.2	0.420
Statin treatment (n,%)	5 (42)	1 (11)	0.178

Data are expressed as mean ± SEM for all variables, for triglycerides data are expressed as median and interquartile range. Categorical variables are expressed as number and percentage.

Table 9. Plasma levels of hsCRP and cytokines in carriers of LCAT deficiency and controls subjects

	LCAT-def	Controls	*P-value*
hsCRP (mg/L)	2.5 (2.0-5.2)	1.5 (0.8-3.4)	0.210
MCP-1 (ρg/mL)	51.3 (38.6-72.1)	74.4 (32.5-101.5)	0.831
TNFα (ρg/mL)	1.6 (1.3-2.2)	1.8 (1.5-2.3)	0.188
IL-6 (ρg/mL)	1.6 (1.3-2.2)	1.2 (0.8-1.6)	0.136

Data are expressed as median and interquartile range for all variables.

3.2. Monocyte subset distribution

Blood monocytes from LCAT deficient carriers and controls were gated based on their CD14 and CD16 expression to analyse their subset distribution. $CD14^{++}/CD16^{-}$ corresponds to the "classical" subset which displays phagocytic activities, while $CD14^{+}/CD16^{+}$ corresponds to the "intermediate" inflammatory subset. $CD14^{+}/CD16^{++}$ ("non classical" monocytes) are the patrolling subset of monocytes (Figure 18). Subset distribution was comparable between carriers of LCAT deficiency and controls. Non classical monocytes were slightly decrease compared to controls, although not significantly (5.1 ± 0.76 vs 8.1 ± 1.4, *P*= 0.058) (Figure 19).

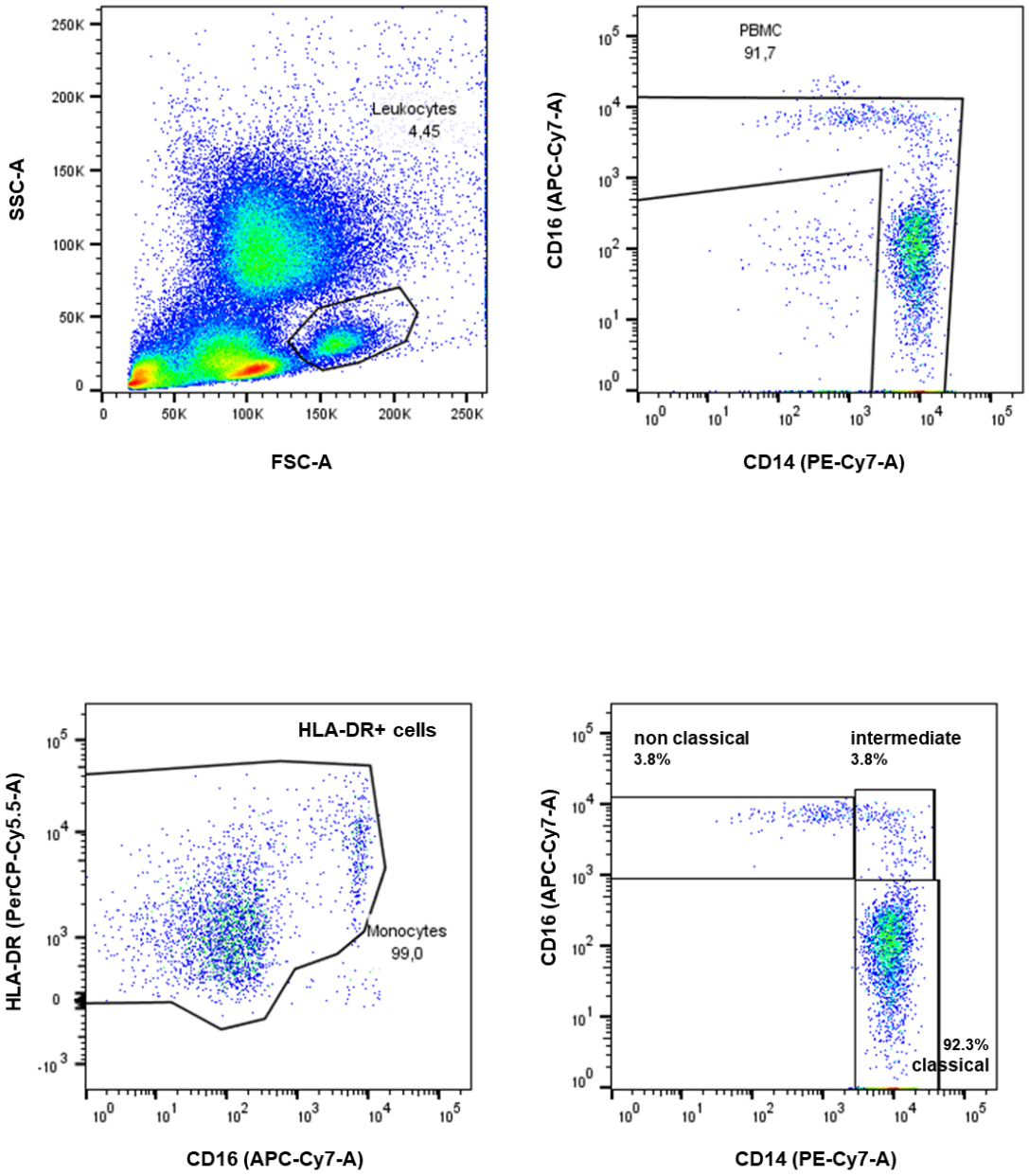

Figure 18. Gating strategy to study monocyte surface expression. CD14+ and/or CD16+ cells were gated, and only HLA-DR positive cells were considered to be monocytes

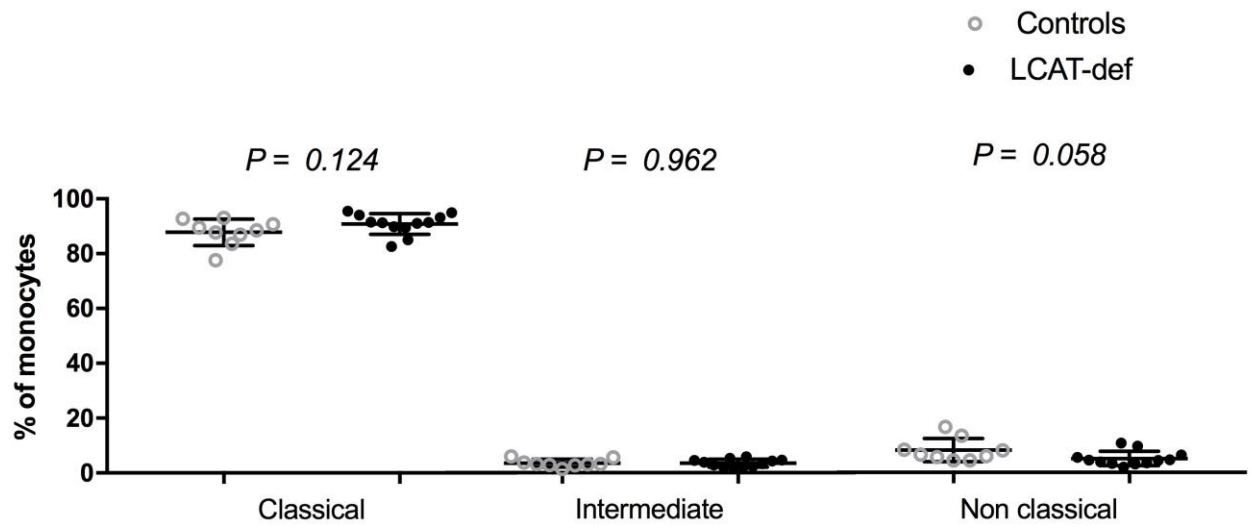

Figure 19. Percentage of monocyte subsets (classical ($CD14^{++}/CD16^{-}$), intermediate ($CD14^{+}/CD16^{+}$), or non-classical ($CD14^{+}/CD16^{+}$) in carriers of LCAT deficiency (n = 12, filled circles) vs. controls (n = 9, open circles).

3.3. Monocyte immunophenotype

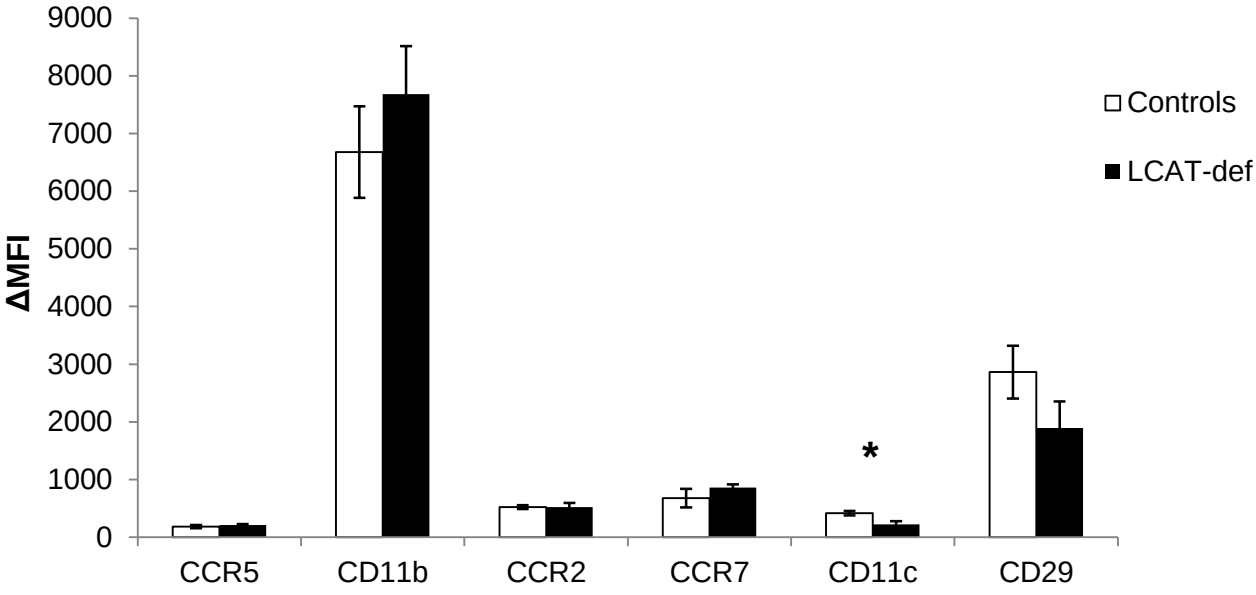

Figure 20. Chemokine, adhesion and transmigration markers on total monocytes as assessed with flow cytometry in carriers of LCAT deficiency (n=12, black bars) compared to controls (n=9, white bars). Bar graphs display the expression (quantified as delta MFI). Data are expressed as media ± SEM. *$P < 0.05$

Expression of chemokine, adhesion and transmigration markers on blood monocytes was comparable between carriers and controls, with the exception of the integrin CD11c that was nearly half in mutations of *LCAT* gene (ΔMFI 220±56 vs 415±38 *P=0.042*) (Figure 20). Decreased CD11c expression was recapitulated in all monocyte subsets (Figure 21).

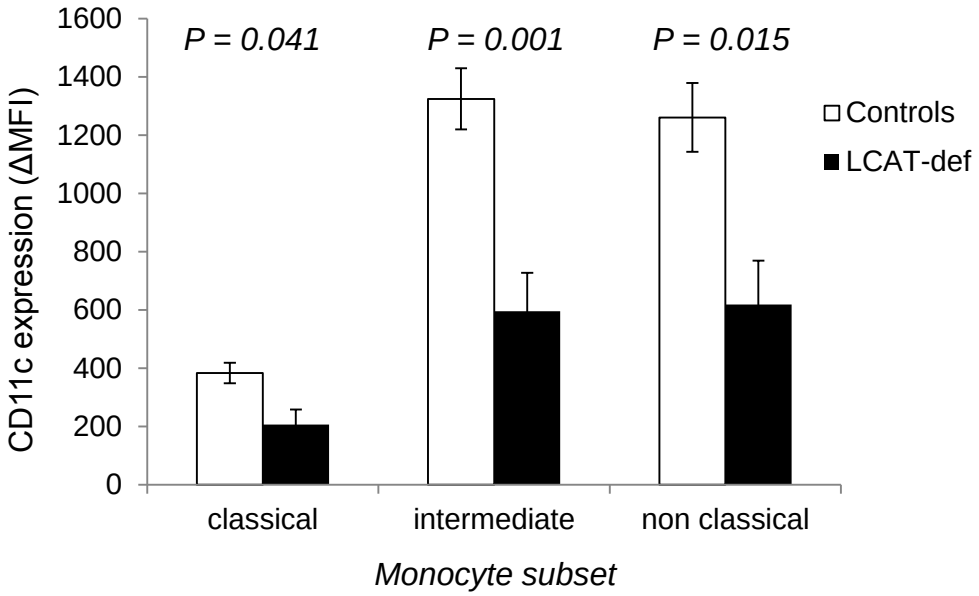

Figure 21. CD11c expression according to monocyte subset as assessed with flow cytometry in carriers of LCAT deficiency (n=12, black bars) compared to controls (n=9, white bars). Bar graphs display the expression (quantified as delta MFI). Data are expressed as media ± SEM.

Interestingly, the reduction of CD11c was gene-dose dependent as shown in Figure 22. The same effect was detected in all monocyte subsets, with the exclusion of the “non classical” one (Figure 23).

