The vicious inflammatory circle in atherosclerosis

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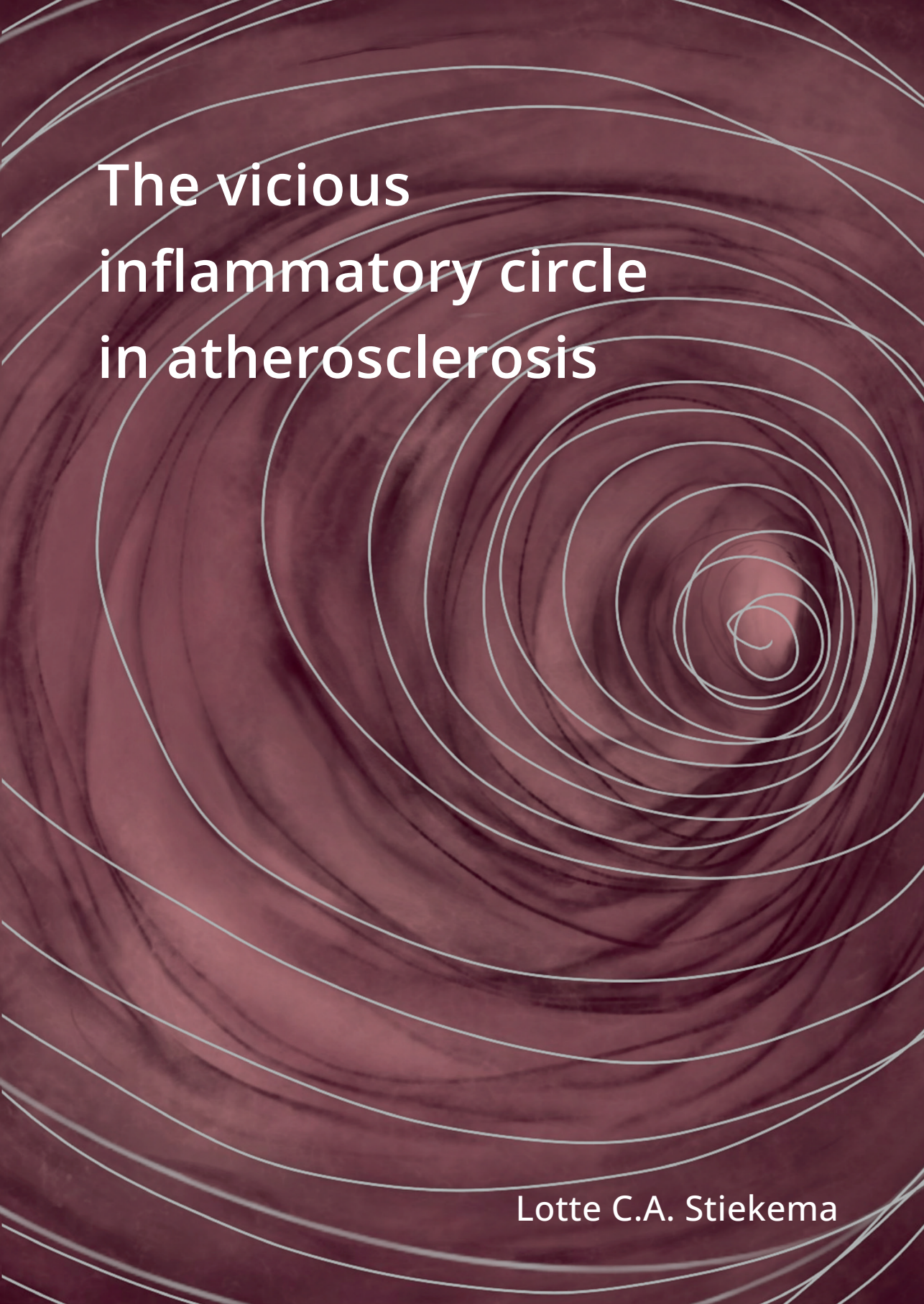

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Lotte C.A. Stiekema

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The vicious inflammatory circle in atherosclerosis

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor

aan de Universiteit van Amsterdam

op gezag van de Rector Magnificus

prof. dr. ir. P.P.C.C. Verbeek

ten overstaan van een door het College voor Promoties ingestelde commissie,

in het openbaar te verdedigen in de Agnietenkapel

op vrijdag 16 december 2022, te 13.00 uur

door Lotte Catharina Albertina Stiekema

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TABLE OF CONTENTS

1

General introduction and outline of thesis

WHY RESEARCH IN CARDIOVASCULAR DISEASE IS (STILL) NEEDED

Cardiovascular disease (CVD) is the leading cause of death and disability worldwide.
World Health Organization, 2021

Each year, 18 million people die from CVD, accounting for one third of all global deaths. When zooming in on the Netherlands, 1.5 million people live with CVD and each day approximately a 100 individuals die from it. A quarter of them die before the age of 75 years; 7 years below the average life expectancy in our country.
Dutch Heart Foundation, 2019

CVD not only costs a lot of lives, it also costs a lot of money. It has been estimated that by 2030, the total costs of CVD in the USA is increased by almost 200 billion US dollars and thereby exceeding the startling trillion-dollar mark.
CDC foundation, 2015

Although promoting a healthy lifestyle prevents CVD in the fast majority of cases, the need for pharmacotherapy is inevitable as current generations will still be faced with the consequences of an unhealthy lifestyle (not in the last part driven by socioeconomic disparities caused by poverty and lack of education).
World Health Organization, 2021

VISION

By providing more insight in the driving factors of CVD and the (lack of) effect of its current treatments, the scientific community can make a significant contribution to the quest of beating the world's most costly and prevalent killer.
This thesis, 2022

BACKGROUND

How atherosclerosis leads to heart attacks and strokes

The formation of *atherosclerotic plaques* that narrow the arteries is a complex process, but in essence it comes down to the buildup of **lipids** and **immune cells** in the arterial wall (**Figure 1**). Over time, the layer of endothelial cells that form the inner coating of the arteries can become 'leaky' under the influence of stressors such as high cholesterol levels (2). This enables lipid particles to penetrate through the endothelial layer and pile up in the arterial wall (3). In turn, this buildup of lipids attracts immune cells (called **monocytes**) from the blood stream (4). After migrating through the endothelial layer, monocytes change into *macrophages* that clean up the fatty deposits in the arterial wall. However, if macrophages consume too much cholesterol, they become *foamy* and die soon after (5). These dead cells need to be cleaned up as well and will attract more and more monocytes (6), eventually forming an instable *necrotic core* (7). Ultimately, the growing plaque containing lipids and (dead) immune cells will rupture (8) causing a *blood clot* which blocks the artery completely (9). Complete blockage of the artery leads to acute organ damage due to low oxygen levels in the tissues downstream of the clot. This phenomenon is called an **ischemic event**. An ischemic event at the level of the heart is termed a *heart attack*. An ischemic event located in the brain is called a *stroke*.

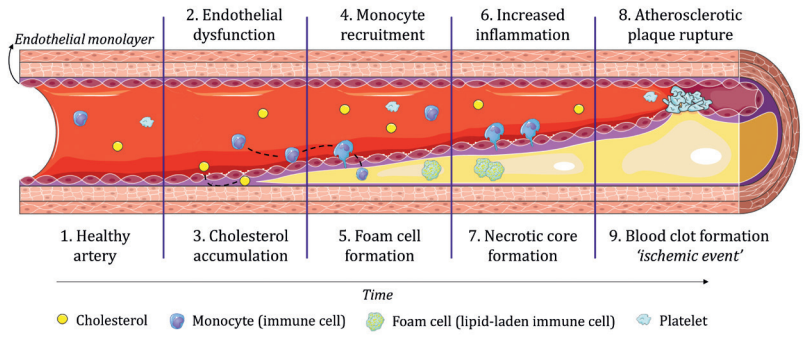


Figure 1. The process of atherosclerotic plaque formation[1]

ATHEROSCLEROSIS

Cardiovascular disease (CVD) is an umbrella term for diseases of the heart and blood vessels including *heart attacks* and *strokes*. The fast majority of CVD is caused by **atherosclerosis**: a narrowing of the blood vessels (*arteries*) that provide oxygen and nutrients to organs and other tissues. When these arteries become too narrow, organs and tissues get damaged. Atherosclerosis develops gradually and usually takes decades to result in organ damage. However, risk factors such as high cholesterol levels can speed up this process.

To date, the treatment of atherosclerotic CVD focuses on reducing lipid accumulation in the arterial wall using *lipid-lowering drugs* and, if present, addressing other modifiable risk factors including smoking, high blood pressure and diabetes mellitus. By this relatively straightforward approach, the worldwide CVD incidence and mortality is steadily dropping since the late 1960's.

However, what holds true for many treatment strategies of multifactorial diseases; one size does not fit all. Three decades after the approval of the revolutionizing lipid-lowering drugs called *statins*, we have learned that reducing lipid levels does not prevent all ischemic events. In fact, in the majority of individuals who are treated with statins re-events are not prevented[2]. The current understanding is that in the context of atherosclerosis it takes two to tango (lipids and immune cells), but just one to mess it up. In other words: even if further lipid buildup in the plaque is limited (by lipid-lowering drugs), **persistent inflammation** in the arterial wall can lead to plaque rupture, and thus ischemic events. This major clinical and therapeutic challenge is referred to as **'residual inflammatory risk'.**

INTERMEZZO

In the last ten years, two important paradigm shifts in the fields of cardiovascular and immunology research regarding inflammation have taken place.

1. As mentioned earlier, for many decades the corner stone of atherosclerotic CVD treatment entailed cholesterol-lowering treatment. However, in 2018, a *double-blind randomized placebo-controlled trial* (the golden-standard to test whether a drug works in patients) showed for the first time that blocking inflammation leads to a lower risk of getting a heart attack or stroke[3]. This drug, *canakinumab*, is a monoclonal antibody that targets *interleukin-1β*, a molecule that helps attracting

immune cells into the plaque. This landmark study proved once and for all that not only lipid reduction, but also reducing inflammation is beneficial for CVD patients.

2. A few years earlier in 2011, Mihai Netea, a Netherlands-based professor in Immunology, proposed the term *trained immunity;* the capacity of innate immune cells (such as monocytes) to remember 'triggers'[4]. Until that point, it was commonly believed that monocytes did not acquire memory like lymphocytes which are immune cells of the *adaptive immune system.* Evolutionary, immune memory gives the advantage of eliciting a faster immune response upon re-infection with, for example, a virus. However, the big downside is a permanent state of 'preparedness' that can accelerate progression of diseases in which innate immune cells play an important role (including atherosclerotic CVD). Potential new drugs targeting the inflammatory response in CVD should take this into account.

TARGETING INFLAMMATION IN CVD

"You don't have to reinvent the wheel; just attach it to a new wagon."
Mark McCormack (died at age 72 after suffering a heart attack)

Although it is still considered a major breakthrough that canakinumab lowers the risk of ischemic events by reducing *residual inflammation*, this drug will probably not be made available on the market for CVD patients due to its side effects. As many anti-inflammatory drugs, canakinumab increases the risk of severe (and even fatal) infections. This unfavourable side effect profile has fuelled the search for treatment strategies that are able to reduce the inflammatory activity in the arterial wall, without hindering the vital immune response against deadly invaders such as bacteria and viruses. To tackle this difficult challenge, better understanding of what determines the persistence of '*residual inflammation'* in CVD will help find a solution.

This brings us back to the second paradigm shift: *innate immune memory*. To link this phenomenon to CVD, understanding of the normal inflammatory response of monocytes is needed. Monocytes are part of the innate immune system that forms the first line of defence in the immune response. In the bloodstream, monocytes patrol along the vessel walls or migrate into tissues, and 'eat' everything that is considered 'foreign'. When monocytes encounter such a foreign invader, they can activate and recruit other immune cells by producing molecules called *cytokines (*including *interleukin-1β*) that are released in the blood stream. When monocytes encounter a second invader, their memory helps them to respond faster and more vigorous, resulting in enhanced cytokine production which will attract even more immune cells.