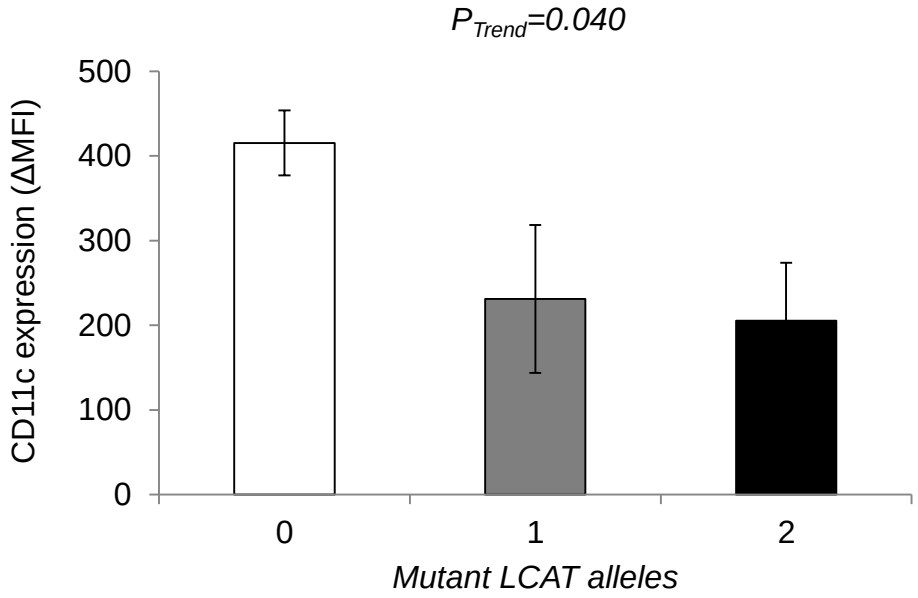

Figure 22. CD11c expression according to mutant *LCAT* alleles as assessed with flow cytometry; n=9 controls, n=7 for heterozygotes and n=5 for homozygotes. Bar graphs display the expression (quantified as delta MFI). Data are expressed as media ± SEM.

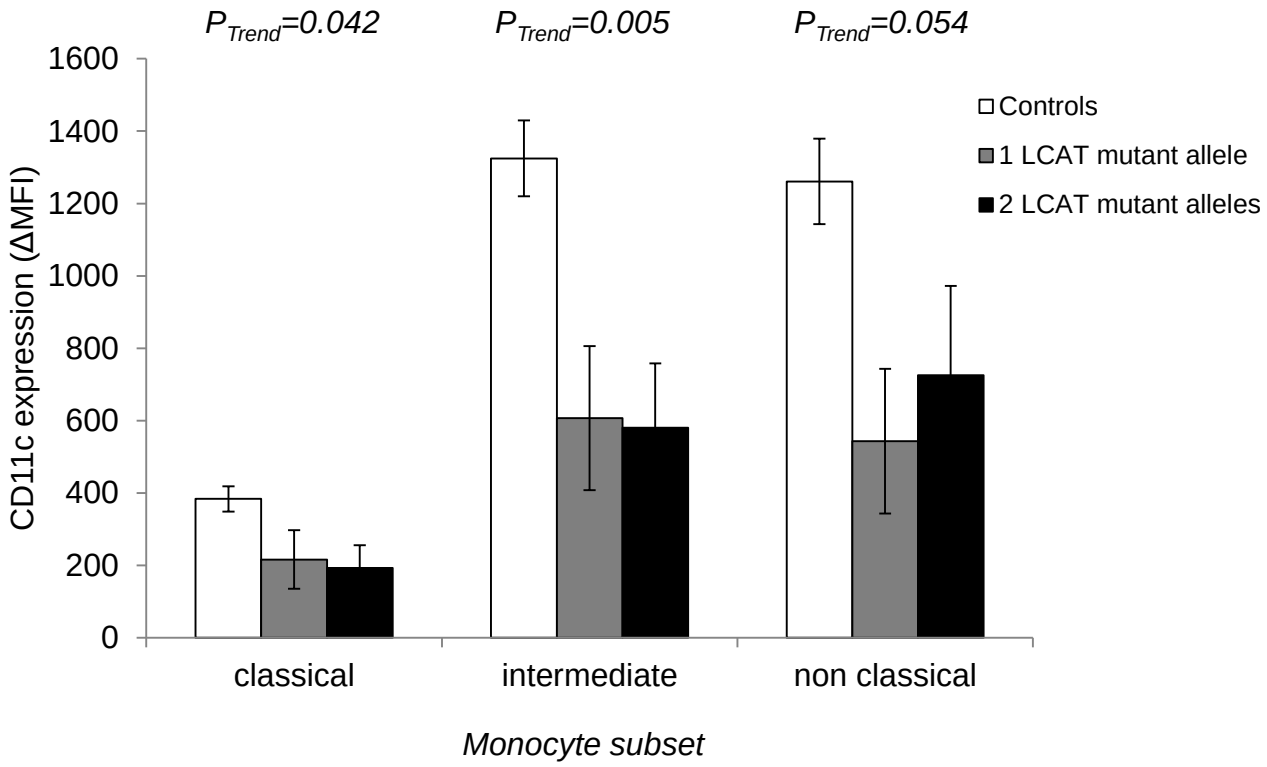

Figure 23. CD11 expression in different monocyte subset as assessed with flow cytometry in carriers of two mutant *LCAT* allele (n=5), one mutant *LCAT* allele (n=7) compared to controls (n=9). Bar graphs display the expression (quantified as delta MFI). Data are expressed as media ± SEM.

CD11c expression on monocytes was found to positively correlate with plasma HDL-C levels (r=0.455,*P=0.038*) but this association was lost when subjects were stratified from the presence or absence of *LCAT* mutation (Figure 24), thus confirming the different properties of HDL in LCAT deficiency.

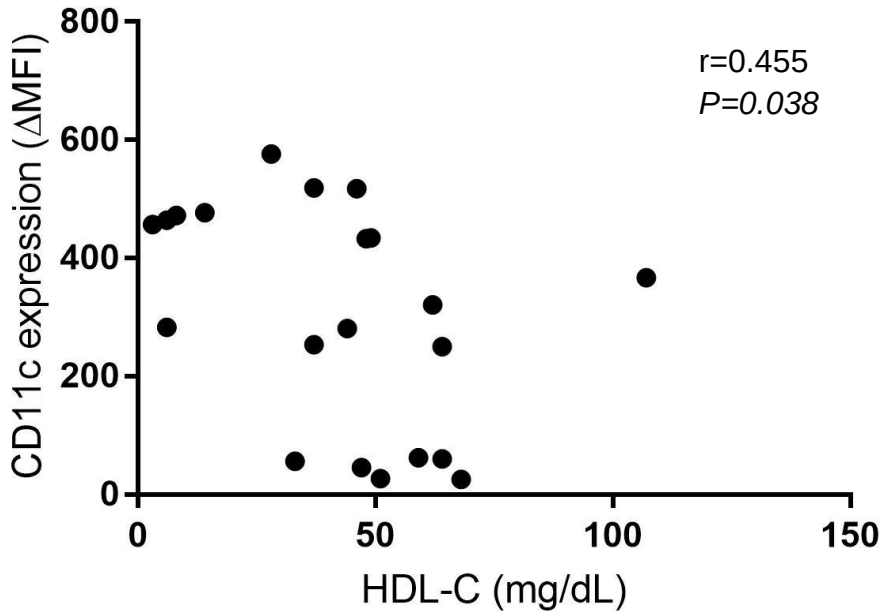

Figure 24. Correlation between HDL-C and CD11c expression on monocytes of study subjects (n=21)

3.4. Expression of cholesterol uptake and activation markers on monocytes

By flow cytometry expression of markers of cholesterol uptake on monocytes was assessed. No difference was found between carriers and controls in the expression of scavenger receptor class A (SRA) and CD36 (Figure 25). Moreover, activation marker expression, i.e. toll-like receptors (TLR) was found to be similar (Figure 26).

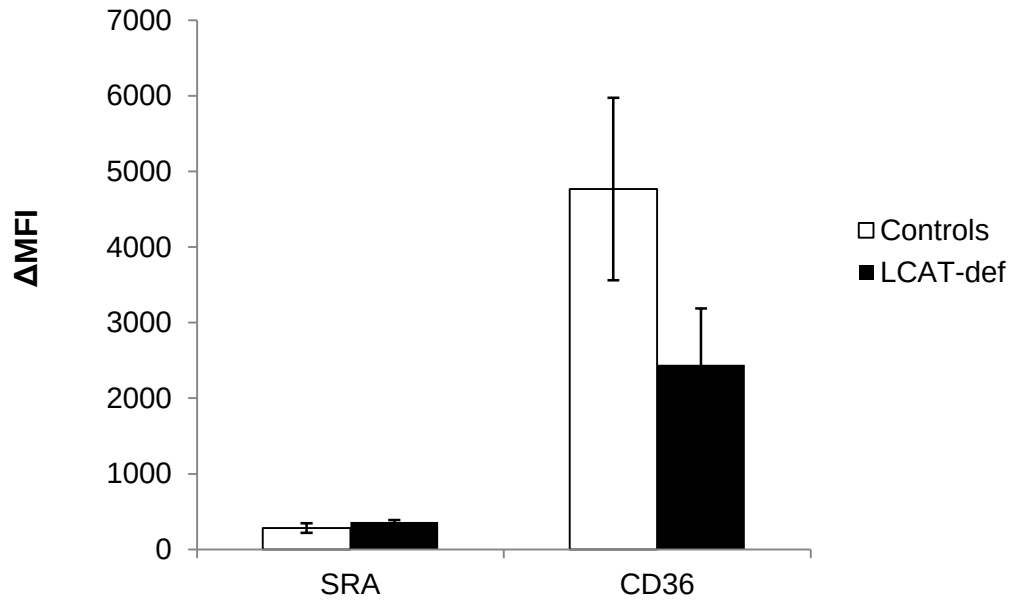

Figure 25. Scavenger receptor expression on total monocytes as assessed with flow cytometry in carriers of LCAT deficiency (n=12, white bars) compared to controls (n=9, black bars). Bar graphs display the expression (quantified as delta MFI). Data are expressed as media ± SEM.

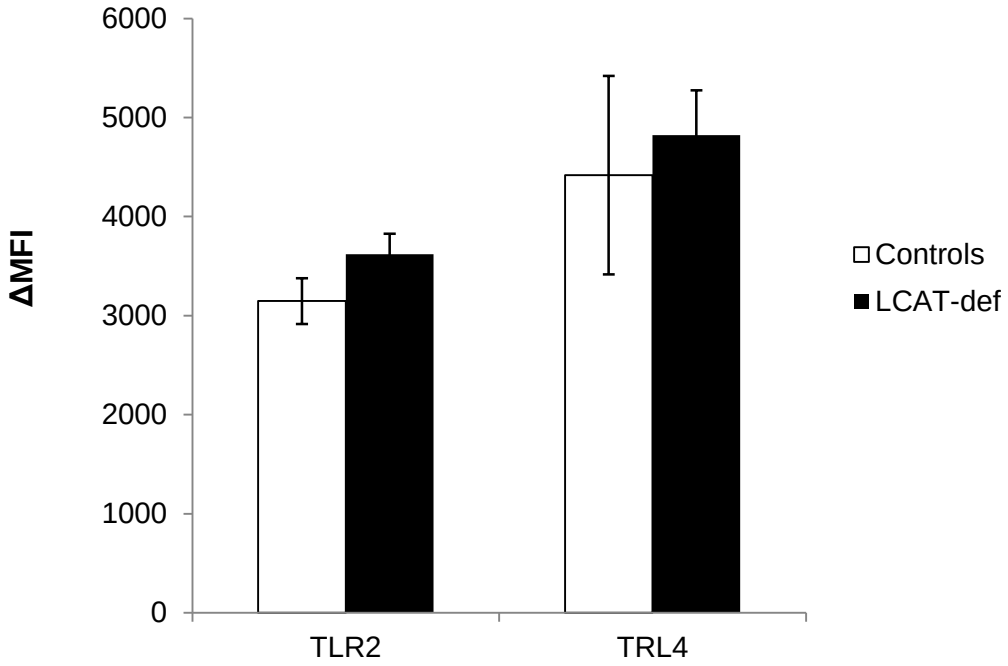

Figure 26. Activation markers expression on total monocytes as assessed with flow cytometry in carriers of LCAT deficiency (n=12, black bars) compared to controls (n=9, white bars). Bar graphs display the expression (quantified as delta MFI). Data are expressed as media ± SEM.

3.5. Monocyte transendothelial migration

Since CD11c is involved in adhesion and migration of monocytes to the vessel wall, we aimed to address functional significance of reduced CD11c expression, by measuring *ex vivo* TEM rate of monocytes isolated from carriers and controls. Monocyte migration rate directly correlates with the expression of CD11c (r=0.725, *P=0.008)* in LCAT-def patients (but also in the total population, *P*<0.001, data not shown)(Figure 27). In accordance, we observed a consistent reduction in monocyte migration in LCAT deficient carriers compared with control subjects (*P=0.006)*, as depicted in Figure 28. Also in this case the effect was gene-dose dependent, with carriers of two mutant *LCAT* alleles showing the lowest migratory capacity (P_{Trend} *= 0.022,* Figure 29).

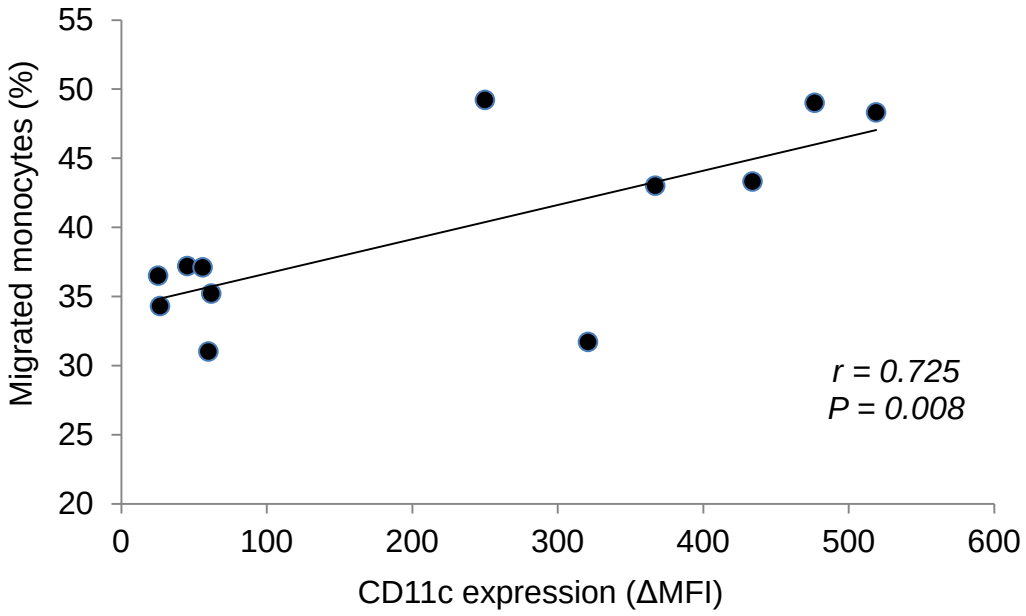

Figure 27. Correlation between CD11c expression and monocyte migration rate in LCAT deficient subjects.

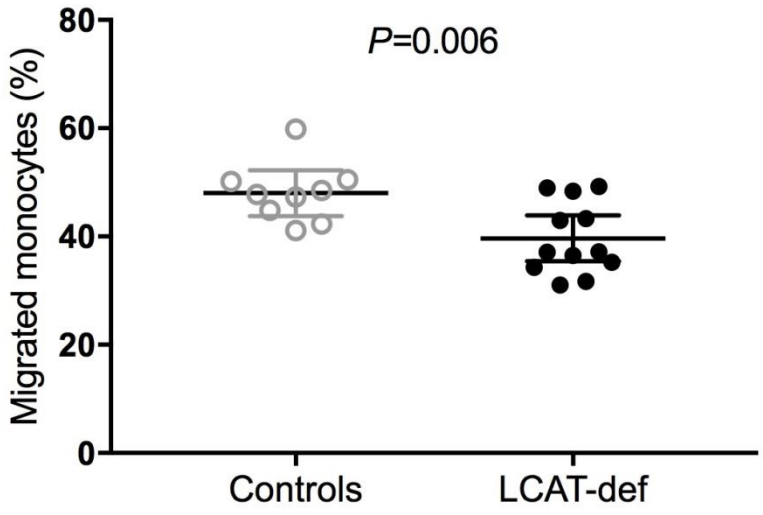

Figure 28. Transendothelial migratory capacity of monocytes isolated from carriers of LCAT mutation (n=12, filled circles) compared to controls (n=9, open circles). Data are presented as percentage of transmigrated cells/total monocytes per field of view.

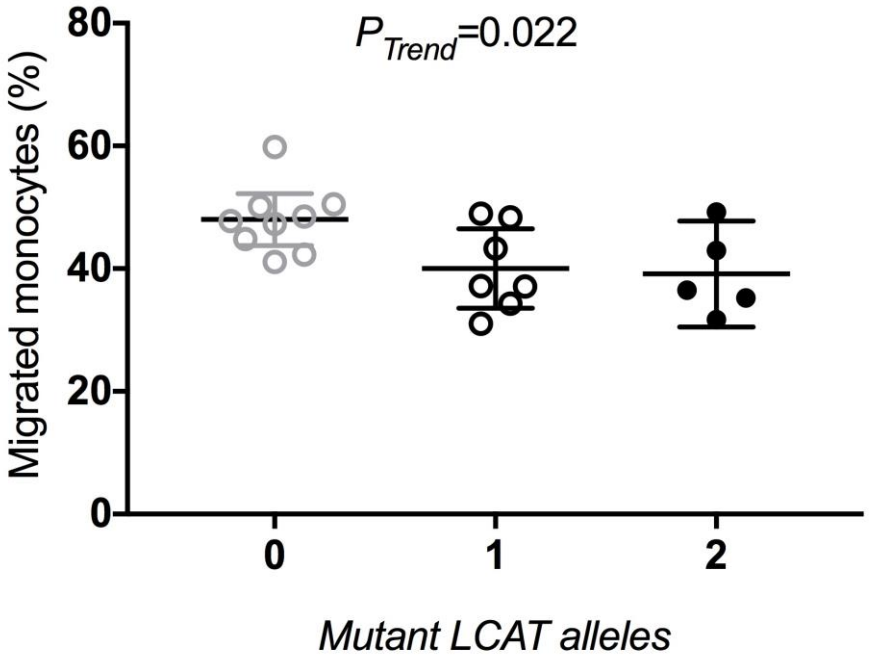

Figure 29. Transendothelial migratory capacity of monocytes according to the number of mutant *LCAT* alleles. Two mutant *LCAT* allele (n=5, filled circles), one mutant *LCAT* allele (n=7, open black circles) compared to controls (n=5, grey open circles). Data are presented as percentage of transmigrated cells/monocytes in field of view

3.6. Monocytes challenge with Toll-like receptor ligands

To assess whether isolated monocytes from patient with genetic *LCAT* mutations have an enhanced inflammatory response, we performed a challenge assay using TLR ligands and then we measured the cytokine production in the supernatant. Cells were stimulated *ex vivo* for 24 hours with TLR4 ligand lipopolysaccharide (LPS). Although a trend was observed, only interleukin 8 (IL-8) production was significantly lower in carriers compared to controls (*P=0.027)* (Figure 30). Upon stimulation with a TLR2 ligand (Pam3Cys) monocytes from LCAT deficient patients produced comparable levels of cytokines, with the exception of interleukin-1 beta (IL-1β) that was decreased compared to controls with a borderline significance (*P*=0.051) (Figure 30). Interestingly, unstimulated monocytes (only RPMI) showed a lower production of monocyte chemo-attractant protein 1 (MCP-1) compared to controls (*P*=0.018) (Figure 30).

In order to evaluate whether these reduced Pam3Cys-induced production of IL-1β and LPS-induced production of IL-8 were gene-dose dependent, we stratified for the number of alleles. As depicted in Figure 32 there was a significant gene-dose effect in the production of IL-1β (A), while this effect was not seen in IL-8 production after LPS stimuli (B). A strong inverse correlation was seen between the number of mutant *LCAT* alleles and the baseline concentration of MCP-1 (C).

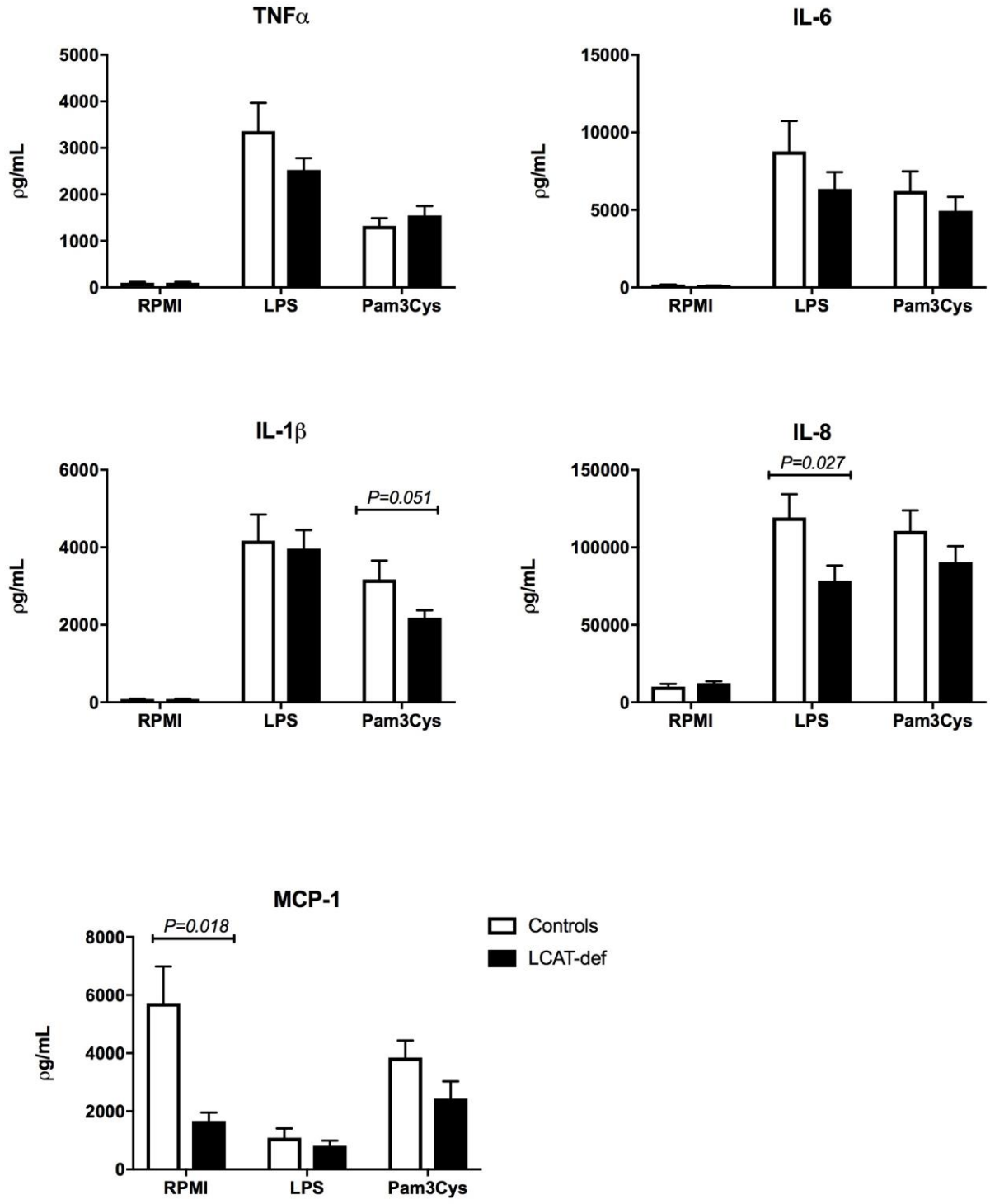

Figure 30. Pro-inflammatory cytokine production in response to a 24-hour challenge with LPS and Pam3Cys (10 µg/mL) in monocytes isolated from LCAT deficient subjects (n=12, black bars) and controls (n=9, white bars). Data are expressed as mean ± SEM.

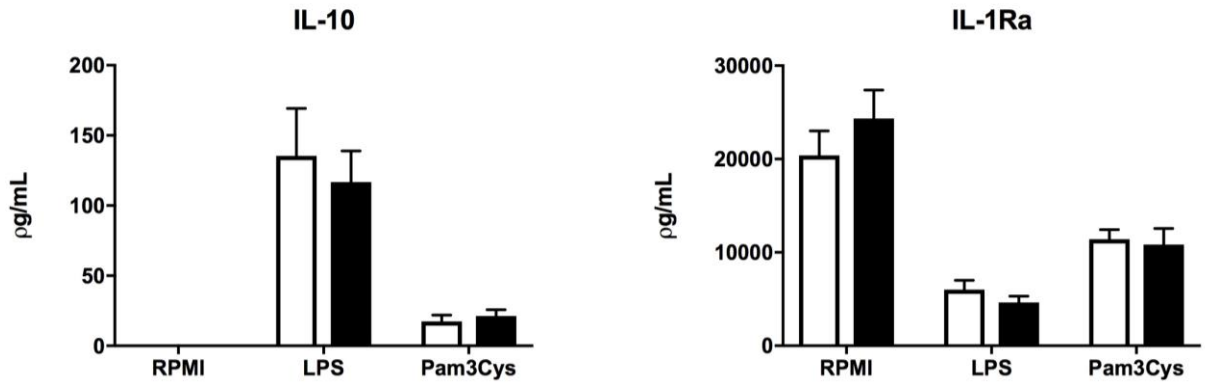

Figure 31. Anti-inflammatory cytokine production in response to a 24-hour challenge with LPS and Pam3Cys (10 µg/mL) in monocytes isolated from LCAT deficient subjects (n=12, black bars) and controls (n=9, white bars). Data are expressed as mean ± SEM.