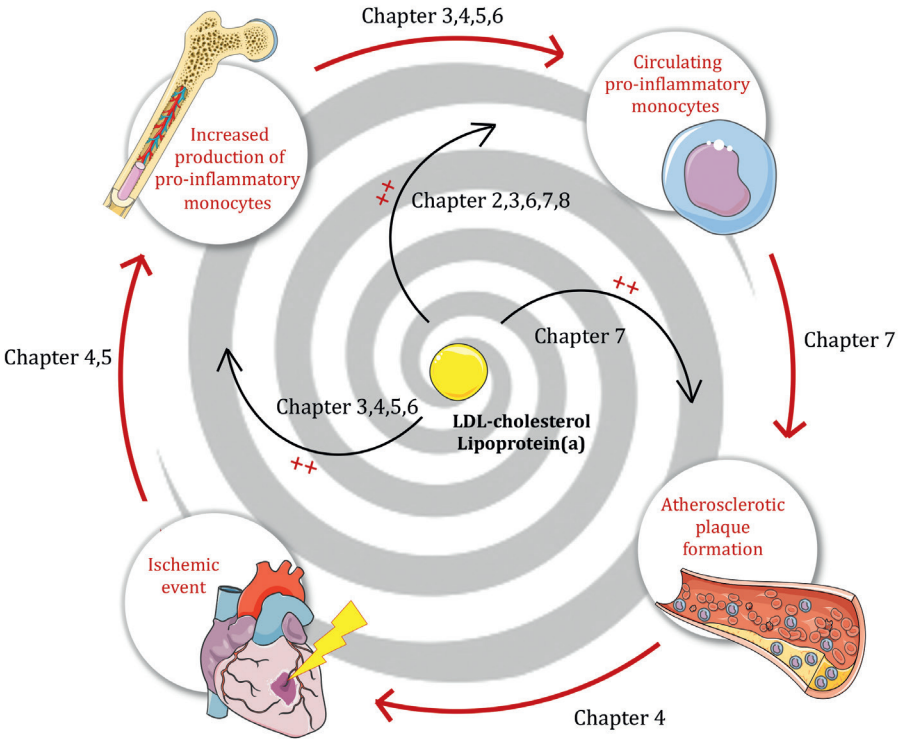
As discussed in the first part of this introduction, foreign invaders are not the only activating triggers for monocytes. Also specific lipoproteins that carry cholesterol (*LDL-cholesterol and lipoprotein(a)*) trigger monocytes to consume them and to produce cytokines. Interestingly, it has been shown in mice that monocytes stay in this triggered hyperactivated state days to weeks after they have last been in contact with these lipoproteins. This is remarkable, since monocytes do not live long: from hours to approximately a few days. The fact that 'freshly' produced monocytes exhibit memory without ever having encountered a first trigger (in this case cholesterol), means that these monocytes are being produced in the bone marrow compartment with a built-in memory.

This built-in memory is considered the motor of the vicious inflammatory circle in CVD and forms the starting point of this thesis. By elucidating the underlying mechanisms, this thesis contributes to a better understanding of residual inflammation in CVD and ultimately helps breaking the vicious circle by finding new treatment targets.

OUTLINE OF THIS THESIS

Residual inflammatory risk in CVD is a major therapeutic challenge; hence a better understanding of its underlying mechanisms is needed (**Summary Figure**). **Part I** of this thesis addresses the complex role of the bone marrow compartment in maintaining systemic inflammation in CVD. First, **chapter 2** examines the role of hypercholesterolemia in inducing innate immune memory, explaining residual inflammatory risk in hypercholesterolemic patients despite optimal lipid-lowering therapy. Training of circulating monocytes is thought to be caused by alterations in the stem cells that produces these cells. Therefore, **chapter 3** investigates whether hematopoietic stem and progenitor cell reprogramming is a possible driver of (persistent) inflammation in hypercholesterolemic patients. Translating the *ex vivo* findings of the previous two chapters to CVD, **chapter 4** uses *in vivo* imaging to link LDL-cholesterol levels to bone marrow activity and arterial wall inflammation; a surrogate marker of cardiovascular risk. Lastly, the final chapter of part I completes the vicious circle, detailing the multi-level inflammatory response following a cardiovascular ischemic event in **chapter 5**.

Part II sheds its light on the inflammatory response in patients with elevated levels of the 'LDL-like' particle lipoprotein(a) (Lp(a)). To determine whether Lp(a) elicits hematopoietic reprogramming as investigated in part I, **chapter 6** describes the effects of Lp(a) on hematopoietic stem cell differentiation in a murine model. Since no specific Lp(a)-lowering therapies are available yet, **chapter 7** studies the effect of *modest* Lp(a)-lowering by PCSK9-ab treatment on arterial wall inflammation in patients with elevated Lp(a). Learned from the observations in the previous two chapters, **chapter 8** gives more insight into whether Lp(a)-associated persistent inflammation is fueled by sub-optimal lowering of Lp(a). Finally, **chapter 9** summarizes the key findings of this thesis and **chapter 10** provides a future perspective on approaching residual inflammatory risk.



Summary Figure. The vicious inflammatory circle in atherosclerosis[1]

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PART 1.

THE VICIOUS INFLAMMATORY CIRCLE FORMED BY THE BONE MARROW-VASCULAR-AXIS

2

Treatment with statins does not revert trained immunity in patients with familial hypercholesterolemia

Siroon Bekkering, Lotte C.A. Stiekema, Sophie Bernelot Moens, Simone L. Verweij, Boris Novakovic, Koen Prange, Miranda Versloot, Jeanine E. Roeters van Lennep, Henk Stunnenberg, Menno de Winther, Erik S.G. Stroes, Leo A.B. Joosten, Mihai G. Netea, and Niels P. Riksen

Cell Metabolism, 2019

Patients with hypercholesterolemia have an increased risk for atherosclerotic cardiovascular disease, and despite lipid lowering with statins, an important residual risk remains[1]. We hypothesize that this is due to persistent pro-inflammatory reprogramming of circulating monocytes. Monocyte-derived macrophages are key components of atherosclerotic plaques. These cells can build a long-term pro-inflammatory phenotype after brief exposure to atherogenic compounds, such as oxidized low-density lipoprotein (oxLDL)[2]. This immune memory is mediated by metabolic and epigenetic reprogramming and is termed trained immunity[3]. In Ldlr-/- mice, a Western-type diet induces trained innate immunity, which persists despite switching back to chow diet, due to reprogramming of myeloid progenitor cells[4]. We propose that monocytes from FH patients have a trained immune phenotype, which persists after treatment with statins despite normalization of circulating cholesterol. In a prospective cohort study in 25 statin-naive patients with definitive or probable familial hypercholesterolemia (FH), we studied the functional and transcriptional reprogramming of circulating monocytes compared to 20 normocholesterolemic individuals and the effect of three months statin treatment (Table S1). See Methods S1 for methodological details. First, flow cytometry revealed a similar distribution of classical, non-classical, and intermediate monocytes in both groups, but monocytes from FH patients had a higher expression of the activation markers CCR2, CD11b, CD11c, and CD29 (Figure S1). Statin treatment reduced CCR2 and CD29 expression without affecting CD11b and CD11c (Figure S1). Cytokine production capacity is the major defining characteristic of trained immunity. Peripheral blood mononuclear cells from patients had a higher production of pro-inflammatory (TNFa, IL-6, and IL-1b) and anti-inflammatory (IL-10 and IL-1Ra) cytokines after Toll-like receptor stimulation (Figure S2), comparable to the oxLDL-induced trained monocytes in vitro[2]. This hyperresponsiveness persisted during statin treatment, although production of the anti-inflammatory IL-1Ra after Candida stimulation was reduced (Figure S2). To investigate the transcriptional reprogramming underlying this functional hyper-responsiveness, we performed RNA sequencing analysis (RNA-seq) of unstimulated monocytes of 5 FH patients with the largest LDL-c reduction by statin treatment and 5 normocholesterolemic controls, matched for age and sex. RNA-seq identified 117 differentially upregulated and 153 downregulated genes in the patients (Figure S3). Pathway analysis of the upregulated genes revealed a significant enrichment of pathways involving activation of the immune system (Figure S3). Unsupervised principle component analysis of all expressed genes showed a separation of patients and controls in PC1 (31% of all variance explained; Figure S3). Pathway enrichment analysis of the top and bottom 200 contributors to PC1 revealed an enrichment in the FH patients of metabolic pathways (oxidative phosphorylation, glycolysis, and amino acid synthesis) and inflammatory pathways

(e.g., NF-kB activation and the TNF signaling pathway), which are important in facilitating trained immunity[5,6,7]. Importantly, statin treatment did not change the RNA expression profile to a great extent, with only 17 genes downregulated by statin treatment, resulting in a persistent clustering in the PCA plot with the patients before statin treatment and not with the normocholesterolemic controls (Figure S3). Epigenetic reprogramming is key in the development of trained immunity[3]. We therefore performed chromatin immunoprecipitation coupled with quantitative PCR in 5 patients with FH and 5 control subjects to assess two histone modifications on the TNFA promoter that are important in trained immunity. There was a significant enrichment of the activating mark histone 3 lysine 4 trimethylation (H3K4me3) as well as a lower presence of the repressive mark histone 3 lysine 9 trimethylation (H3K9me3), which were not affected by statin treatment (Figure S4). In this study, we introduce trained immunity as a mechanism that might contribute to the persistently increased cardiovascular risk in patients with hypercholesterolemia. Trained immunity refers to the persistent non-specific pro-inflammatory status of innate immune cells following brief stimulation with micro-organisms, which can offer profound protection against reinfection. However, it can also be induced by endogenous atherogenic particles, such as oxLDL, which might be detrimental in the context of chronic inflammatory diseases such as atherosclerosis[8]. The long-term persistence of the trained immune phenotype is explained by the fact that reprogramming occurs at the level of myeloid progenitors in the bone marrow niche[4,9]. Monocytes from patients with FH are characterized by a trained immune phenotype, in terms of enhanced cytokine production capacity, and pro-inflammatory transcriptional reprogramming, associated with persistent epigenetic changes of histone modifications. This mirrors the monocyte reprogramming in Ldlr-/- mice on a Western-type diet[4], which persisted at least four weeks after normalization of plasma cholesterol by switching to chow diet[4]. Here, we lowered cholesterol levels in FH patients with a three-month statin treatment. Previously, we showed that trained immunity induced by b-glucan is dependent on activation of the mevalonate pathway, and both b-glucan-induced and oxLDL-induced trained immunity are prevented by statins[6]. Despite this in vitro effect, the monocyte hyper responsiveness persisted during statin treatment, with an even lower production of the anti-inflammatory IL-1Ra following Candida stimulation. Thus, despite statins preventing oxLDL-induced trained immunity in vitro, they cannot reverse training in vivo. These data might provide an explanation for the residual risk in FH patients treated with statins and for the observation that anti-inflammatory treatment is effective in lowering cardiovascular risk, even on top of statin treatment[10]. The clinical relevance of this mechanism was recently illustrated by our findings that in vivo in patients with severe coronary atherosclerosis, monocytes show a trained

immunity phenotype in terms of pro-inflammatory cytokine production and epigenetic remodeling[11]. We acknowledge that our cohort is relatively small, but the well-matched control group and the paired experimental design with regard to the effect of statins increase the power, and the observed differences were robust. Further elucidation of the mechanism of the persistent pro-inflammatory reprogramming might reveal novel targets for pharmacotherapy to lower cardiovascular risk in patients with hyperlipidemia.

Supplemental information

Supplemental Information can be found online at https://doi.org/10.1016/j.cmet.2019.05.014.

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Conflict of interest

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Author contributions

Conceptualization, S.B., M.G.N., and N.P.R.; Methodology, S.B., S.J.B.M., E.S.G.S., and N.P.R.; Investigation, S.B., S.J.B.M., S.L.V., M.V., L.C.A.S., B.N., and K.P.; Formal Analysis, S.B. and B.N.; Data Curation, S.B. and B.N.; Visualization, S.B.; Resources, N.P.R, E.S.G.S., and J.R.v.L.; Writing – Original Draft, S.B.; Writing – Review & Editing, all other authors; Supervision, N.P.R. and E.S.G.S.; Funding Acquisition, N.P.R., M.G.N., L.A.B.J., H.S., and E.S.G.S.

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SUPPLEMENTAL INFORMATION

TABLES

Table S1. Baseline characteristics of the two groups

	Patients *(n=25)*	**Normocholesterolemic controls** *(n=20)*	**P-value**
Age, yrs	39 ± 3	38 ± 3	0.80
Sex, n (%male)	13 (52)	12 (61)	0.56
BMI, kg/m²	25 ± 3.5	22 ± 2.9	0.003
Smoking, %current **SBP, mmHg**	26 123 ± 17	30 120 ± 9	0.78 0.47
DBP, mmHg	80 ± 10	78 ± 5	0.57
TChol, mmol/L	8.7 ± 1.9	5.0 ± 0.8	<0.001
LDL-C, mmol/L	6.8 ± 1.7	2.8 ± 0.8	<0.001
HDL-C, mmol/L	1.3 ± 0.4	1.8 ± 0.4	<0.001
TG, mmol/L	1.5 ± 0.8	0.8 ± 0.4	0.04
Leukocytes, *10⁹/L	6.7 ± 2.3	5.8 ± 1.8	0.19
Neutrophils, *10⁹/L **Lymphocytes, *10⁹/L**	4.0 ± 2.0 2.1 ± 0.6	3.1 ± 1.5 1.9 ± 0.5	0.18 0.44
Monocytes, *10⁹/L	0.5 ± 0.1	0.5 ± 0.1	0.80

Values are n (%) or mean ± SD. *BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TChol, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; IQR, inter-quartile range; SD, standard deviation.*

	Patients untreated *(n=19)*	**Patient treated** *(n=19)*	**P-value**
TChol, mmol/L	8.6 ± 1.7	5.5 ± 1.1	<0.001
LDL-C, mmol/L	6.5 ± 1.5	3.7 ± 1.1	<0.001
HDL-C, mmol/L	1.4 ± 0.4	1.4 ± 0.4	0.66
TG, mmol/L	1.4 ± 0.9	0.9 ± 0.4	0.04
Leukocytes, *10⁹/L	6.2 ± 2.0	6.0 ± 1.0	0.78
Neutrophils, *10⁹/L **Lymphocytes, *10⁹/L**	3.4 ± 1.7 2.1 ± 0.5	3.3 ± 0.9 1.9 ± 0.4	0.73 0.93
Monocytes, *10⁹/L	0.5 ± 0.1	0.5 ± 0.1	0.90

Change of baseline parameters from patients before and after statin treatment. Values are n (%), mean ± SD or median [IQR] for skewed data. *BMI, body mass index; CRP; c-reactive protein; TChol, total cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; TG, triglycerides; IQR, inter-quartile range; SD, standard deviation.*

Table S2. Prescribed statin treatment and dose

Statin + dose	Number of patients (%)
Simvastatin 10mg once daily	1 (5)
Simvastatin 40mg once daily	1 (5)
Atorvastatin 20mg once daily	6 (32)
Atorvastatin 40mg once daily	6 (32)
Rosuvastatin 5mg once daily	1 (5)
Rosuvastatin 10mg once daily	3 (16)
Rosuvastatin 40mg once daily	1 (5)

Values are n (%), all doses are dd.

FIGURES

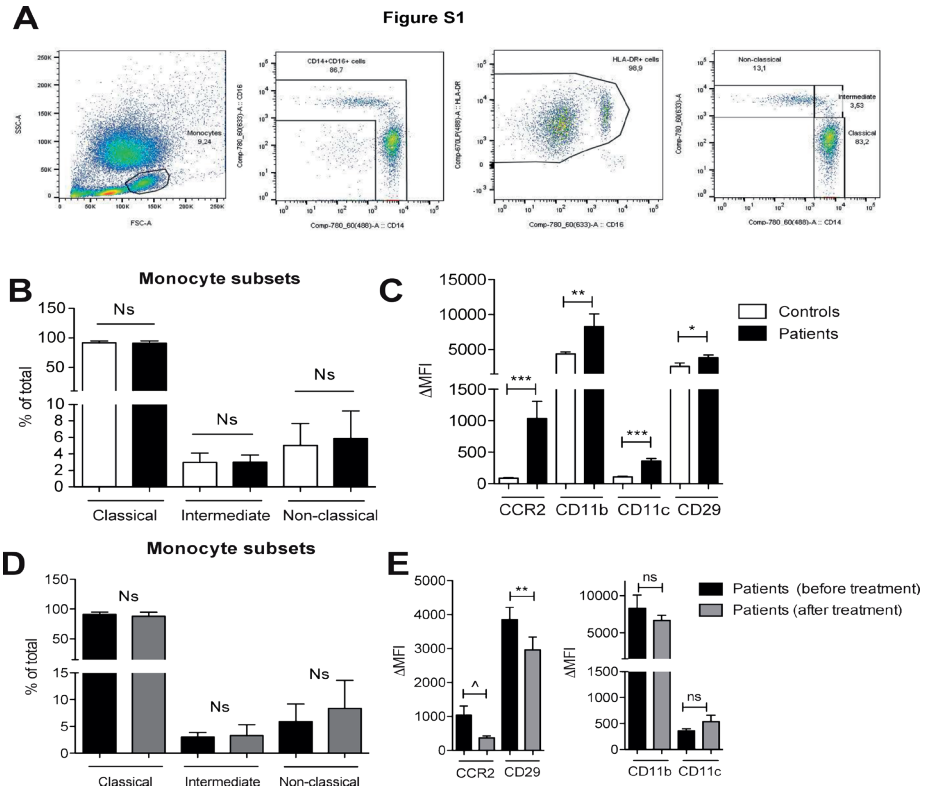

Figure S1. Circulating monocytes from patients with FH have a long-term inflammatory phenotype, which persists during treatment with statins

Flow cytometry was used to analyze activation markers on circulating monocytes. Data are represented as mean ± SEM. (A) Gating strategy of the three monocytes subsets. Only HLA-DR positive cells were considered to be monocytes. (B) Percentage of monocyte subsets (i.e. classical (CD14++CD16-), intermediate, (CD14++CD16+) and non-classical (CD14dimCD16+)) in FH patients (n=25, black bars) vs normocholesterolemic controls (n=20, white bars). (C) Surface expression of monocyte CCR2, CD11b, CD11c and CD29 represented as delta median fluorescence intensity. (D) Percentage of monocyte subsets of patients before (black bars) and during (grey bars) statin treatment (n=25 and n=19 respectively). (E) Surface expression of monocyte chemokine and adhesion receptors in patients before and after statin treatment. Statins decrease CCR2 and CD29 but not CD11b and CD11c. Data are shown as delta MFI and mean ± SEM. ^ p=0.06, * p<0.05, ** p<0.005, *** p<0.001

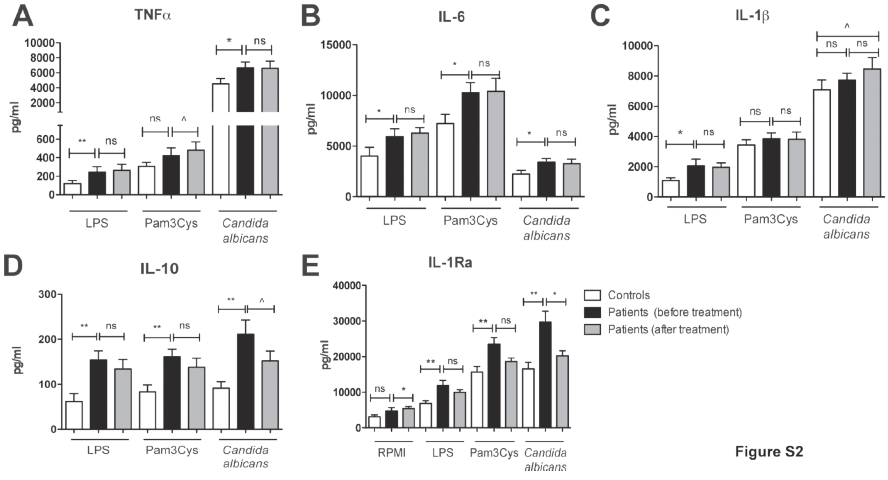

Figure S2. Hypercholesterolemia due to FH is associated with persistent proinflammatory cytokine production in circulating monocytes

Cytokine production capacity was measured in isolated PBMCs from patients with FH before (black bars, n=25) and after statin treatment (n=19, grey bars) as well as PBMCs from matched normocholesterolemic controls (n=20, white bars) upon stimulation with several TLR-ligands for 24h. Cytokines TNFα (A), IL-6 (B), IL-1β (C), IL-10 (D) and IL-1Ra (E) were measured in the supernatants. Data are shown as mean ± SEM. LDL-c lowering by statins does not lower ex vivo cytokine production capacity of PBMCs. ^ p=0.06, * p<0.05, ** p<0.005, *** p<0.001

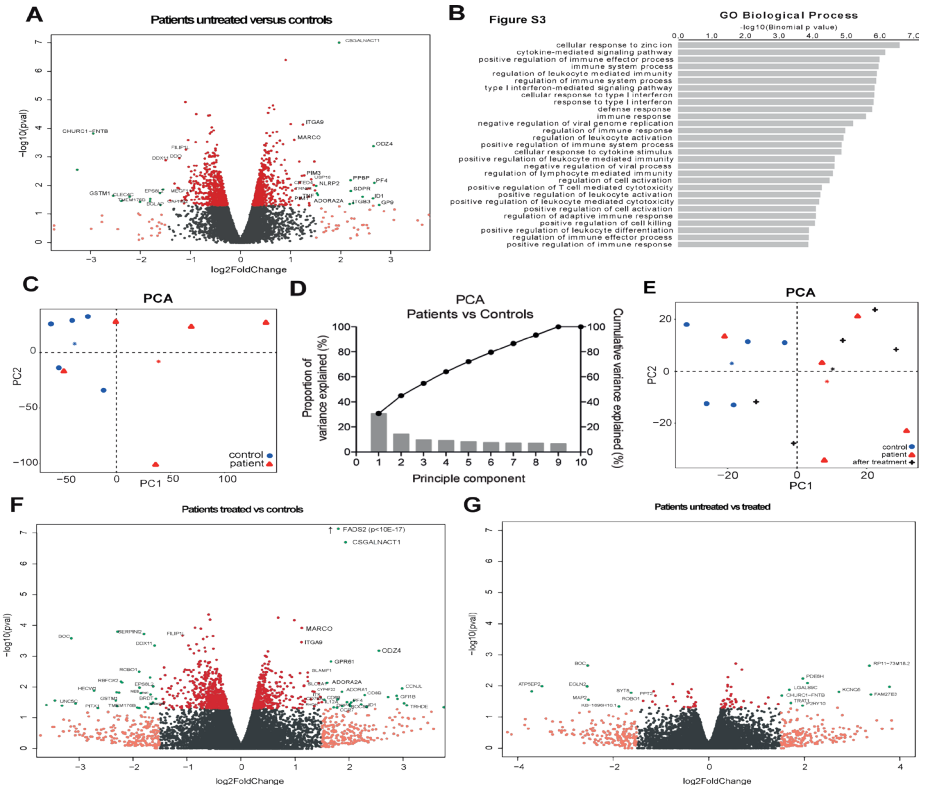

Figure S3. Persistent pro-inflammatory transcriptional reprogramming in FH patients
Isolated CD14+ monocytes from five FH patients before and after treatment as well as from five normocholesterolemic controls were analyzed using RNAseq. (A) Volcano plot of all expressed genes from FH patients vs matched controls (n=5 for both). Red and green dots indicate significant differentially expressed genes (-log10[pval]>1). Pink and grey dots indicate non-significant differentially expressed genes (log10[pval]<1). Green dots indicate the most significantly up and downregulated genes (logFC>1.5 & -log10[pval]>1). (B) GO Pathway analysis of all significantly upregulated genes shows an inflammatory phenotype. (C) Unsupervised Principle Component Analysis (PCA) of all transcribed genes shows a clear distinction between patients and controls. * indicates the mean of each group (controls in blue, patients in red). (D) The explained variation for each principle component is shown in a spree plot. (E) Three-month statin treatment does not change the transcriptome of monocytes in patients, as shown with PCA. * indicates the mean of each group (controls in blue, patients before treatment in red, patients after treatment in black). (F) Volcano plot of all expressed genes from FH patients after treatment vs matched controls (n=5 for both). Red and green dots indicate significant differentially expressed genes (-log10[pval]>1). (G) Volcano plot of all expressed genes from FH patients untreated vs treated (n=5 for both). Red and green dots indicate significant differentially expressed genes (-log10[pval]>1).