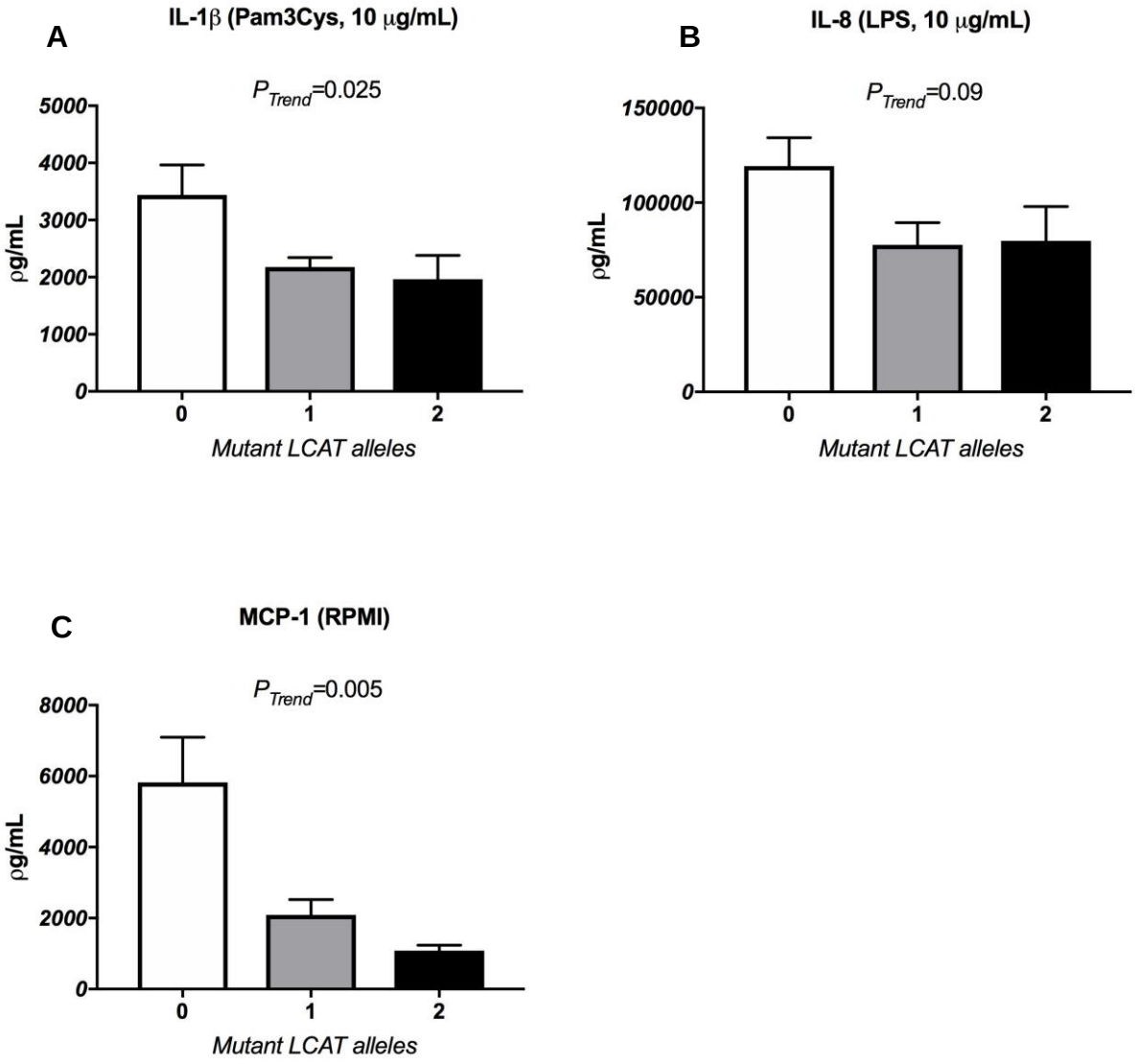

Figure 32. Gene dose effect in reduction of IL-1β production after Pam3Cys stimuli (10 μg/mL) (A), IL-8 after LPS stimuli (10 μg/mL) (B) and MCP-1 production in unstimulated cells (C). Two mutant *LCAT* allele (n=5, black bars), one mutant *LCAT* allele (n=7, grey bars) compared to controls (n=9, white bars). Data are expressed as mean ± SEM.

3.7. Baseline mRNA expression of cytokine in monocytes

In addition we measured baseline mRNA expression of cytokines and gene involved in cholesterol metabolism. While increased mRNA expression of CD11c was found, other gene were expressed similarly between LCAT deficient patients and controls. No difference in gene expression was found between carriers and controls, although a trend in *abca1* and *abcg1* was observed, with higher *abca1* and *abcg1* in genetic *LCAT* mutations (0.72 vs 2.10, *P=0.068* and 0.65 vs 2.33, *P=0.055* in controls vs LCAT-def) (Figure 34).

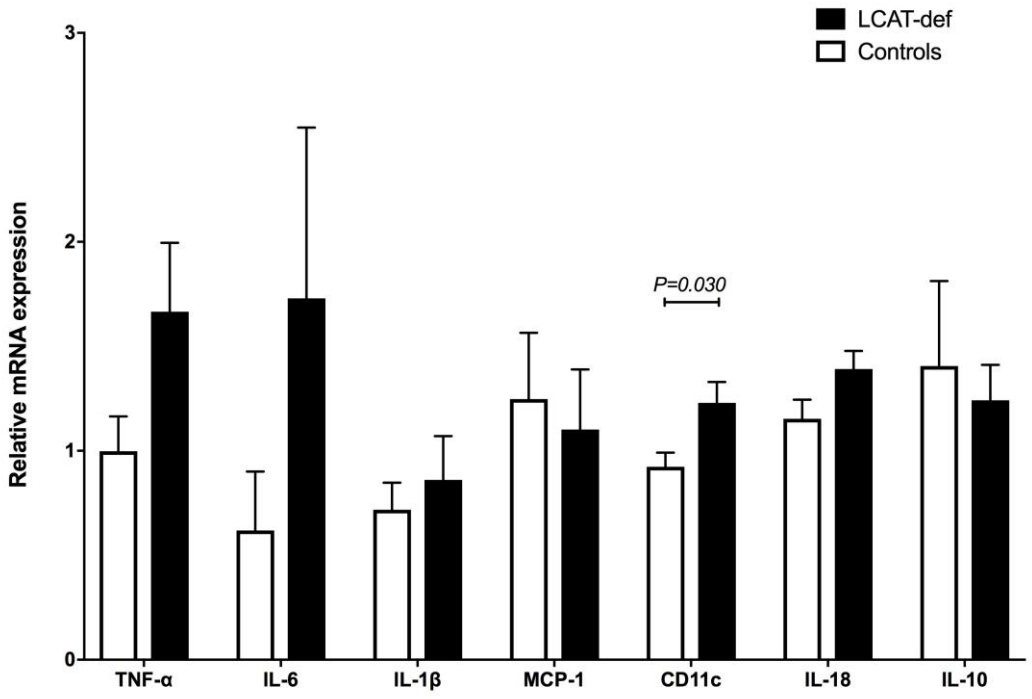

Figure 33. Baseline mRNA expression levels of inflammation genes. Data are expressed as mean ± SEM.

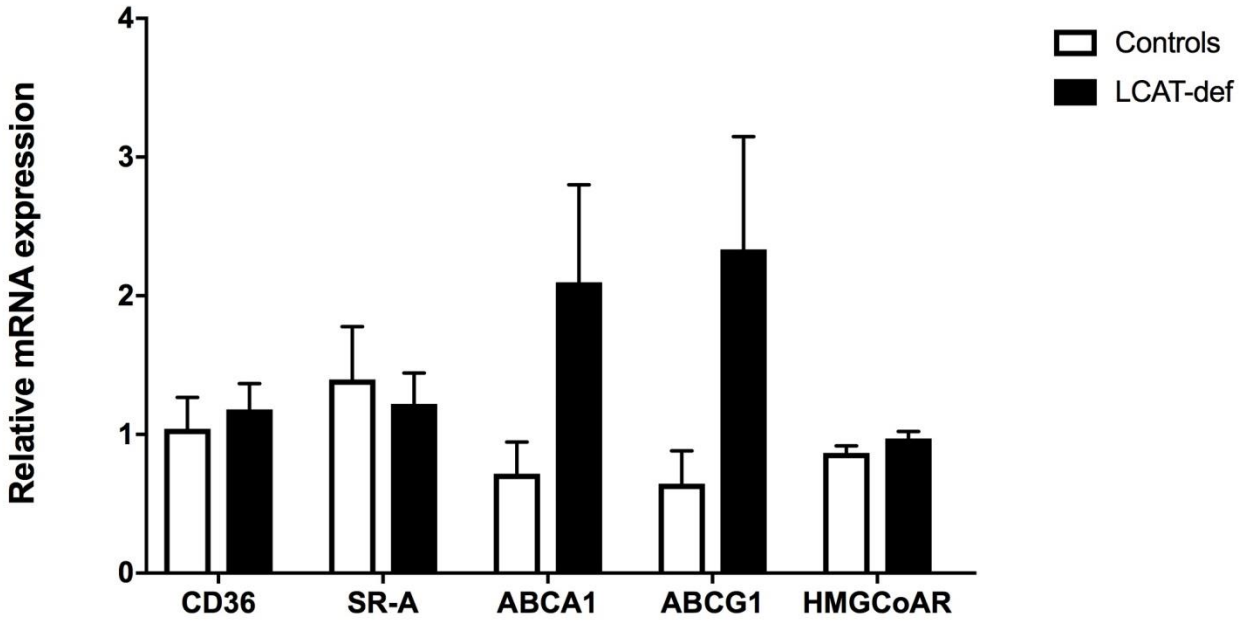

Figure 34. Baseline mRNA expression levels of gene involved in cholesterol metabolism. Data are expressed as mean ± SEM.

3.8. Comparison with other genetic hypoalphalipoproteinemias

To evaluate whether the effect on monocytes was dependent on low HDL-C in LCAT deficient subjects, we added to the study a comparison with other genetically determined hypoalphalipoproteinemias, as ABCA1 and APOA1 deficiency. Exclusion criteria were the previously mentioned. Briefly, absence of cardiovascular or renal disease, diabetes or any chronic/acute inflammatory condition.

In Table 10 are presented the clinical characteristics of the populations and of the control group matched by age, gender and BMI.

Table 10. Clinical characteristics of carriers of ABCA1 and APOA1 deficiency and control subjects

	ABCA1-def	**APOA1-def**	**Controls**	P_{Anova}
n	10	9	9	
Age (years)	44.7 ± 4.2	45.2 ± 5.2	41.2 ± 4.0	0.801
Gender (male/female)	3/7	3/6	5/4	0.793
BMI (kg/m^2)	28.4 ± 2.0	27.5 ± 1.9	24.5 ± 1.3	0.165
Smoking (n,%)	5 (50)	6 (67)	5 (44)	0.615
TC (mg/dL)	166.2 ± 12.6	125.7 ± 14.1	208.5 ± 9.6	<0.0001
TG (mg/dL)	155 (81-265)	157 (119-223)	113 (93-128)	0.280
LDL-C (mg/dL)	109.7 ± 5.5	87.4 ± 15.8	121.5 ± 7.7	0.086
HDL-C (mg/dL)	31.7 ± 2.7	20.0 ± 1.8	63.4 ± 5.9	<0.0001
ApoA-I (mg/dL)	76.7 ± 3.4	58.2 ± 5.7	121.1 ± 7.9	<0.0001
ApoB (mg/dL)	111.2 ± 10.1	84.7 ± 9.0	93.9 ± 2.9	0.077
Glucose (mg/dL)	90.8 ± 4.2	87.4 ± 6.0	87.1 ± 4.2	0.841
Statin treatment (n,%)	6 (60)	5 (56)	1 (11)	0.064

Data are expressed as mean ± SEM for all variables.

As for LCAT-deficiency, ABCA1 and APOA1 deficiency were not associated with increased circulating levels of hsCRP, as well as of other cytokines (Table 11).

Table 11. Plasma levels of hsCRP and cytokines in carriers of ABCA1 and APOA1 deficiency and controls subjects

	ABCA1-def	APOA1-def	Controls	*P-value*
hsCRP (mg/L)	2.2 (0.5-10.3)	1.8 (0.7-7.2)	1.5 (0.8-3.4)	0.485
MCP-1 (ρg/mL)	42.9 (36.2-59.1)	62.0 (46.6-85.2)	74.4 (32.5-101.5)	0.254
TNFα (ρg/mL)	2.1 (1.5-2.2)	2.3 (1.7-2.7)	1.8 (1.5-2.3)	0.446
IL-6 (ρg/mL)	1.0 (0.6-2.2)	1.4 (1.0-3.3)	1.2 (0.8-1.6)	0.346

Data are expressed as median and interquartile range for all variables.

Notably, hsCRP inversely correlates with the plasmatic concentration of apoA-I in APOA1-def carriers (Figure 35), but not in ABCA1-def or in the total study populations.