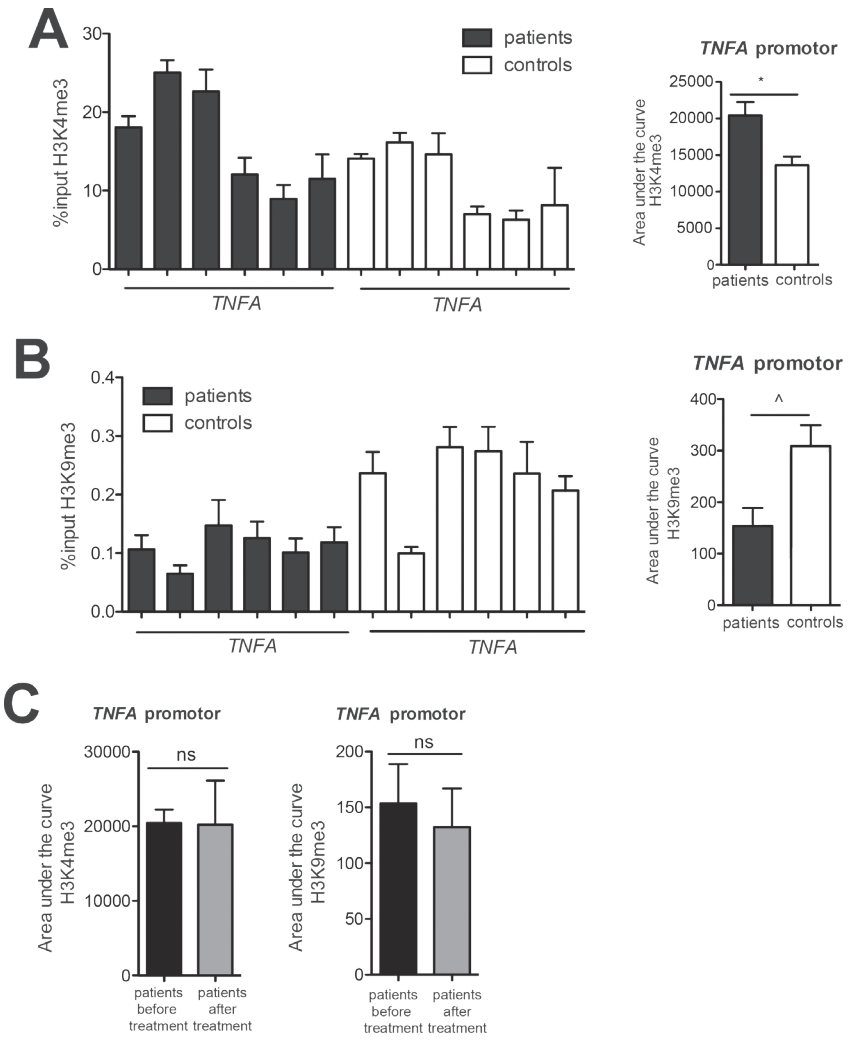

Figure S4. Hypercholesterolemia is associated with long-term activating reprogramming of histone marks in the promoter of TNFA

(A) ChIP-PCR of the TNFA promoter shows a significantly increased H3K4me3 in patients at baseline compared to controls. (B) H3K9me3 ChIP-PCR shows a significantly decreased H3K9me3 on the promoter of TNFA in patients compared to controls. Black bars indicate patients (n=5) and white bars indicate controls (n=5). (C) LDL lowering by statins does not revert epigenetic changes in patients. ChIP-PCR of the TNFA promoter shows a similar pattern of H3K4me3 in patients before and after treatment. H3K9me3 ChIP-PCR also shows no difference before and after treatment. Black bars indicate patients before (n=5) and grey bars indicate patients after treatment (n=5). Data shown are mean AUC ± SEM. ^ p=0.06, * p<0.05, ** p<0.005, *** p<0.001

STAR METHODS

Contact for reagent and resource shring

Further information and requests for resources and reagents should be directed to Siroon Bekkering (Siroon.bekkering@radboudumc.nl)

Experimental model and subject details

In this multicenter, observational study, we recruited 25 patients (age≥18 years) with definite or probable FH based on the Simon Broome criteria (13 with definite FH according to a pathogenic mutations in LDL-R or ApoB, 12 with possible FH, based on cholesterol levels and family history). Patients had LDL cholesterol levels of ≥4.9 mmol/l and were not treated with lipid lowering therapy for at least one year at time of inclusion. We also recruited 20 age- and sex-matched healthy controls with a LDLC<3.5 mmol/l. Patients were recruited in three university hospitals in the Netherlands (Radboud university medical center, Nijmegen; Amsterdam UMC, location AMC, Amsterdam; Erasmus University Medical Center, Rotterdam). Both patients and control subjects did not have any history of established cardiovascular disease. All patients with FH were treated by their treating physician with statins, and after at least three months we repeated the blood drawing. Exclusion criteria for both patients and controls included the use of lipid lowering drugs in the past year, known malignant disorders, auto-immune disorder, or diabetes mellitus, clinical signs of acute infection and the use of anti-inflammatory medication. Subjects visited the hospital after an overnight fast for physical examination, medical history recording and blood withdrawal. Patients were treated according to their treating physician with statins for approximately 3 months. The specific statins and dosages used are summarized in table S2. Six patients had to be excluded from the second timepoint due to meeting of exclusion criteria during the study, a non-compliance to statin therapy or were lost to follow-up. Mean duration of statin treatment was 100±30 days, which led to a mean percent LDL-C reduction of 44±13% (P<0.001), reaching a mean posttreatment LDL-C of 3.66±0.4 mmol/L.

The study protocol was approved by the local institutional review board (CMO regionArnhem-Nijmegen, #2299 2010/104) as well as the local hospital review board in theAmsterdam UMC, location AMC, Amsterdam and the Erasmus University Medical Center Rotterdam. The study was conducted according to the principles of the Declaration of Helsinki, and written informed consent was obtained from each participant.

Method details

Flow cytometry

Red blood cells were lysed with red blood cell lysis buffer for 15 min with subsequent washing with PBS. After centrifugation (387 g, 5 min, 4°C) and decantation of the supernatant, leukocytes remained. First, the leukocytes were suspended in 200 µL PBS, and incubated with fluorochrome labelled antibodies (per well: 25 µL leukocytes and 25 µL antibody; see material and methods table for details and dilutions) for 15 min at room temperature in the dark. After incubation, the leukocytes were washed again with PBS. The cells were centrifuged (441 g, 3 min, 4°C), the supernatant was decanted and the cell pellet was suspended in 200 µL PBS. Samples were analysed using BD FACS Canto II (Becton, Dickinson, Fanklin Lakes, NJ, USA). Per patient, 5000 monocyte events were acquired. Monocyte subsets (i.e. classical, intermediate, and non-classical monocytes) were classified according to HLA-DR, CD14, and CD16 expression. The gating strategy is outlined in Figure S1A. Subsequently, the presence of various chemokine receptors and vascular adhesion molecules as well as scavenger receptors and Toll-like receptors on these monocytes was determined. Samples were analysed using FlowJo software. Delta median fluorescence intensity (ΔMFI = MFI surface staining − MFI isotype control) was obtained.

Plasma storage and analysis

Within 4 hours after blood withdrawal, blood vacutainers were centrifuged for 10 minutes at 3800 rpm, 4°C. Plasma was removed sterile and immediately stored at - 80°C until analysis. Repeated freeze-thaw cycles were prevented by storing several aliquots per subject. Analysis of blood cholesterol levels and white blood cell counts were performed immediately upon blood withdrawal. Lipid levels were measured via a colorimetrical test using enzyme reactions (cholesterol esterase and oxidase), and LDL cholesterol is calculated with the Friedewald formula. In the patients where LDL cholesterol could not be calculated with the Friedewald formula, FPLC analysis was performed to measure cholesterol and TG content of VLDL, LDL and HDL. Leukocyte count and differentiation were determined using automated cell counters.

PBMC and monocyte isolation

PBMC isolation was performed by dilution of blood in pyrogen-free PBS supplemented with 2 mM EDTA and differential density centrifugation over Ficoll-Paque. Cells were washed thrice in PBS+EDTA, resuspended in RPMI and counted. Monocytes were isolated using anti human CD14 magnetic beads and MACS® cell separation columns according to the manufacturer's instructions.

PBMC stimulation assays

5x105 PBMCs/well were stimulated ex vivo in duplicate for 24h with several TLR ligands: 10 ng/ml LPS (TLR4), 10 ug/ml Pam3Cys (TLR2) or 106/ml heat-killed Candida albicans (C-type lectins) to study ex vivo cytokine production capacity.

Cytokine measurements

Cytokine production was determined in supernatants using commercial ELISA kits for human IL-1β, TNFα, IL-6, IL-10 and IL-1Ra following the instructions of the manufacturer. Cytokine measurements and analysis were performed blinded and at the same day for all samples.

RNA isolation and qPCR

For qRT-PCR, 1x106 monocytes were stored in TRIzol reagent. Total RNA purification was performed according to the manufacturer's instructions. RNA concentrations were measured using NanoDrop software, and isolated RNA was reverse-transcribed using the iScript cDNA Synthesis Kit. qPCR was performed using the SYBR Green method. Samples were analyzed following a quantitation method with efficiency correction, and 18S was used as a housekeeping gene.

Chromatin immunoprecipitation followed by qPCR

For the assessment of H3K4me3 (activating histone mark) and H3K9me3 (repressive histone mark), purified monocytes were fixed with 1% formaldehyde at a concentration of approximately 15 million cells/ml. Fixed cell preparations were sonicated using a Diagenode Bioruptor Pico sonicator using 7 cycles of 30 seconds on, 30 seconds off. One µg of chromatin (approx. 0.5-1 million cells) was incubated with dilution buffer, 12 µl protease inhibitor cocktail, protein A/G agarose beads and 1µg of H3K9me3 or H3K4me3 antibody to a final volume of 300 µl, and incubated overnight at 4°C with rotation. Beads were washed with 400µl buffer for 5 minutes at 4°C with five rounds of washes. After washing, chromatin was eluted using 200 µl elution buffer for 20 minutes. Supernatant was collected, 8 µl 5M NaCl, 3 µl proteinase K were added and samples were incubated for 4h at 65°C. Finally samples were purified using Qiagen Qiaquick MinElute PCR purification Kit and eluted in 20 µl elution buffer. ChIPed DNA was processed further for qPCR analysis. Primers used in the reaction are listed in the materials and methods section. Samples were analyzed following a comparative Ct method, myoglobin was used as a negative control for H3K4me3, GAPDH as negative control for H3K9me3. H2B was used as a positive control for H3K4me3, the untranslated region of ZNF for H3K9me3 according to the manufacturer's instructions. We selected six primers covering the *TNFA* promoter

to find differences across the whole promoter region and to be able to analyze the area under the curve.

RNA sequencing

Monocytes isolated from FH patients and controls were lysed in Trizol reagent and stored at -80°C. Total RNA was extracted from cells according to the manufacturer's instructions and DNAse I treatment was performed using a DNA free kit Sequencing library preparation was done using the RNA HyperPrep Kit with RiboErase (KAPA Biosystems) and using the KAPA Single-Indexed Adapter Kit (KAPA Biosystems) following manufacturer directions, with the following study-specific parameters: 300ng starting RNA material and 6.5 minute fragmentation. Sequencing was performed using the Illumina NextSeq500 machine and a 75bp 400M reads kit (Illumina).

Quantification and statistical analysis

Data are expressed as mean (standard deviation), median (inter-quartile range) or number (percentage), unless stated otherwise in the figure legends. Normality checks were performed using qq-plots and GraphPads normality checks. Differences in clinical characteristics and monocyte phenotype and function between patients and controls were assessed with Student's t-tests or Mann–Whitney U tests. Correlations were assessed using Pearson's for normally distributed data and Spearman's Rho for non-normaly distributed data. A two-sided P-value <0.05 was considered statistically significant. Data were analysed using Prism version 5.0 (GraphPad software, La Jolla, CA, USA), SPSS version 22.0 (SPSS Inc., Chicago, IL, USA) or R-studio version 1.1.485 *p < 0.05, **p < 0.01, ***P<0.001.

RNA-seq data analysis

To infer gene expression levels, RNA-seq reads were aligned to the Ensembl v68 human transcriptome using Bowtie 1. Quantification of gene expression wasperformed using MMSEQ. Differential expression was determined using DEseq. Differentially expressed genes were determined using comparison between patients and controls, with a fold change >1.5, adjusted p value of <0.1, and RPKM >1. PCA analysis and visualization was performed using R-studio version 1.1.485 package MixOmics. Gene Ontology analysis was performed using GREAT. KEGG pathway analysis was performed using DAVID.

Data and software availability

The accession number for the raw data file reported in this paper is GEO: GSE126352

KEY RESOURCES TABLE

Reagent or Resource	Source	Identifier
Antibodies		
APC-anti-CCR2 (1/50)	BD	558406
APC-anti-CD163 (1/25)	Biolegend	326507
APC-anti-CD11c (1/50)	BD	559877
APC-anti-CD36 (1/25)	BD	550956
APC-anti-CD29 (1/50)	BD	559883
APC isotype control	BD	550854
FITC-anti-CCR5 (1/10)	BD	555992
FITC-anti-CD32 (1/50)	BD	552883
FITC-anti-TLR2 (1/100)	Biolegend	121805
FITC isotype control	BD	551954
PerCP5.5-anti-HLADR (1/50)	BD Pharmingen	560652
PEcy7-anti-CD14 (1/50)	BD	557742
APC-Cy7-anti-CD16 (1/50)	BD	560195
IVIG	Sanquin	
PE-anti-CD11b (1/25)	BD	555388
PE-anti-CD45RA (1/100)	BD	304107
PE-anti-CCR7 (1/25)	Biolegend	353203
PE-anti-TLR4 (1/10)	Biolegend	312805
PE isotype control	BD	551436
Anti-human-H3K4me3 polyclonal antibody premium	Diagenode	C15410003 (pab-003-050)
Anti-human-H3K9me3 polyclonal antibody Premium	Diagenode	C15410193 (pAb-193-050)
Chemicals, Peptides and recombinant proteins		
Lipopolysaccharide	Sigma-Aldrich	From E.coli serotype 055:B5, L2880
Pam3Cys	EMC Microcollections	L2000
Candida albicans		strain ATCC MYA-3573, UC 820
Percoll	Sigma-Aldrich	P1644
Ficoll-Paque	GE Healthcare	17-1440-03
Roswell Park Memorial Institute medium (RPMI)	Invitrogen	22406031
Bovine Serum Albumin (BSA)	Sigma-Aldrich	A7030

0.5M EDTA sterile	Life Technologies	15575020
CD14+ beads	MACS Miltenyi	130-050-201
TRIzol reagent	Life Technologies	15596018
SYBR Green	Applied Biosciences	4368708
16% Formaldehyde	Fisher Scientific	28908, 11835835
Protein A/G Plus-Agarose (IP reagent) (Santa Cruz sc-2003)	AntibodyChain	ABCA0770861
Red blood cell lysis buffer	Affymetrix eBioscience	00-4300-54
Phosphate Buffered Saline	Melsumgen	
Critical Commercial Assays		
Human IL-1β ELISA	R&D systems	DY201
Human TNFα ELISA	R&D systems	DY210
Human IL-6 ELISA	R&D systems	DY206
Human IL-10 ELISA	IBL international	M9310
Human IL-1Ra ELISA	R&D systems	DY280
DNA free DNAse kit	Ambion	AM1906
iScript cDNA Synthesis Kit	Bio-Rad	1708891
KAPA Single-Indexed Adapter Kit, Set A (30 µM)	Illumina	KK8701
KAPA RNA HyperPrep with RiboErase	Illumina	KK8561
NextSeq 500/550 High Output v2 kit (75 cycles)	Illumina	FC-404-2005
Qubit RNA HS assay kit	Life Technologies	Q32852
Ribozero Gold Kit	Illumina	MRZG12324
Rneasy Mini Kit	Qiagen	74106
Software		
FlowJo software	FlowJO	version 10.0 FlowJO, LLC, Ashland, OR, USA
GREAT		
R Studio	R	R version 3.5.1 (2018-07-02)
GraphPad Prism	Graphpad Software	https://www.graphpad.com
Bowtie 1	Lan mead - 2010 PMID: 21154709	http://bowtiebio.sourceforge.net/index.shtml
MMSEQ	Turro - 2011 Genome Biology	https://github.com/eturro/mseq

DEseq	Love - 2014 Genome Biology	(http://bioconductor.org/packages/release/bioc/html/DESeq.html)
HOMER	PMID: 20513432	http://homer.salk.edu/homer/motif/
DAVID	PMID: 17784955	https://david.ncifcrf.gov/
Deposited Data		
RNAseq data	This Study	GSE126352
Oligonucleotides		
MYO forward AGCATGGTGCCACTGTGCT	Biolegio	Primer for ChIP-qPCR
MYO reverse GGCTTAATCTCTGCCTCATGAT	Biolegio	Primer for ChIP-qPCR
H2B forward TGTACTTGGTGACGGCCTTA	Biolegio	Primer for ChIP-qPCR
H2B reverse CATTACAACAAGCGCTCGAC	Biolegio	Primer for ChIP-qPCR
GAPDH forward CACCGTCAAGGCTGAGAACG	Biolegio	Primer for ChIP-qPCR
GAPDH reverse ATACCCAAGGGAGCCACACC	Biolegio	Primer for ChIP-qPCR
ZNF UTR forward AAGCACTTTGACAACCGTGA	Biolegio	Primer for ChIP-qPCR
ZNF UTR reverse GGAGGAATTTTGTGGAGCAA	Biolegio	Primer for ChIP-qPCR
TNFa 1 forward AGAGGACCAGCTAAGAGGGA	Biolegio	Primer for ChIP-qPCR
TNFa 1 reverse AGCTTGTCAGGGGATGTGG	Biolegio	Primer for ChIP-qPCR
TNFa 2 forward CAGGCAGGTTCTCTTCCTCT	Biolegio	Primer for ChIP-qPCR
TNFa 2 reverse GCTTTCAGTGCTCATGGTGT	Biolegio	Primer for ChIP-qPCR
TNFa 3 forward GTGCTTGTTCCTCAGCCTCT	Biolegio	Primer for ChIP-qPCR
TNFa 3 reverse ATCACTCCAAAGTGCAGCAG	Biolegio	Primer for ChIP-qPCR
TNFa 4 forward TGTCTGGCACACAGAAGACA	Biolegio	Primer for ChIP-qPCR
TNFa 4 reverse CCCTGAGGTGTCTGGTTTTC	Biolegio	Primer for ChIP-qPCR

TNFa 5 forward AGCCAGCTGTTCCTCCTTTA	Biolegio	Primer for ChIP-qPCR
TNFa 5 reverse TTAGAGAGAGGTCCCTGGGG	Biolegio	Primer for ChIP-qPCR
TNFa 6 forward TGATGGTAGGCAGAACTTGG	Biolegio	Primer for ChIP-qPCR
TNFa 6 reverse ACTAAGGCCTGTGCTGTTCC	Biolegio	Primer for ChIP-qPCR

3

Impact of cholesterol on proinflammatory monocyte production by the bone marrow

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European Heart Journal, 2021

ABSTRACT

Aim

Preclinical work indicates that low-density lipoprotein cholesterol (LDL-C) not only drives atherosclerosis by directing the innate immune response at plaque level but also augments proinflammatory monocyte production in the bone marrow (BM) compartment. In this study, we aim to unravel the impact of LDL-C on monocyte production in the BM compartment in human subjects.

Methods and results

A multivariable linear regression analysis in 12 304 individuals of the EPIC-Norfolk prospective population study showed that LDL-C is associated with monocyte percentage ($\beta = 0.131$ [95% CI: 0.036–0.225]; $P = 0.007$), at the expense of granulocytes ($\beta = -0.876$ [95% CI: −1.046 to −0.705]; $P < 0.001$). Next, we investigated whether altered haematopoiesis could explain this monocytic skewing by characterizing CD34+ BM haematopoietic stem and progenitor cells (HSPCs) of patients with familial hypercholesterolaemia (FH) and healthy normocholesterolaemic controls. The HSPC transcriptomic profile of untreated FH patients showed increased gene expression in pathways involved in HSPC migration and, in agreement with our epidemiological findings, myelomonocytic skewing. Twelve weeks of cholesterol-lowering treatment reverted the myelomonocytic skewing, but transcriptomic enrichment of monocyte-associated inflammatory and migratory pathways persisted in HSPCs post-treatment. Lastly, we link hypercholesterolaemia to perturbed lipid homeostasis in HSPCs, characterized by lipid droplet formation and transcriptomic changes compatible with increased intracellular cholesterol availability.

Conclusions

Collectively, these data highlight that LDL-C impacts haematopoiesis, promoting both the number and the proinflammatory activation of circulating monocytes. Furthermore, this study reveals a potential contributory role of HSPC transcriptomic reprogramming to residual inflammatory risk in FH patients despite cholesterol-lowering therapy.

INTRODUCTION

Atherosclerotic cardiovascular disease (CVD) is the leading cause of death worldwide, despite improved preventive strategies and survival[1]. Low-density lipoprotein cholesterol (LDL-C) is the key driver of atherosclerotic CVD, making cholesterol-lowering treatment the cornerstone of CVD prevention[2,3]. Although a substantial proportion of cardiovascular (CV) events is prevented by cholesterol-lowering treatment, residual CV risk is considerable, even in patients who reach very low plasma LDL-C levels[4]. One of the contributors to residual CV risk is inflammation, which is suggested to be partly driven by enhanced monocyte activation[5]. Targeting the proinflammatory monocyte response is therefore considered a potential strategy to reduce inflammatory risk with minimal systemic immunosuppression[6–8].

Mechanistically, atherosclerosis is the result of an unresolved inflammatory response of monocytes and monocyte-derived macrophages to cholesterol retention in the arterial wall[9]. Over the last decade, it has become increasingly clear that hypercholesterolaemia aggravates this inflammatory process by enhancing the production of proinflammatory monocytes in the bone marrow (BM) compartment[10,11]. This is of clinical interest since epidemiological studies have identified monocyte count as an important CV risk factor[12,13], whereas translational research has confirmed the association of a proinflammatory monocyte phenotype with increased inflammatory activity in the arterial wall[14].