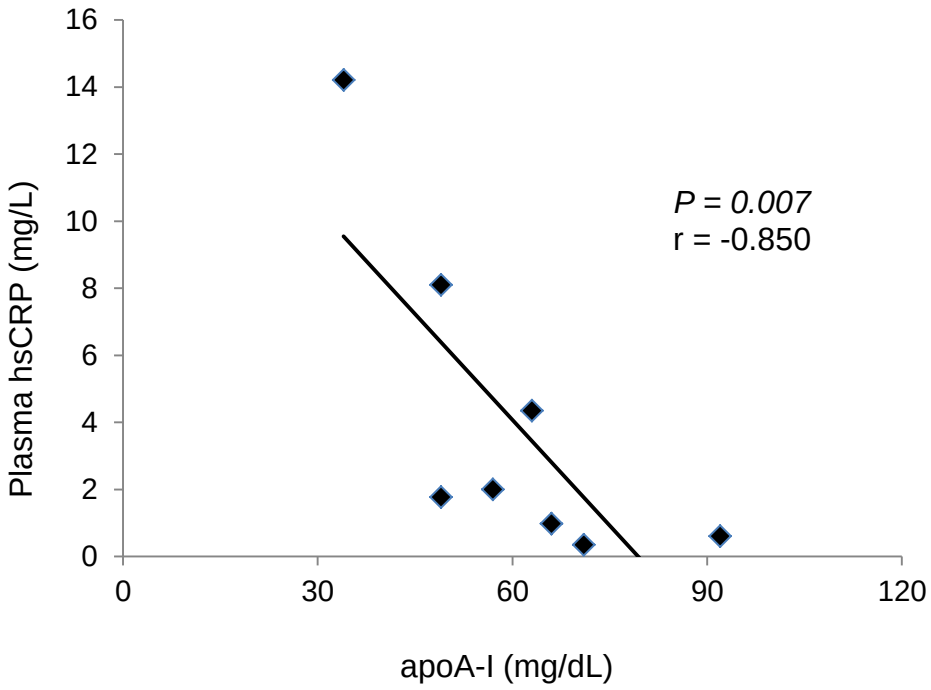

Figure 35. Correlations between plasma hsCRP and apoA-I in carriers of *APOA1* mutation

Monocyte subsets distribution were comparable in all genotypes, as shown in Figure 36.
Expression of chemokine, adhesion, transmigration and cholesterol uptake markers on blood monocytes (and in all monocyte subsets) was not different between mutations carriers and controls, including CD11c expression (Figure 37-Figure 39).
Interestingly, when activation markers expression on monocytes was assessed with flow cytometry, TLR2 expression was higher in *APOA1* mutation carriers compared to *ABCA1* and controls, although it was statistically significant only when intermediate and non-classical monocytes were considered (Figure 40). In a post-hoc analysis, TLR2 expression in APOA1-def was higher compared to ABCA1-def (*P=0.028* and *P=0.034* for intermediate and non-classical subset respectively after Bonferroni's multiple comparisons), while was similar compared to controls (Figure 40).

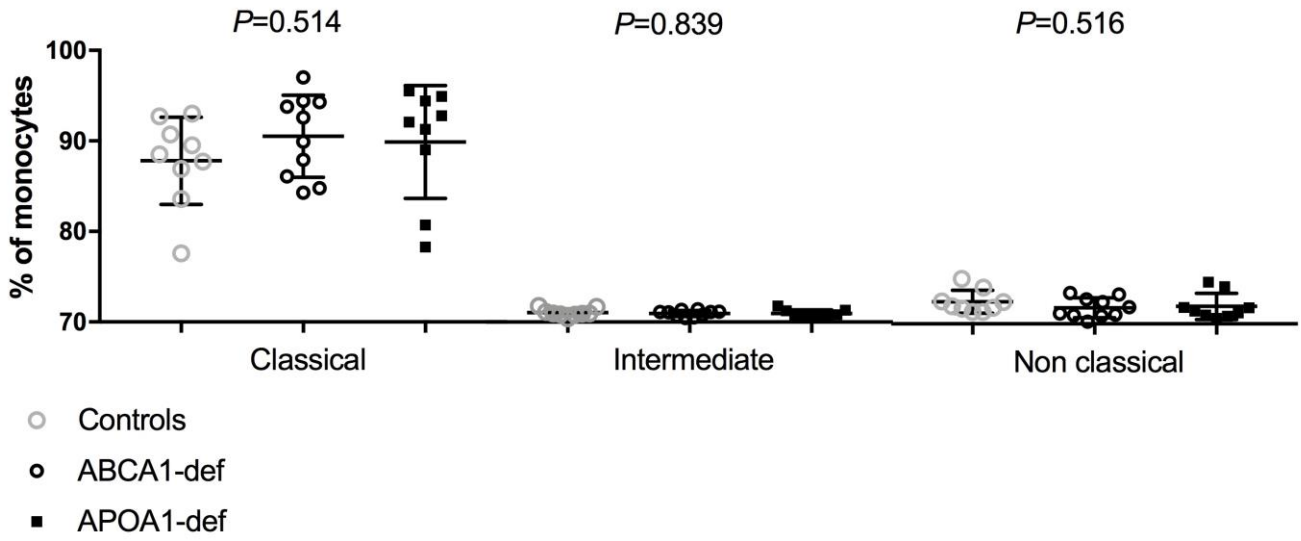

Figure 36. Percentage of monocyte subsets (classical ($CD14^{++}/CD16^{-}$), intermediate ($CD14^{+}/CD16^{+}$), or non-classical ($CD14^{+}/CD16^{++}$) in carriers of ABCA1 deficiency (n = 10, black open circles), APOA1 deficiency (n=9, filled squares) vs. controls (n = 9, grey open circles).

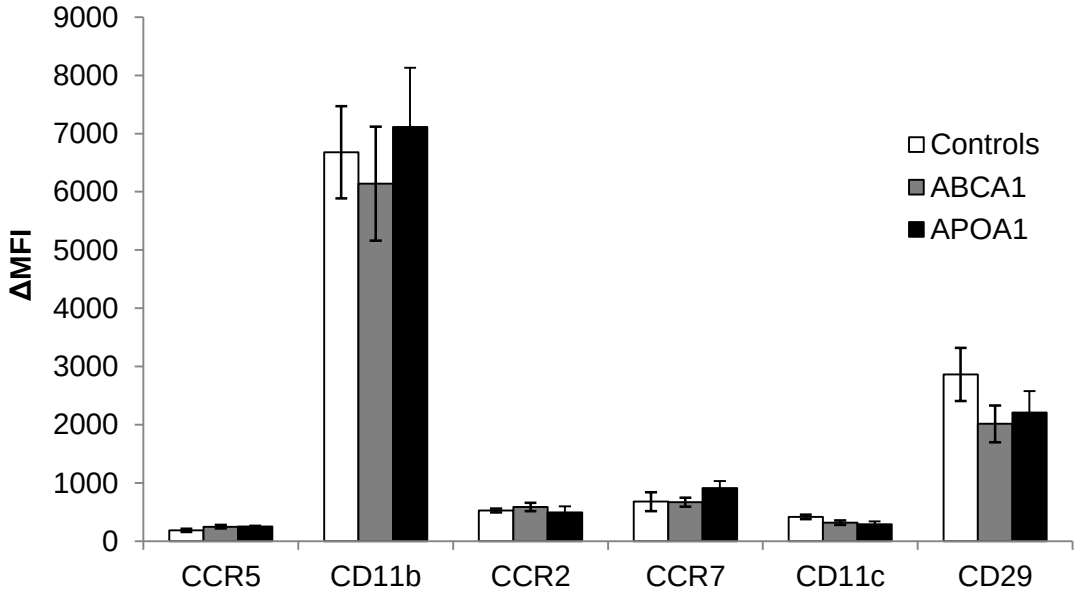

Figure 37. Chemokine, adhesion and transmigration markers on total monocytes as assessed with flow cytometry in carriers of ABCA1 (n=10, grey bars) e APOA1 (n=9, black bars) deficiency compared to controls (n=9, white bars). Bar graphs display the expression (quantified as delta MFI). Data are expressed as media ± SEM. * $P < 0.05$.

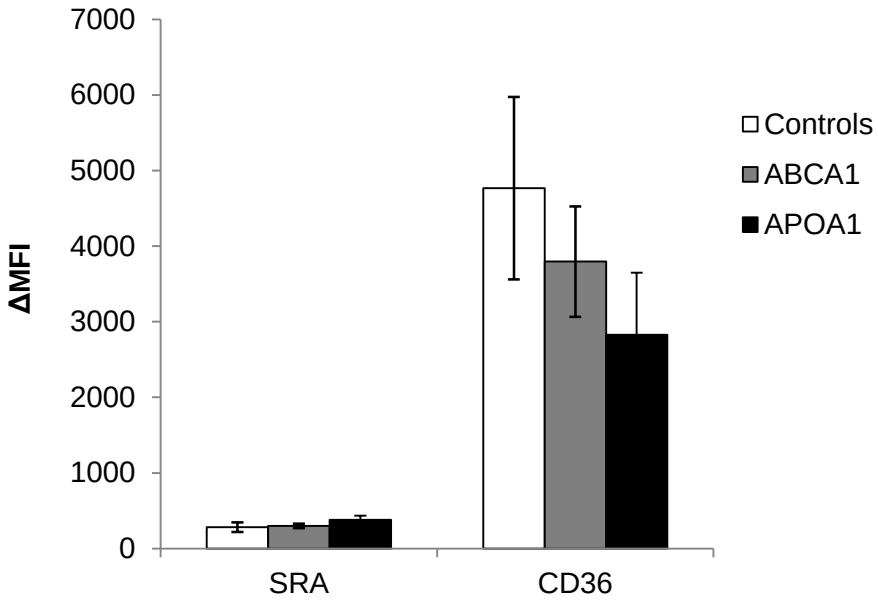

Figure 38. Scavenger receptor expression on total monocytes as assessed with flow cytometry in carriers of ABCA1 (n=10, grey bars) and APOA1 (n=9, black bars) mutations compared to controls (n=9, white bars). Bar graphs display the expression (quantified as delta MFI). Data are expressed as media ± SEM.

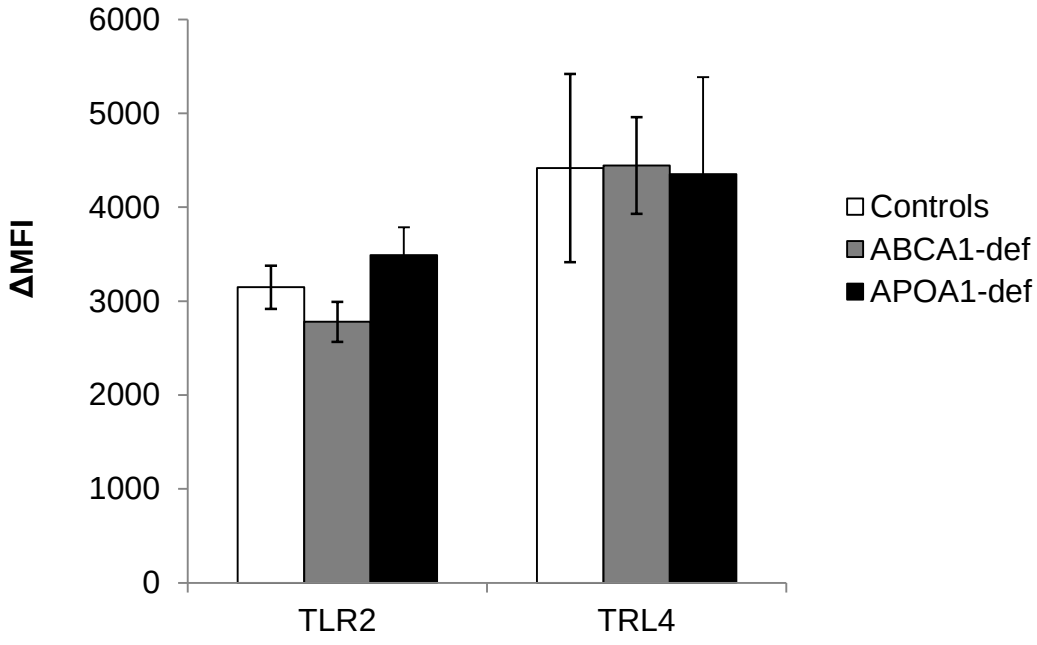

Figure 39. Activation markers expression on total monocytes as assessed with flow cytometry in carriers of ABCA1 (n=10) and APOA1 (n=9) mutations compared to controls (n=9). Bar graphs display the expression (quantified as delta MFI). Data are expressed as media ± SEM

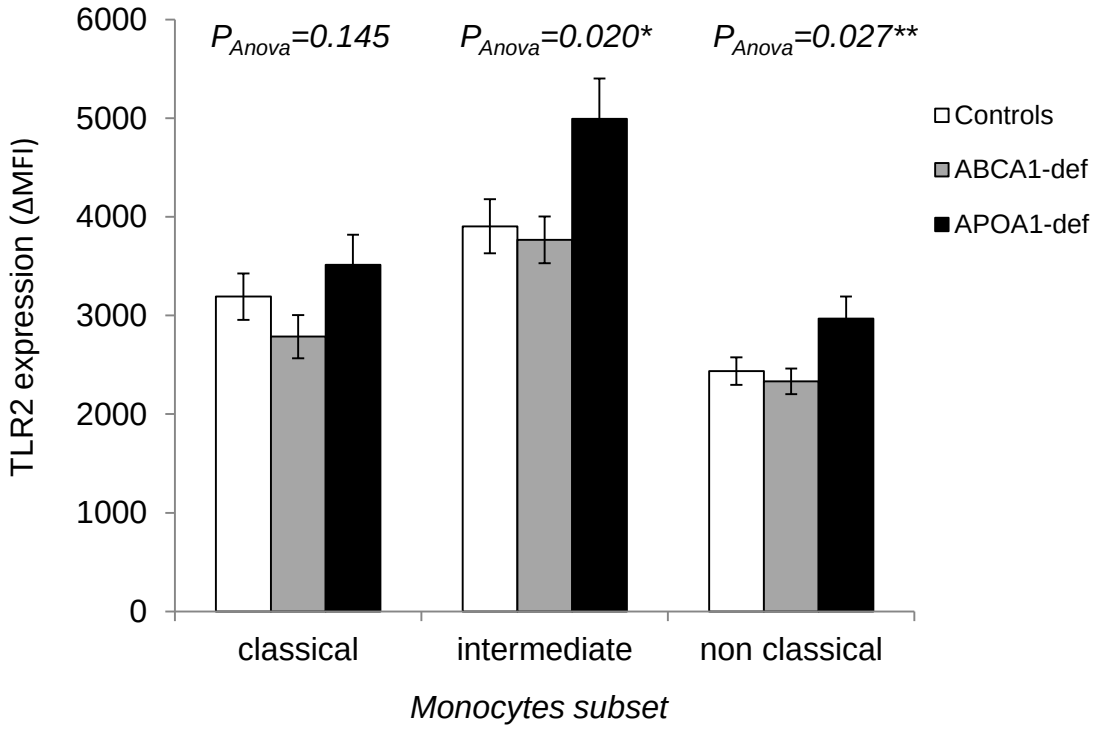

Figure 40. TLR2 expression in different monocyte subset as assessed by flow-cytometry in carriers of ABCA1 (n=10, white bars), APOA1 (n=9, grey bars) mutations and controls (n=9, black bars). Bar graphs display the expression (quantified as delta MFI). Data are expressed as media ± SEM. * *P=0.028* and ***P=0.034* after Bonferroni's multiple comparisons.