In steady state, all types of mature blood cells—including monocytes—are produced in the BM compartment via a process called haematopoiesis. Following specific stimuli, multipotent haematopoietic stem and progenitor cells (HSPCs) undergo lineage commitment steps while proliferating and differentiating into leukocytes, erythrocytes or thrombocytes[15]. Animal studies have shown that hypercholesterolaemia influences this process by promoting HSPC proliferation and myeloid commitment, ultimately leading to monocytosis and accelerated atherosclerosis[16–18]. Also, alterations in lipid metabolism in HSPCs themselves following, for example, activation of cholesterol synthesis[19] or blocking of cholesterol efflux[20–22], enhances HSPC expansion and myeloid skewing. Together, these studies substantiate that plasma cholesterol levels and intracellular cholesterol homeostasis have impact on HSPC proliferation and differentiation[23]. We therefore hypothesized that the enhanced monocyte response in hypercholesterolemic patients could be traced back to LDL-C-mediated disruption of cholesterol homeostasis in HSPCs and subsequent altered haematopoiesis.

To examine our hypothesis, we performed two human studies. First, we conducted a mechanistic study in which we performed ex vivo unbiased RNA sequencing (RNAseq) and functional analyses of CD34+ BM HSPCs (hereafter HSPCs) of patients with untreated familial hypercholesterolaemia (FH) before and after cholesterol-lowering treatment, and compared these results to normocholesterolemic healthy control subjects. Next, we used epidemiological data to determine the relationship between LDL-C and leucocyte count and differential in the EPIC-Norfolk study.

METHODS

Study population and design

Study population and design for mechanistic analyses in hypercholesterolemic patients

For mechanistic validation, we conducted a single-centre observational study between July 2017 and May 2019 at the Amsterdam UMC (location AMC), The Netherlands. We included untreated FH patients who had an indication to start lipid-lowering therapy [statin, proprotein convertase subtilisin/kexin type 9 (PCSK9), and/or ezetimibe] according to their treating physician. FH was defined as having a mutation in one of the known FH-causing genes (LDLR, PCSK9, APOB) or, in the absence of such mutation after genetic testing, having a Dutch Lipid Clinic Network score ≥ 6 (=probable or definite FH)[24]. Exclusion criteria included active smoking, established CVD and recent use (<3 months) of cholesterol-lowering drugs. The healthy controls were age, sex, and body mass index (BMI) matched with the FH patients. After inclusion, FH patients underwent blood withdrawal and a sternal bone marrow aspiration at baseline and after 12 weeks of lipid-lowering therapy. The healthy controls underwent these procedures once. All participants provided written informed consent. The study protocol was approved by the ethics committee of the Amsterdam UMC and was conducted according to the principles of the Declaration of Helsinki.

Study population and design for epidemiological analysis in the EPIC-Norfolk cohort

For the assessment of the correlation between LDL-C, apolipoprotein B (ApoB) and leucocyte count and differential, we used data from the European Prospective Investigation into Cancer in Norfolk (EPIC-Norfolk) study[25]. Between 1993 and 1997, 639 subjects were recruited from general practices and included in this study. The study protocol was approved by the ethics committee of the Norwich District Health Authority and all study participants gave written informed consent prior to enrolment.

Bone marrow experiments

All the laboratory experiments and bioinformatics analyses regarding HSPC characterization are available in detail in the Supplementary material online.

Statistical approach

Statistical analysis of the EPIC-Norfolk data

After excluding subjects with C-reactive protein (CRP) ≥10 g/L [to minimize bias caused by (acute) infections] and missing leucocyte count and differential values, we performed a univariate regression analysis for LDL-C on leucocyte count, and monocyte, lymphocyte and granulocyte percentage in 12 304 individuals. In addition, we performed multivariable analyses to adjust for age, sex, BMI, smoking, and CRP.

Quantification and statistical analyses FH study

All data were analysed using R version 3.6.3 (R Core Team, Vienna, Austria), SPSS version 25 (SPSS Inc., Chicago, IL, USA), and Graphpad Prism 8 (La Jolla, CA, USA). Data are presented as mean ± standard deviation for normally distributed data, median (interquartile range) for non-normally distributed data, or as a number with percentage from total (%) for categorical variables. Changes in biochemical measurements after cholesterol-lowering treatment were assessed using a paired Students t-test or Wilcoxon signed-rank test for normally and non-normally distributed data, respectively. Unpaired analyses to compare patients with the healthy control group were performed using an unpaired Student's t-test or Mann–Whitney U-test for normally and non-normally distributed data, respectively.

RESULTS

HSPCs of hypercholesterolaemic patients exhibit myelomonocytic skewing and a promigratory profile

In an ex vivo study, we evaluated the impact of hypercholesterolaemia on HSPCs in the BM compartment at a cellular level. We included 10 untreated FH patients (mean baseline LDL-C 6.0 ± 2.5 mmol/L; corresponding with Dutch reference LDL-C > 99th percentile[26]) and 9 age, sex and BMI matched normocholesterolemic healthy controls (mean LDL-C 3.3 ± 0.6 mmol/L; corresponding with Dutch reference LDL-C < 50th percentile26). Leukocyte (differential) count did not significantly differ between the two groups (additional baseline characteristics are shown in Supplementary material online, Table S1). In all study participants, a sternal BM aspirate was obtained, from which we isolated and purified CD34+ HSPCs.

First, principle component analysis of the HSPC RNAseq data showed a separation of the untreated FH patients and healthy controls (Figure 1A). Differential gene expression analysis revealed 1892 differentially expressed genes (DEGs) with a false discovery rate (FDR) of <0.05 (Figure 1B), of which 1642 genes were up- and 250 genes were downregulated in the untreated FH patients vs. healthy controls. Gene ontology (GO) term analysis of the significantly upregulated genes showed predominantly enrichment in pathways related to cell migration (Figure 1C). Most genes in these upregulated pathways, including FLT1, NRP1, CCL2, and CXCL12 (Figure 1B), are members of the vascular endothelial growth factor (VEGF) and chemokine family, respectively. Interestingly, these migratory associated genes promote myeloid progenitor and monocyte mobilization from the BM compartment and increase macrophage and foam cell content in atherosclerotic lesions[27–29]. Gene set enrichment analysis (GSEA) underlined this finding, showing enrichment of the gene set 'monocyte chemotaxis' (FDR = 0.007) in untreated FH patients (Figure 1D). Of note, VEGF receptors (VEGFRs) belong to the large superfamily of receptor tyrosine kinases (RTKs) that play a central role in fundamental cellular functions including proliferation, differentiation, metabolism and migration[30]. In line, pathway analysis showed enrichment in the RTK signalling pathway (Figure 1C) and the small GTPase mediated signalling pathway (Figure 1C), of which the latter are important downstream effectors for many cell surface receptors including RTKs[31]. The top significantly upregulated genes in these two pathways included KDR, a gene encoding VEGFR 2, but also the non-VEGFR RTKs ALK, AXL, and EGFR (Figure 1E). Interestingly, up-regulation of PDK4, a gene encoding a key metabolic mitochondrial protein promoting the switch from glucose to fatty acid oxidation, suggests that fatty acids are the preferred substrates for oxidation in HSPCs of untreated FH patients[32] (Figure 1E).

GO term analysis of the significantly downregulated genes in untreated FH patients demonstrated enrichment of the pathways 'myeloid leucocyte activation' and 'antibacterial humoral response' (Figure 1F). In accordance with our epidemiological data showing a negative association of LDL-C with granulocyte percentage, these two pathways predominantly consisted of downregulated granulocytic associated genes (15 out of 16 genes), which was also reflected by a down-regulation of the gene set 'transcriptional regulation of granulopoiesis' in untreated FH patients (FDR = 0.000) (Figure 1G). In addition to these monocytic-skewed transcriptomic changes in HSPCs of untreated FH patients, an up-regulation of KITLG, encoding an important regulator of stem cell survival and myelopoiesis called stem cell factor (SCF)[33] was observed, in addition to down-regulation of lymphoid-associated gene IL7[34] (Figure 1H).

HSPCs give rise to mature blood cells through cell proliferation and differentiation[35]. Interestingly, GSEA showed that gene sets related to cell cycle and differentiation were negatively enriched in untreated FH patients (FDR = 0.000 for both) (Figure 1I–J). Metabolically, this was in line with a concordant negative enrichment of the oxidized phosphorylation (OXPHOS) gene set (FDR = 0.000) (Figure 1K), which is a metabolic programme used in more proliferating and mitochondrial active HSPCs[36,37]. The decreased gene expression associated with HSPC differentiation, in addition to the up-regulation of stem cell survival regulator KITLG, coincided with a 1.4-fold increase of the percentage CD34+ HSPCs in the BM compartment (P = 0.004) (Figure 1L), measured by flow cytometry. Taken together, the HSPC transcriptome of untreated FH patients differs from healthy controls, hallmarked by global up-regulation of promigratory pathways and myelomonocytic skewing.

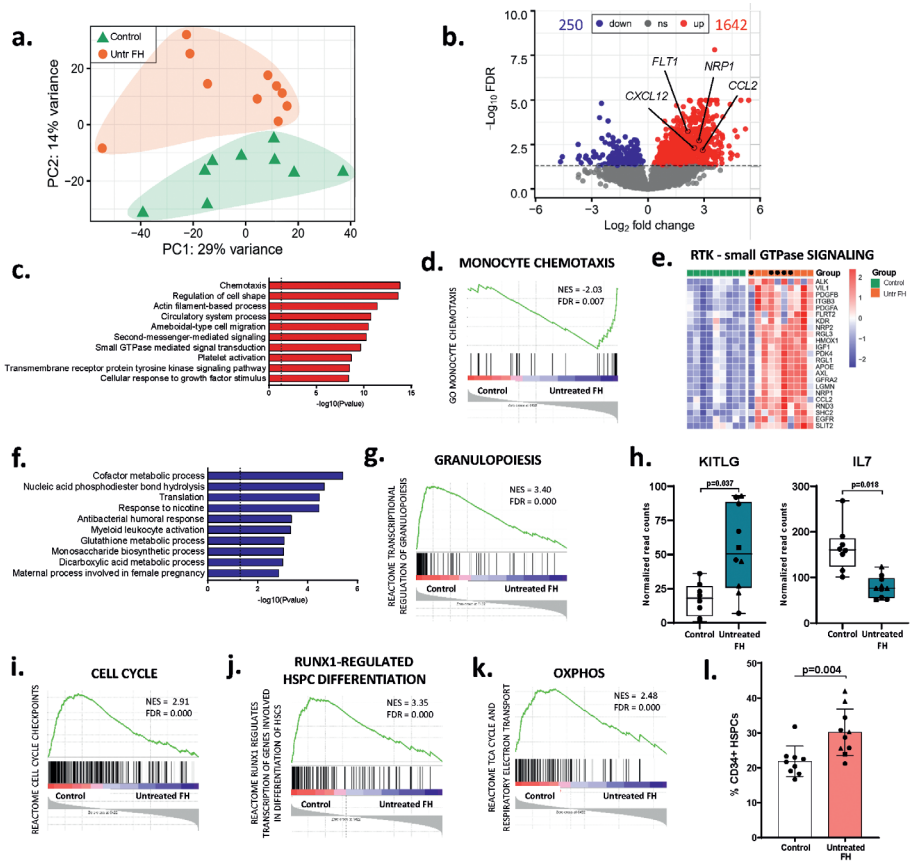


Figure 1. HSPCs of hypercholesterolemic patients exhibit myelomonocytic skewing and a promigratory profile

(A) Principle component analysis plot of RNAseq data of isolated CD34+ HSPCs. (B) Volcano plot showing differentially expressed genes. (C) Top 10 most significant enriched pathways using gene ontology (GO) term analysis of significantly upregulated genes in untreated FH patients vs. healthy controls. (D) Enrichment of gene set 'GO monocyte chemotaxis'. (E) Heatmap of most significant upregulated genes in GO RTK signalling and small GTP-ase mediated signalling in untreated FH patients; black dot indicates mutation proven FH. (F) Top 10 most significant enriched pathways using GO term analysis of significantly downregulated genes in untreated FH patients vs. healthy controls. (G) Enrichment of gene set 'REACTOME transcriptional regulation of granulopoiesis'. (H) Normalized gene counts for KITLG and IL7. P-values are adjusted for multiple testing using Bonferroni–Hochberg correction. Triangle symbol indicates proven LDLR mutation, a square indicates proven APOB mutation, a dot indicates no FH mutation. (I–K) Enrichment of gene sets 'REACTOME cell cycle checkpoints', 'REACTOME RUNX1 regulates transcription of genes involved in differentiation of HSCs', and 'REACTOME TCA cycle and respiratory electron transport'. (L) Percentage CD34+ HSPC in bone marrow compartment measured by flow cytometry. Data are mean ± SD. Triangle symbol indicates proven LDLR mutation, a square indicates proven APOB mutation, and a dot indicates no FH mutation.

Cholesterol-lowering treatment mitigates decreased HSPC differentiation and myelomonocytic skewing in the BM compartment

Following the first BM aspiration, FH patients received maximally tolerated cholesterol-lowering treatment by either a statin, a PCSK9 antibody or a combination, with or without ezetimibe (Supplementary material online, Table S2). After 12 weeks of treatment, a mean 66% reduction in plasma LDL-C levels was achieved ($P < 0.001$), resulting in a mean post-treatment plasma LDL-C level of 1.89 ± 1.16 mmol/L (Supplementary material online, Table S2). No significant changes in leucocyte (−0.02 [1.25]; $P = 0.961$) and monocyte count (0.12 [1.48]; $P = 0.803$) were observed.

After 12 weeks of treatment, HSPC gene expression demonstrated a trend towards normalization of the transcriptomic profile (Figure 2A). Pairwise comparison of the transcriptomic profile before vs. after treatment showed 2462 significantly DEGs, of which 940 genes were upregulated and 1522 genes were downregulated after treatment (Figure 2B). GO term analysis of the significantly downregulated genes showed predominantly enrichment of pathways involved in cell motility (Figure 2C). In addition, GO term analysis of the significantly upregulated genes and GSEA showed predominantly enrichment of pathways implicated in OXPHOS (Figure 2D–F). To functionally validate these findings, we measured the oxygen consumption rate in HSPCs by Seahorse Flux Analysis. As expected and while not significant, basal respiration was almost 70% lower ($P = 0.067$) in untreated FH patients vs. healthy controls, and was significantly increased after cholesterol-lowering therapy ($P = 0.029$) (Figure 2G), following the OXPHOS gene expression pattern seen in the RNAseq.

Since OXPHOS fluxes are higher in more proliferative and differentiated HSPCs[36,37], we examined whether gene sets involved in proliferation and HSPC differentiation were concomitantly increased after cholesterol-lowering therapy. Indeed, GSEA confirmed enrichment of the cell cycle and RUNX1-mediated differentiation gene sets in FH patients after treatment (FDR = 0.000 and FDR <0.001, respectively) (Figure 2H and I). Earlier we noted that the attenuation of genes involved in granulocyte differentiation was most prominent in untreated FH patients compared to healthy controls. Interestingly, both GO term analysis and GSEA revealed reversibility of this effect, showing significantly increased gene expression associated with neutrophil activation (Figure 2D) and granulocyte differentiation (Figure 2J), respectively. Alleviation of decreased gene expression in pathways involved in HSPC differentiation was further supported by significant enrichment of gene sets involved in lymphopoiesis, erythropoiesis, and megakaryopoiesis, whereas the gene set involved in monocytopoiesis was unaffected (FDR = 0.994) (Supplementary material online, Figure S1). More specifically, expression of key transcriptional determinants of myeloid progenitor commitment GATA1 (inducing megakaryo-erythroid commitment), and CEBPA with its target gene EVI2B (inducing granulocytic over monocytic commitment) significantly increased after cholesterol-lowering treatment (Figure 2K)[35,38,39]. These data, including the up-regulation of CEBPA in the presence of non-significant change in PU.1 expression, indicate reduced myelomonocytic skewing in HSPCs of treated FH patients (Figure 2K)[39]. A significant decrease in functional ex vivo progenitor capacity of the colony-forming unit of granulocytes and monocytes (CFU-GM) (−26.2%; P = 0.028) in the presence of a significant increase of CEPBA, and no effect on progenitor capacity of the burst-forming unit of erythrocytes (BFU-e) (−5.3%, P = 0.751; Figure 2L), further supports reduced myelomonocytic skewing after cholesterol-lowering treatment, as observed in the gene expression data.

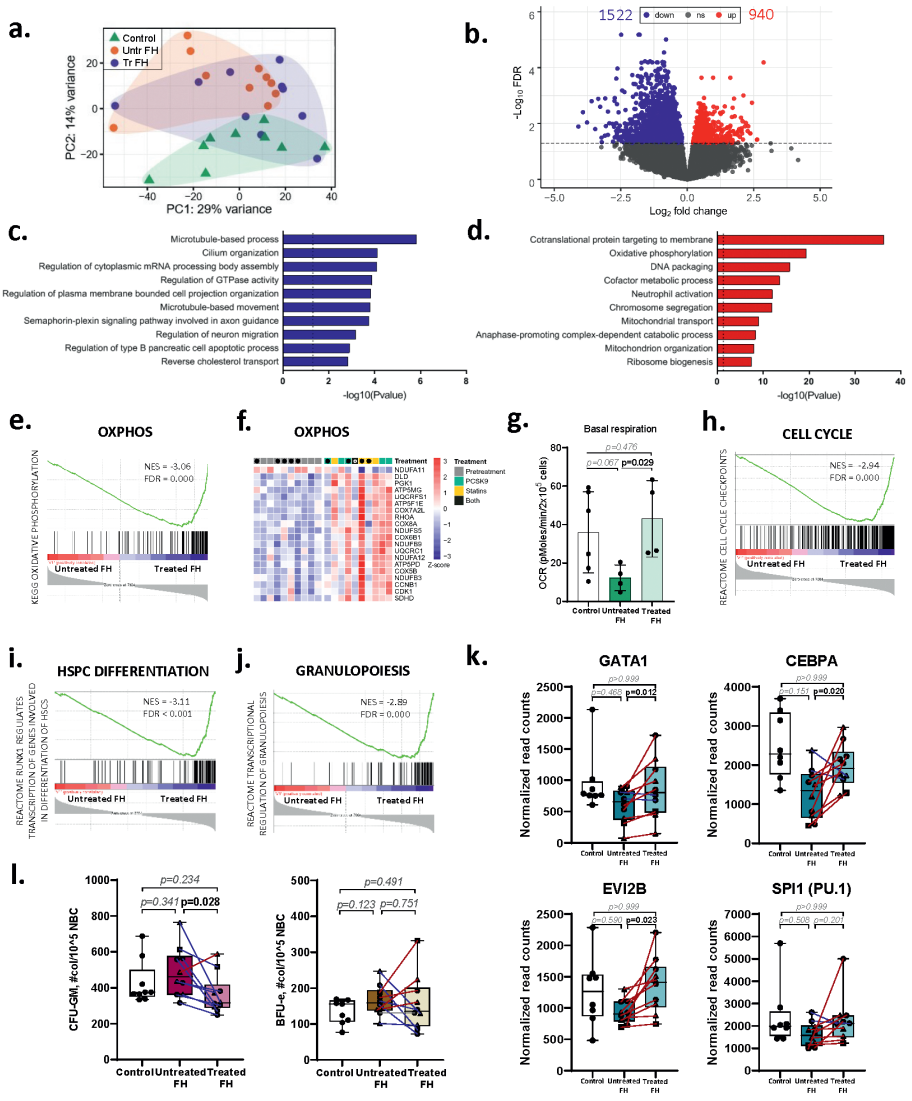


Figure 2. Cholesterol-lowering treatment mitigates decreased HSPC differentiation and myelomonocytic skewing

(A) Principle component analysis plot of RNAseq data of isolated CD34+ HSPCs. (B) Volcano plot showing differentially expressed genes before vs. after cholesterol-lowering treatment in FH patients. (C) Top 10 most significant enriched pathways using gene ontology (GO) term analysis of significantly downregulated genes in treated FH patients vs. untreated FH patients. (D) Top 10 most significant enriched pathways using GO term analysis of significantly upregulated genes in treated FH patients vs. untreated FH patients. (E) Enrichment of gene set 'KEGGoxidative phosphorylation'. (F) Heatmap of most significantly differentially expressed gene in KEGG pathway OXPHOS in treated FH patients, black dot indicates mutation proven

FH. (G) Seahorse extracellular flux analysis of Oxygen Consumption Rate (OCR) in CD34+ HSPCs. (H–J) Enrichment of gene sets relating to HSPC proliferation and differentiation. (K) Normalized read counts for several genes encoding regulators of myeloid HSPC differentiation. P-values are adjusted for multiple testing using Bonferroni–Hochberg correction. Triangle symbol indicates proven LDLR mutation, a square indicates proven APOB mutation, and a dot indicates no FH mutation. (L) Granulocyte monocyte colony forming unit (CFU-GM) and burst-forming unit-erythroid (BFU-e) assay. Data are mean ± SD. Triangle symbol indicates proven LDLR mutation, a square indicates proven APOB mutation, and a dot indicates no FH mutation.

Persistent proinflammatory and promigratory gene expression in HSPCs after cholesterol-lowering treatment

We previously described a persistent hyper responsiveness of circulating monocytes in treated FH patients termed 'trained immunity'[5]. Since this immune memory persists beyond the short lifespan (hours to days) of circulating monocytes, it has been hypothesized that cholesterol-induced reprogramming of the long-lived progenitors of monocytes maintain this innate immune memory[5,18]. To examine this hypothesis, we compared the HSPC transcriptional profile of the FH patients post-treatment to healthy controls.

Whereas we found 1892 significantly DEGs in untreated FH patients vs. healthy controls (Figure 1B), only 133 genes were differentially expressed after treatment compared to healthy controls (Figure 3A). Interestingly, 128 out of the 131 upregulated genes were also significantly upregulated before treatment (Figure 3B). GO term analysis of these genes showed enrichment of the 'chemotaxis' and 'acute inflammatory response' pathway and GSEA revealed enrichment of the gene set 'regulation of lipopolysaccharide (LPS) mediated signalling pathway' (FDR = 0.019) (Figure 3C and D). This is in line with our previous findings showing that the trained immunity phenotype of circulating monocytes of FH patients is hallmarked by persistent enhanced cytokine production after LPS stimulation ex vivo[5]. Among the most upregulated inflammatory genes were plaque macrophage marker CD163 (Klco et al., 2011)[40] and PLA2G7, which encodes lipoprotein-associated phospholipase A2 (Lp-PLA2) (Figure 3E). Lp-PLA2 is an enzyme produced by plaque macrophages, serves as a marker for vulnerable plaques and is a strong predictor of CVD[41,42]. Also, genes involved in chemotaxis were persistently elevated in HSPCs of treated FH patients, including CCL2, CXCL12, and VCAM1 (Figure 3E), with concomitant enrichment of the gene set 'monocyte chemotaxis' (Figure 3F). This gene expression profile coincided with persistent decreased cell surface expression of BM homing receptor CXCR4 on CD34+ HSPCs (Figure 3G and Supplementary material online, Figure S2), and sustained increased CCR2 cell surface expression on both BM (Figure 3H and Supplementary material online, Figure S2) and circulating CD14+ monocytes (Figure 3I) after treatment. Analysis of the monocyte subset distribution in the BM

compartment did not show any significant differences (Figure 3J). Also, we did not find significant correlations between CCR2 expression on circulating monocytes and CCR2 expression on bone marrow monocytes, nor with CXCR4 expression on CD34+ HSPCs (Supplementary material online, Figure S3). Altogether, these results indicate that transcriptomic reprogramming of HSPCs could contribute to the trained immunity phenotype found in circulating monocytes of FH patients.