TEM was measured in HAEC on isolated monocytes in carriers of *ABCA1* and *APOA1* mutations. As expected we found no difference between groups as depicted in Figure 41.

Ex vivo stimulation of monocytes isolated from carriers and controls with LPS and Pam3Cys did not show any difference in the cytokine production, neither pro-inflammatory (Figure 42, Figure 44), nor anti-inflammatory (Figure 43, Figure 45).

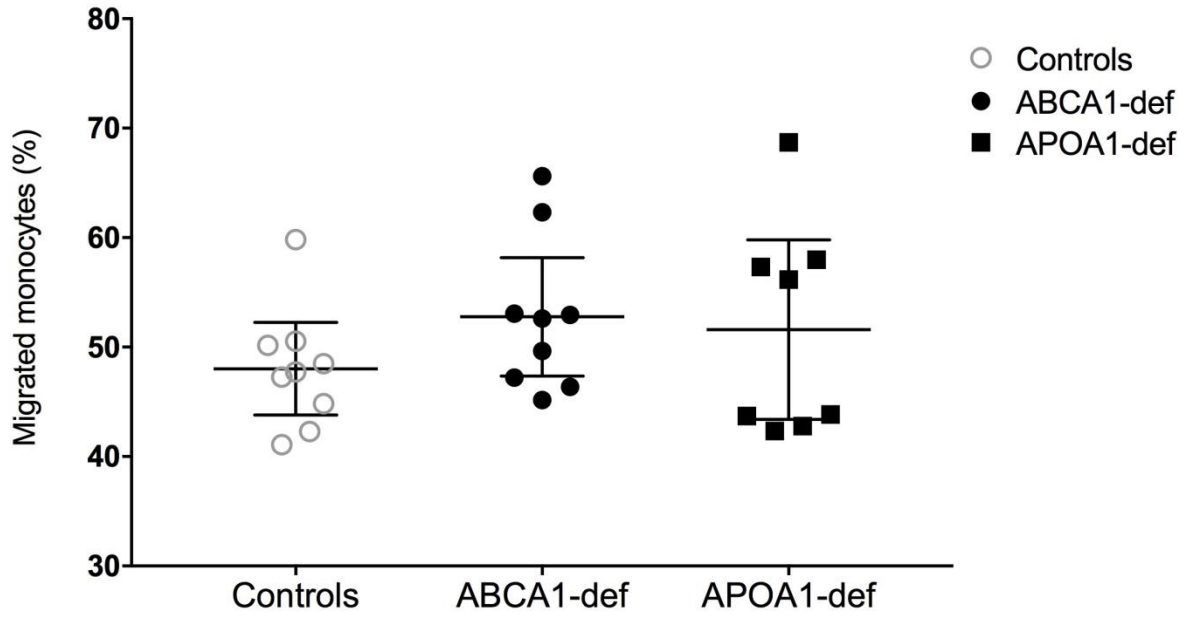

Figure 41. Transendothelial migratory capacity of monocytes isolated from carriers of *ABCA1* (n=9, black circles) and *APOA1* mutations (n=8, black squares) and controls (n=9, grey circles). Data are presented as percentage of transmigrated cells/total monocytes per field of view.

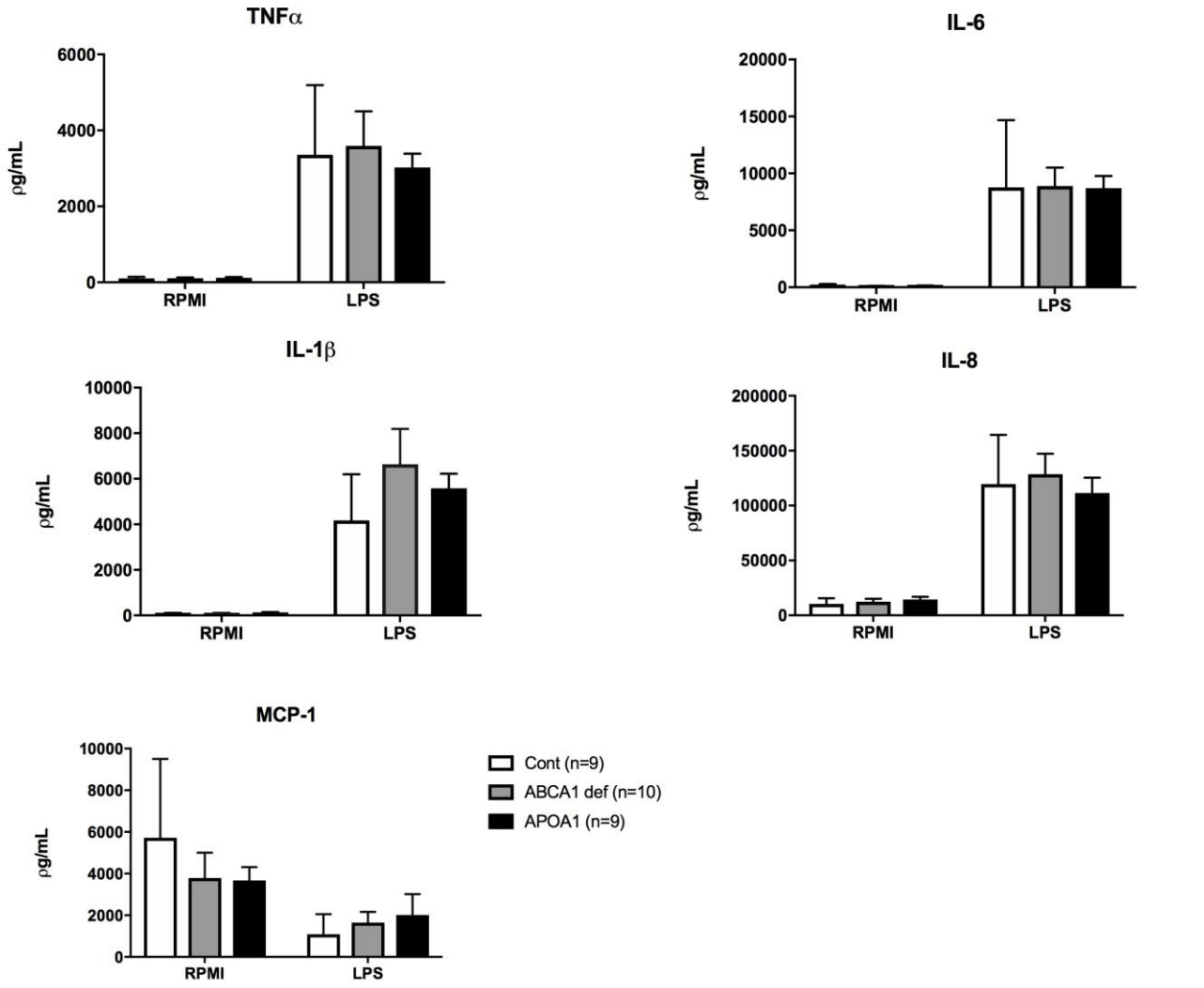

Figure 42. Pro-inflammatory cytokine production in response to a 24-hour challenge with LPS (10 µg/mL) in monocytes isolated from ABCA1 (n=9, grey bars) and APOA1 (n=9, black bars) deficient subjects compared to controls (n=7, white bars).

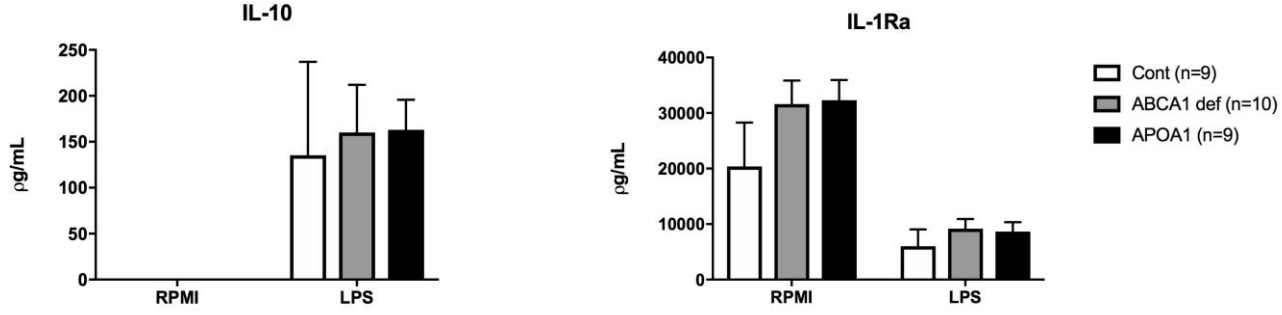

Figure 43. Anti-inflammatory cytokine production in response to a 24-hour challenge with LPS (10 µg/mL) in monocytes isolated from ABCA1 (n=9, grey bars) and APOA1 (n=9, black bars) deficient subjects compared to controls (n=7, white bars).

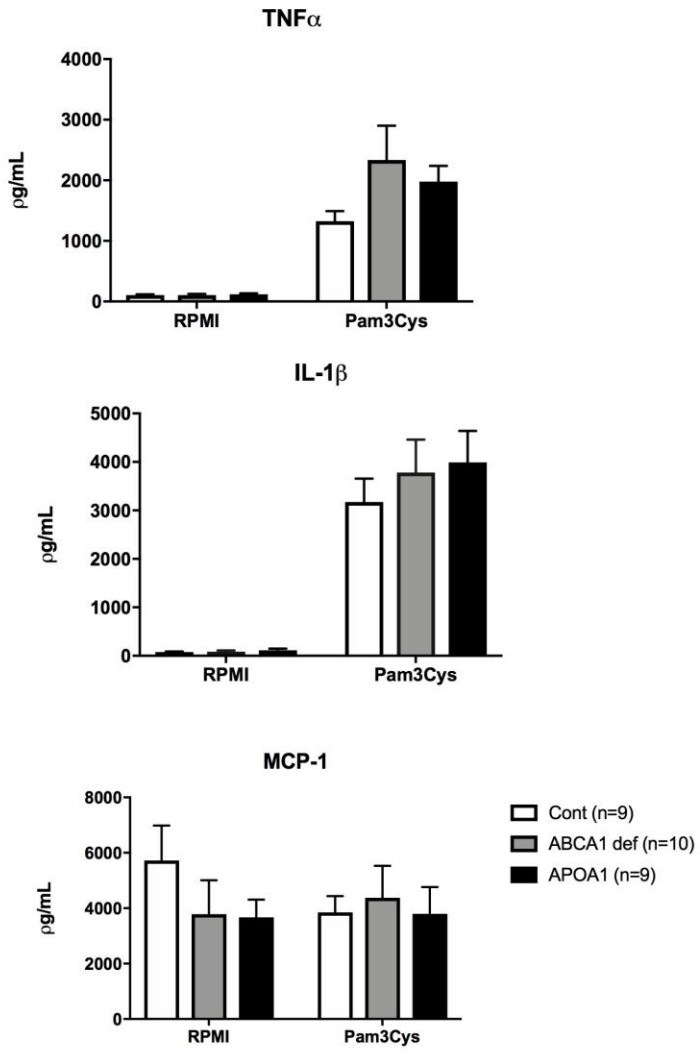

Figure 44. Pro-inflammatory cytokine production in response to a 24-hour challenge with Pam3Cys (10 µg/mL) in monocytes isolated from ABCA1 (n=9, grey bars) and APOA1 (n=9, black bars) deficient subjects compared to controls (n=7, white bars).

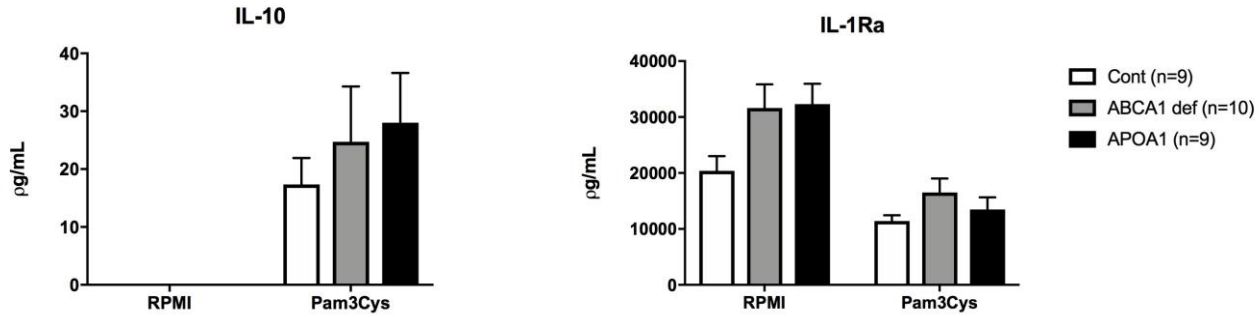

Figure 45. Anti-inflammatory cytokine production in response to a 24-hour challenge with LPS (10 µg/mL) in monocytes isolated from ABCA1 (n=9, grey bars) and APOA1 (n=9, black bars) deficient subjects compared to controls (n=7, white bars).

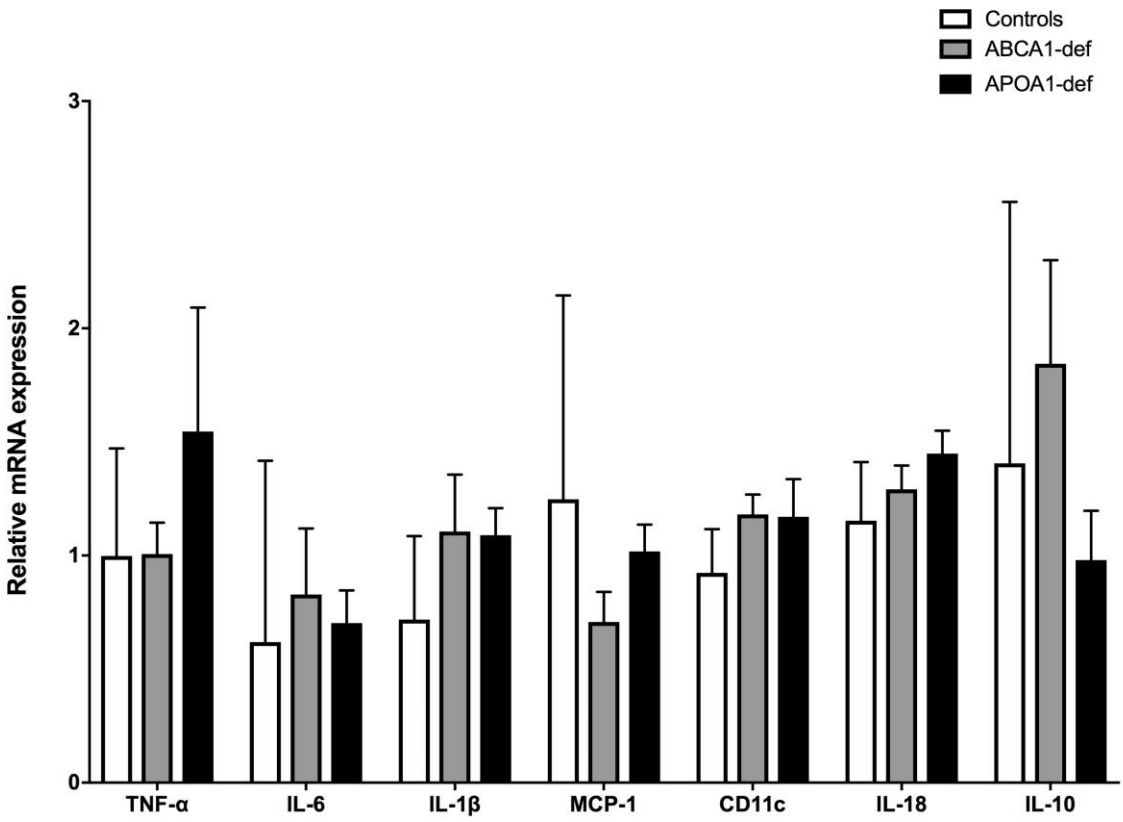

Figure 46. Baseline mRNA expression levels of inflammation genes. Data are expressed as mean ± SEM.

Baseline expression of genes involved in inflammation (Figure 46) and cholesterol metabolism were also measured and no difference was found between carriers and controls. Conversely, mRNA of *hmgcoar* was significantly higher in ABCA1-def and APOA1-def compared to controls (*P=0.016)*. The significance was maintained also after correction for statin treatment (*P=0.034)* (Figure 47)

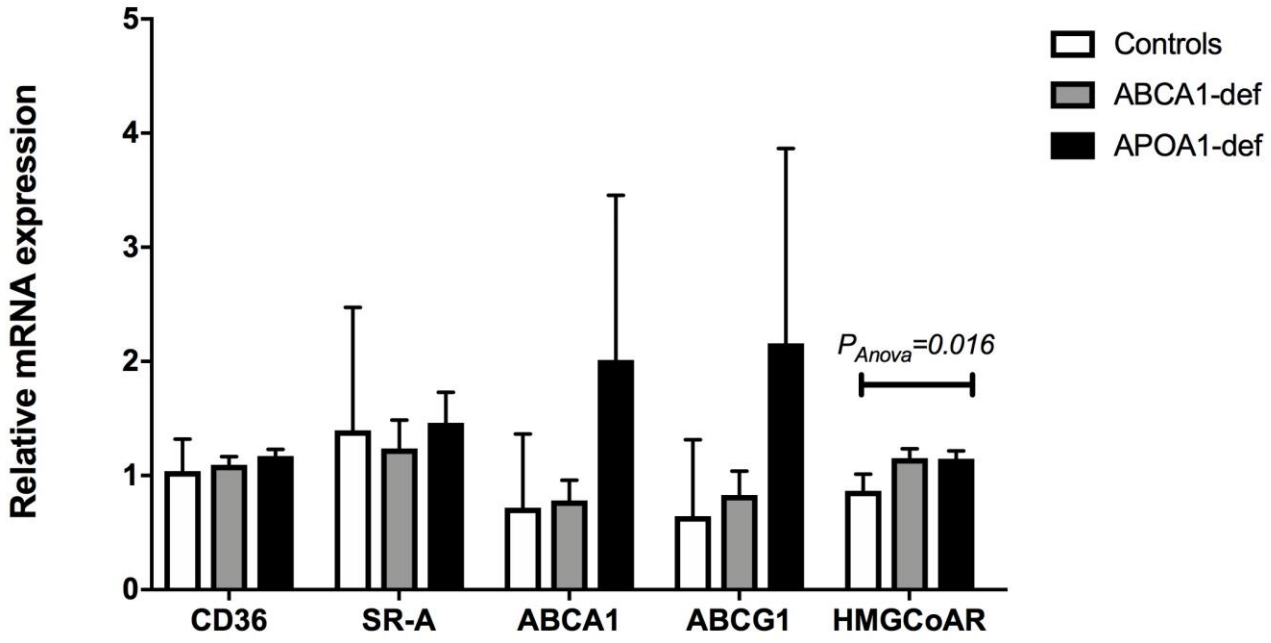

Figure 47. Baseline mRNA expression levels of gene involved in cholesterol metabolism. Data are expressed as mean ± SEM. **P=0.034* after correction for statin treatment.

3.9. Correlations between mRNA expression of inflammatory markers, HDL-C and apoA-I

Although the number of subjects were too small, we ran some correlations to assess whether HDL-C or apoA-I concentrations are associated with mRNA expression of inflammatory markers in our study populations.

Table 12. Correlations between inflammation parameters and HDL-C

	Total		*ABCA1*		*APOA1*		*LCAT*		*CONTROLS*	
Parameter	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
il1β	-0.412	0.009	-0.674	0.023	-0.008	0.983	-0.364	0.272	0.643	0.086
il18	-0.335	0.037	-0.456	0.159	0.234	0.544	-0.236	0.484	0.095	0.823

Table 13. Correlations between inflammation parameters and apoA-I

	Total		*ABCA1*		*APOA1*		*LCAT*		*CONTROLS*	
Parameter	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
il1β	-0.325	0.047	-0.354	0.316	-0.301	0.431	-0.191	0.574	0.786	0.021
il18	-0.323	0.048	-0.305	0.392	0.109	0.781	-0.227	0.502	-0.095	0.823

mRNA expression of IL-1β and IL-18 inversely correlated with plasma HDL-C (r=-0.412, *P*=0.009 and r=-0.335, *P*=0.037 respectively) as well as with apoA-I (r=-0.325, *P*=0.047 and r=-0.323, *P*=0.048). In addition HMGCoAR expression negatively correlates with HDL-C (r=-0.391, *P*=0.014) and apoA-I (r=-0.416, *P*=0.009). Correlation significance was lost when stratified for different genotype.

4. VASCULAR STRUCTURE AND FUNCTION IN CARRIERS OF LCAT DEFICIENCY

4.1. Carotid intima media thickness

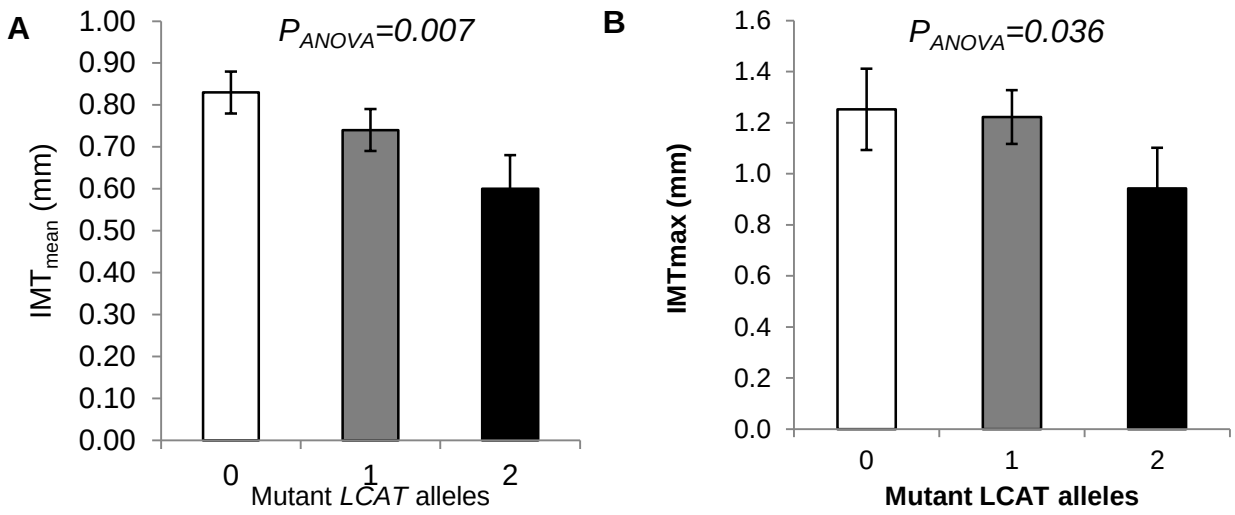

Figure 48. Carotid intima media thickness measurement in LCAT deficient and control subjects. Data are expressed as mean ± SEM; two mutant *LCAT* allele carriers (n=11, black bars), one mutant *LCAT* allele carriers (n=36, grey bars) compared to controls (n=21, white bars); P_{Anova} adjusted for age, gender and family.

To evaluate whether a defect in *LCAT* gene, leading to low HDL-C concentration, is associated to cardiovascular disease, carotid intima-media thickness (IMT), as an index of subclinical atherosclerosis, was measured on a representative subgroup of subjects,. Despite the atherogenic lipid profile, carrying *LCAT* mutation is not associated with an increased atherosclerosis degree. Indeed, maximum carotid IMT was even decreased in carriers of two mutant *LCAT* alleles, compared to controls (0.943 ± 0.224 vs 1.252 ± 0.159), while carriers of one mutant allele displayed a comparable degree of atherosclerosis (1.222 ± 0.105). Moreover, presence of *LCAT* mutation in our population is not associated with differences in mean carotid IMT (Figure 48).

4.2. Flow-mediated vasodilation

As an *in vivo* indicator of nitric oxide production and endothelial functionality (195), flow-mediated vasodilation (FMD) was measured in a small subgroup of patients with LCAT deficiency. Heterozygous (n=10) and homozygous (n=5) carriers were pooled together for statistical analysis requirement. Characteristics of the included subjects are listed in Table 14. In accordance with the *in vitro* findings, despite low HDL-C, carriers of dysfunctional LCAT did not show impaired FMD (Figure 49).

Table 14. Clinical and biochemical characteristics of carriers of *LCAT* gene mutations included in the FMD assessment

	Carriers	Controls	*P*
n	15	35	
Age (years)	43.9 ± 3.0	44.1 ± 1.9	0.97
Gender (male/female)	8/7	19/16	0.95
BMI (kg/m^2)	23.4 ± 0.8	23.7 ± 0.5	0.75
TC (mg/dL)	160.8 ± 12.0	199.1 ± 6.8	0.005
LDL-C (mg/dL)	105.2 ± 9.8	124.0 ± 5.3	0.07
HDL-C (mg/dL)	32.9 ± 5.4	56.1 ± 2.2	<0.0001
Triglycerides (mg/dL)	113.1 ± 12.4	95.1 ± 9.2	0.27

Data are presented as mean ± SEM.

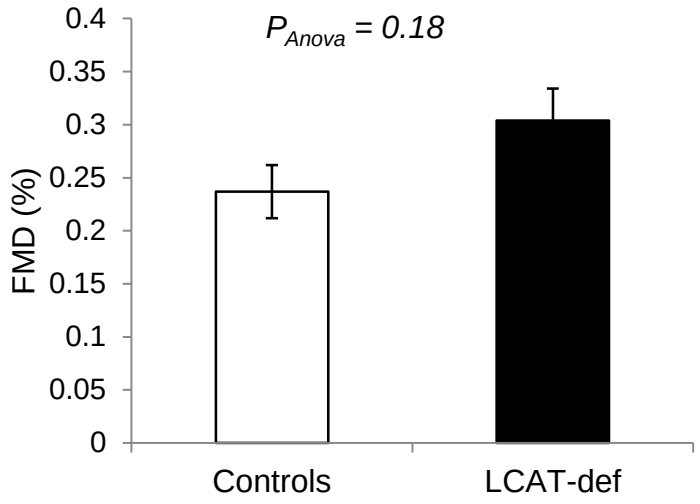

Figure 49. Flow-mediated vasodilation in LCAT deficient and control subjects. Data are expressed as mean ± SEM; n=35 controls, n=21 carriers (n=10 heterozygotes and n=5 homozygotes); P_{Anova}=0.18; P_{Anova}=0.22 after adjustment for HDL-C.

DISCUSSION

Endothelial dysfunction is a peculiar aspect of atherosclerosis and is characterized by disruption of the vascular homeostasis, mediated by altered balance of substances that modulate vascular tone, inflammation and hemostasis. A peculiar switch of endothelial surface from non-adhesive to pro-adhesive is also seen (51). A number of studies have shown a direct protective effect of HDL on endothelial cells, by promoting the production of NO, the most important regulator of vascular tone, and by downregulating CAM expression (51, 196). HDL subpopulations have demonstrated different efficiency in maintaining endothelial cells homeostasis (197) and in some conditions can lose their positive properties (198). Although genetic LCAT deficiency is characterized by extremely low HDL-C, there is no clear evidence of increased atherosclerosis associated with the disease and some studies have even shown a protective effect of the lack of LCAT (199). Thus we hypothesized that this increased protection was dependent on an enhanced functionality of HDL in protecting against endothelial dysfunction.

The project was split in two parts: (i) studies on endothelial cells and (ii) studies on monocytes.

(i) Studies on endothelial cells

HDL particles distribution is altered in carriers of *LCAT* mutations, that have a reduced content of large HDL_2 and accumulate small HDL_3 particles, mainly immature preβ-HDL (18, 99). Selective depletion in HDL containing apoA-II can be also appreciated (200). It has already been demonstrated that HDL in LCAT deficiency are more capable in promoting ABCA1 efflux from peripheral cells, since preβ-HDL content is more prominent in the serum of patients (59), but not data were available so far on the ability of HDL in LCAT to promote NO production. NO plays a central role in maintaining vascular tone and HDL can stimulate eNOS expression and activation in endothelial cells (201-203). This process involves the binding of apoA-I to SR-BI (78) and thus the activation of the PI3K/Akt signalling pathway, with the subsequent phosphorylation of eNOS (78, 204). HDL isolated from carriers showed an increased ability in eNOS activation, resulting in an increased NO production. Interestingly the effect was gene-dose dependent. The selective (and gene-dose dependent) depletion of LpA-I:LpA-II particles compared to LpA-I could account for this increased capacity. Indeed, while the role of apoA-I is crucial for eNOS activation, as demonstrated by the suppression of NO production in endothelial cells with anti-apoA-I antibody (78), apoA-II presence is detrimental, since anti-apoA-II antibody further enhance eNOS stimulation (78). Accordingly, HDL in CETP deficiency are enriched in LpA-I:A-II and showed reduced ability in stimulating NO production (87). We have

shown that synthetic HDL containing only apoA-I are more efficient in promoting endothelial NO production, likely due to the change in conformation of apoA-I induced by apoA-II (205).

In addition to the presence of apoA-II itself, other protein transported by HDL could account for the enhance efficacy of HDL in LCAT deficiency.

Among other, PON1 has been suggested to have a major impact on endothelial function and PON1 inactivation results in decreased eNOS phosphorylation (193) confirmed in animal studies where PON1-deficient mice fail to stimulate NO production that was restored after PON1 supplementation (193). PON1 is preferentially found in LpA-I particles and apoA-I seems to contribute to PON1 stability (206) while apoA-II can displace this enzyme. Carriers of LCAT deficiency have normal PON1 activity despite the low HDL-C levels (207).

It must be underlined that the large part of patients included in this study had FLD, thus the result of this study may not apply to FED. Indeed in other study, where mainly FED were included, an increased arterial stiffness was measured (161), supporting the clinical distinction between FLD and FED (183).

(ii) Studies on monocytes

In the second part of the project, to complete the assessment on endothelial functionality, the effect of genetic LCAT deficiency on monocytes phenotype and functionality was evaluated for the first time. Endothelial cells activation leads to monocyte recruitment from the blood stream, that represents the initial stage in the atherosclerotic plaque formation and it is mediated by chemoattractants, cell adhesion molecules and their receptors (208). Many studies suggested that the proportion of intermediate monocytes is increased in CVD patients (209), inversely associated with apoA-I and HDL-C levels (210, 211), and independent predictors of major clinical event (209). More recently researchers suggested a role in endothelial dysfunction of non-classical $CD14^{+}/CD16^{++}$ monocytes (212). Hypoalphalipoproteinemia was previously associated with a different prevalence of classical $CD14^{++}/CD16^{-}$ but not of intermediate $CD14^{++}/CD16^{+}$ monocytes (213). Despite low HDL-C, we found no difference in circulating monocyte subset distribution in carriers of LCAT deficiency, which supports absence of increased atherosclerosis in *LCAT* mutation carriers.

Murphy and colleagues have shown that *in vitro* incubation of monocytes with HDL reduces both CD11b expression and monocyte adhesion to endothelial cells, similarly to

lipid-free apoA-I (214) supporting the role of HDL in preventing monocyte recruitment. Opposite observation came from our study, where monocyte of LCAT deficient patients showed a comparable expression of CD11b, but a reduced expression of the similar integrine, CD11c. The reduction was recapitulated throughout all monocyte subset and was gene dose dependent, supporting a discriminatory role of the presence/absence of LCAT on monocyte immunophenotype. CD11c is a β_2 integrin that promotes the firm arrest of monocytes on endothelial cells, through the interaction with vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). Deficiency of CD11c has been shown to decrease monocytes adhesion in mouse, reducing monocyte/macrophage accumulation in atherosclerotic lesion and decreasing atherosclerosis development in apoE$^{-/-}$ mice on high fat diet (215). Moreover, CD11c expression is increased in inflammatory condition, e.g. during hypertriglyceridemia (216). Interestingly, the decrease in CD11c expression seen in our study was accompanied by a reduced migratory capacity of isolated monocytes in HAEC pre-stimulated with TNF-α, to mimic endothelial dysfunction, supporting once again the less inflammatory phenotype of monocyte in genetic *LCAT* deficiency.

In this study we provided additional characterization of monocyte by challenging them with TLR2 and TRL4 ligands (LPS and Pam3Cys) and measuring cytokine production. It has been previously shown that HDL are able to inhibit TLR-induced production of proinflammatory cytokines from macrophages, acting at transcriptional levels. Activating transcription factor (ATF3) was identified as an HDL-inducible negative regulator of macrophage activation (217). Once again, despite the severe HDL deficiency, monocytes from carriers stimulated with TLR-ligands did not show increased production of TNF-α and IL-6, while IL-8 production was even reduced. Notably unstimulated monocytes displayed a significantly reduced MCP-1 production compared to controls, although comparable circulating levels of MCP-1. Very few reports are present so far about monocyte in LCAT deficiency. The studies are limited to murine model that are not fully translatable to human. Petropoulou and colleagues showed that mice lacking LCAT had on one hand increased systemic responsiveness to LPS, likely due to a decrease LPS-neutralizing ability of immature HDL, but on the other hand an increased number of circulating monocytes associated with a reduced pro-inflammatory phenotype (218). The mechanism by which HDL and apoA-I inhibit monocyte activation was demonstrated to be independent from HDL-stimulating NO production (219-221) and dependent on cholesterol efflux capacity, since the non-specifically cholesterol removal exerts similar anti-inflammatory effect (214).

Accordingly, the ability of subject's HDL to inhibit monocyte chemotaxis was highly correlated with its capacity to promote cholesterol efflux (222).

Thus we speculated that the decreased inflammatory activation of LCAT deficiency monocyte can be possibly explained by the increased cholesterol efflux capacity of the HDL in this genetic disorders. The higher concentration of immature preβ-HDL can enhance the ABCA1 cholesterol efflux from cells (59) and the consequent cholesterol depletion from monocytes can disrupt the intracellular signalling in specific membrane microdomains enriched in cholesterol and sphingolipids, called lipid rafts (214, 223). In support to this hypothesis Thacker et al. demonstrated that HDL reduces inflammation in response to cholesterol crystals in THP-1 cells and in human-monocyte-derived macrophages, but the reduction with rHDL (phospholipid and apoA-I only) was greater (224), suggesting also in this case a possible importance for the conformation of apoA-I in mediating the anti-inflammatory effects.

Since LCAT is not expressed in monocytes, but monocyte isolated from LCAT deficient patients have been in a LCAT-poor milieu, characterized by a peculiar lipoprotein profile, it might be possible that these cells could have been "primed". Epigenetics modification could have occurred, as has been already observed for other lipid disorders (225), but the exact mechanisms by which LCAT deficiency supports an "anti-inflammatory phenotype" still have to be unravelled.

Finally, we compared findings about monocytes in LCAT deficiency with other genetic HDL deficiency as ABCA1 and apoA-I deficiency. As mentioned before, cholesterol efflux seems to largely account for the reduction in anti-inflammatory potential exerted by HDL, since modification in cholesterol trafficking in rafts disrupt the inflammatory signalling in cells. Macrophages lacking ABCA1 exhibit enlarged cholesterol-rich lipid rafts, with higher content of TLR4 and increased responsiveness to TLR2 and TLR4 stimulation (226, 227).

ApoA-I, by promoting cholesterol efflux through ABCA1 may contribute to the modulation of inflammatory response (214). In fact, apoA-I reduces the migration of TLR4 into lipid rafts, thus preventing TLR4-mediated NF-kB activation in endothelial cells (227).

In the recent work by Westerterp and collegues it was reported that ABCA1/ABCG1 deficiency, leading to intracellular cholesterol accumulation, activates the NLRP3 inflammasome promoting neutrophil infiltration in LDLR -/- mice (228). In addition patients with Tangier, carrying a completely dysfunctional ABCA1 are unable to support cholesterol efflux to apoA-I (214) and display elevated plasma levels of inflammatory cytokine (228).

Surprisingly, taking into account what was known so far about HDL and monocytes, we

didn’t find any striking difference in monocyte phenotype and functionality in this HDL-related disorders. Total monocytes TLR2 expression was comparable between *ABCA1*, *APOA1* mutation carriers and controls, but was significantly increased in APOA1-def when monocyte subset expression was considered. Probably this inconsistence was dependent on patient selection that represents one of the major limitations of the study. All patients of *ABCA1* and *APOA1* mutations were carriers of only one mutant allele, that imply a residual number of effective HDL particles that might be sufficient to maintain an healthy immunophenotype or other pathway might be involved (214). This finding was however in line with genetic studies, where lower plasma levels of HDL-C due to heterozygosity for loss-of-function mutations in ABCA1 were not associated with an increased risk of ischemic heart disease (229).

To summarized, in the present work we showed that (i) HDLs in LCAT deficiency have an increased capacity in protecting against endothelial dysfunction (ii) the enhance ability to promote NO production was dependent on the selective depletion in LpA-I:A-II particles (iii) monocytes from patients with LCAT deficiency do not display pro-inflammatory immunophenotype and migratory changes.

Together, these findings contribute to explain the lack of association of genetic LCAT deficiency with cardiovascular disease and indeed, confirmatory findings are shown *in vivo*, where carotid IMT, as an index of atherosclerosis, is not increased in carriers compared to controls despite the reduced HDL-C levels. Actually, maximum IMT was lower in two mutant *LCAT* carriers compared to non carriers, while one mutant *LCAT* carriers display comparable thickness. In addition FMD, a marker of endothelial dysfunction is comparable in carriers and controls. Thus suggesting that the increased functionality of HDL might overcome their reduced concentration. However, how it can be translatable to the general populations is still matter of debate. The EPIC-Norfolk prospective study found no association between LCAT concentrations and CV risk (171), while others associated increased LCAT levels with preclinical atherosclerosis in female (173). LCAT activity is increased in disease associated with high CV risk, e.g. metabolic syndrome (174, 177, 230), but others report showed the opposite (181) (231).

Limitations:

The results should be interpreted with caution. A possible limitation of our approach is that our conclusions are based on data obtained from *ex vivo* experiments from a small number of low HDL-C subjects. Selection bias have to be taken into account, particularly for the monocyte study, since, due to the rareness of the disease, we included mainly

patient with only one mutant allele. For the monocyte study we have selected patients free of CVD, diabetes, renal disease or other inflammatory condition, hence our considerations could not be applicable to advance status of the disease.

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