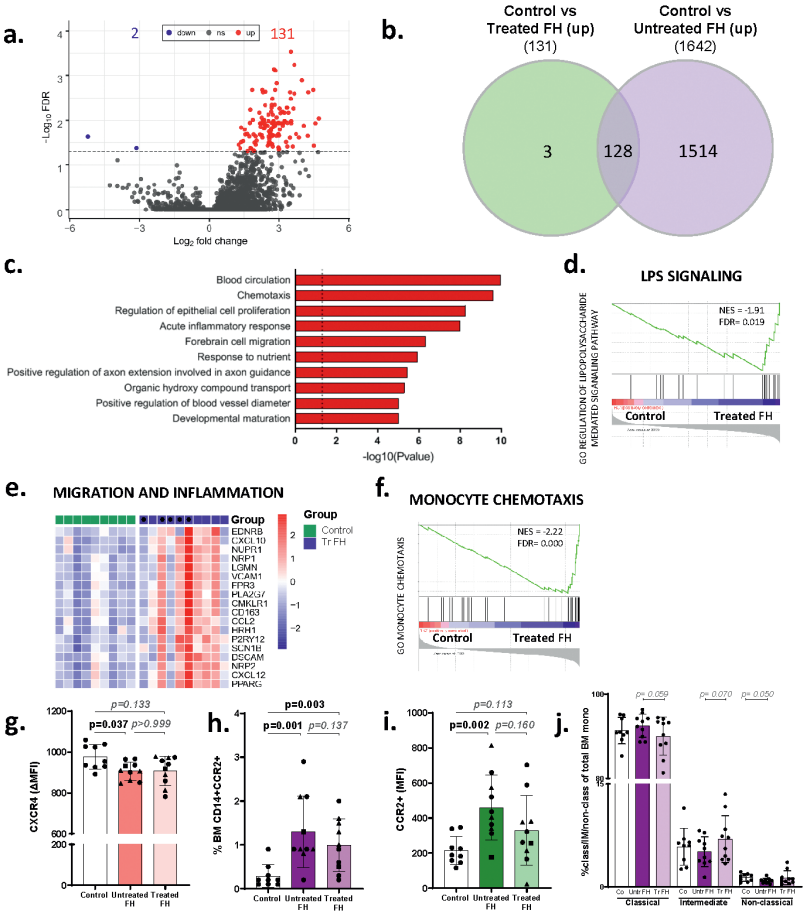


Figure 3. Persistent proinflammatory and promigratory gene expression in HSPCs after cholesterol-lowering treatment

(A) Volcano plot showing differentially expressed genes in treated FH patients vs. healthy controls. (B) Venn diagram indicating the number of significantly upregulated genes in untreated FH patients vs. healthy controls, and in treated FH patients vs. healthy controls. (C) Top 10 most significant enriched pathways in treated FH patients vs. healthy controls. (D) Enrichment of gene set 'GO regulation of lipopolysaccharide mediated signalling pathway'. (E) Heatmap of most significant differentially expressed genes in GO chemotaxis and GO acute inflammatory response pathways in treated FH patients; black dot indicates mutation

proven FH. (G) Enrichment of gene set 'GO monocyte chemotaxis'. (G–J) CXCR4 expression on CD34+ HSPC, CCR2 expression on circulating and bone marrow CD14+ monocytes, and monocyte subsets (CD14, CD16) in BM measured by flow cytometry. Data are mean ± SD. Triangle symbol indicates proven LDLR mutation, a square indicates proven APOB mutation, and a dot indicates no FH mutation.

Both hypercholesterolaemia and cholesterol-lowering treatment affect lipid homeostasis in HSPCs of FH patients

Disrupted lipid homeostasis in HSPCs has major impact on HSPC behaviour[10,23]. Moreover, preclinical studies have linked altered lipid metabolism in myeloid progenitors to trained immunity[18,43]. Indeed, the top 25 of most significantly overexpressed gene sets in untreated FH patients compared to healthy controls included gene sets 'cholesterol storage', 'lipid metabolism', and 'lipid digestion mobilization and transport' (FDR = 0.007, 0.005, and 0.038, respectively) (Figure 4A–C). In parallel, staining of intracellular lipid droplets (LDs) by Nile Red demonstrated an increased number of LDs in HSPCs of untreated FH patients compared to healthy controls (Figure 4D and E). Cells form LDs in reaction to lipid overload to prevent lipotoxicity[44]. In line with GSEA, also other compensatory pathways to lower intracellular cholesterol content were observed in HSPCs of untreated FH patients, including significant up-regulation of cholesterol efflux transporter gene expression of ABCA1 and ABCG1 (Figure 4F). In turn, cholesterol-lowering treatment led to normalization of intracellular LD number (Figure 4D and E). This coincided with a significant reduction of ABCA1 and ABCG1 (Figure 4F). Lastly, PPARD and PPARG, encoding lipid sensors peroxisome proliferator-activated receptor delta and gamma, respectively, were significantly upregulated before treatment (Figure 4G). After cholesterol-lowering treatment, PPARD expression decreased, whereas PPARG remained significantly upregulated (Figure 4G). This persistent up-regulation of PPARG post-treatment corresponded with enrichment of the gene set 'lipid metabolism' in treated FH patient vs. healthy controls (Figure 4H).

LDL-C is positively associated with monocyte percentage but inversely associated with granulocyte percentage

To assess the impact of plasma LDL-C level (mmol/L) on leucocyte count (10^9/L) and differential (% and count), we performed a linear regression analysis using data from 12 304 individuals participating in the EPIC-Norfolk prospective population study (Supplementary material online, Table S3). LDL-C was not significantly associated with leucocyte count (β = −0.017, 95% CI (−0.046 to 0.012); P = 0.251); whereas we did find a significant positive association between LDL-C and monocyte percentage, also after adjustment for age, sex, BMI, smoking, CRP and leucocyte count [β = 0.131, 95% CI (0.036–0.225); P = 0.007]. Conversely, LDL-C was inversely associated with granulocyte percentage [β = −0.876, 95% CI (−1.046 to −0.705);

P < 0.001] (Table 1). The discrepancy between the positive vs. negative association of monocyte and granulocyte percentage with LDL-C respectively suggests that LDL-C skews haematopoiesis favouring monocyte over granulocyte production, since both immune cells arise from the same bipotential haematopoietic granulocyte–monocyte progenitor (GMP)[35]. We found a similar association pattern between LDL-C and monocyte and granulocyte count (Supplementary material online, Tables S4 and S5). Interestingly, ApoB also shows a reciprocal association with monocytes and granulocytes, whereas ApoA1 does not show these associations (Supplementary material online, Table S6).

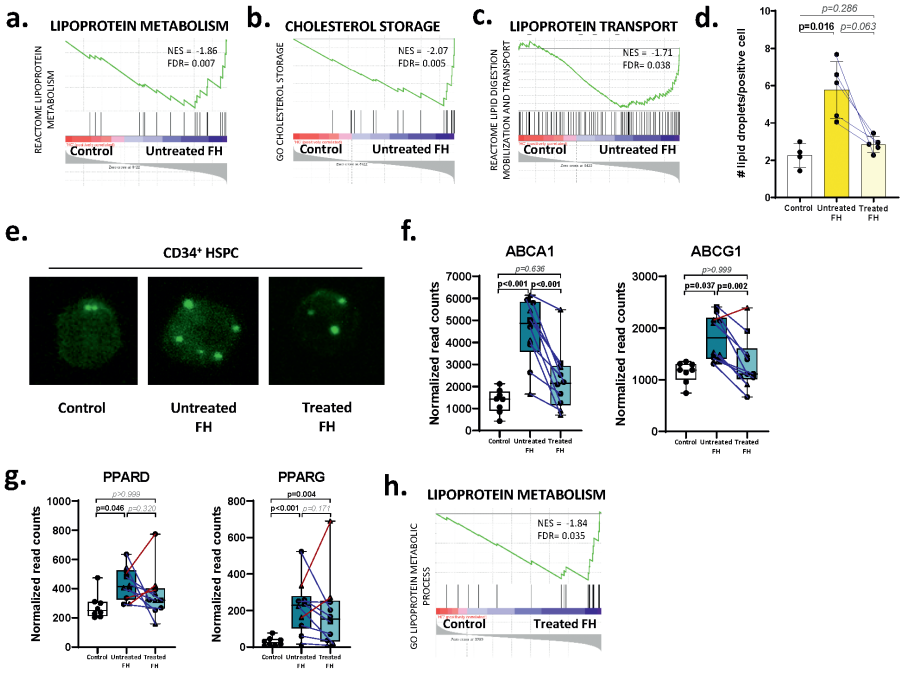


Figure 4. Both hypercholesterolaemia and cholesterol-lowering treatment affects lipid homeostasis in HSPCs of FH patients

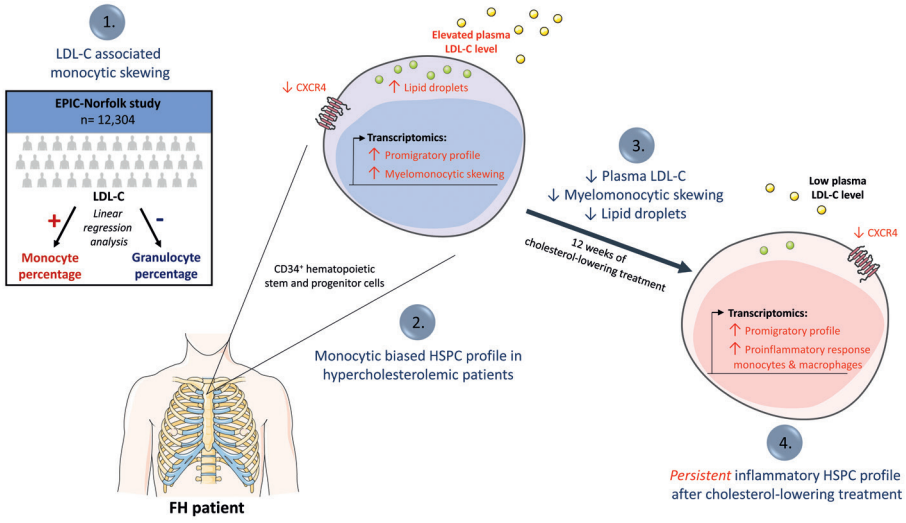
(A–C) Enrichment of gene sets related to lipid homeostasis. (D) Lipid droplet count in lipid droplet positive CD34+ HSPCs. Data are mean ± SD. (E) Representative images of CD34+ HSPC stained with Nile red. (F and G) Normalized read counts for genes related to lipid homeostasis. P-values are adjusted for multiplicity using Bonferroni correction. Triangle symbol indicates proven LDLR mutation, a square indicates proven APOB mutation, and a dot indicates no FH mutation. (H) Enrichment of gene set 'GO lipoprotein metabolic process'.

Table 1. Relationship between LDL-C and leukocyte differential count

	Monocytes, %		Granulocytes, %		Lymphocytes, %	
	β (95% CI)	*p-value*	*β (95% CI)*	*p-value*	*β (95% CI)*	*p-value*
Unadjusted model	0.197 (0.105 – 0.289)	<0.001	-0.910 (-1.076 – 0.745)	<0.001	0.742 (0.613–0.870)	<0.001
Model 1	0.126 (0.032 – 0.220)	0.009	-0.884 (-1.054 –0.715)	<0.001	0.787 (0.655–0.918)	<0.001
Model 2	0.131 (0.036 – 0.225)	0.007	-0.876 (-1.046 –0.705)	<0.001	0.752 (0.622–0.882)	<0.001

N=12,304 individuals (general population EPIC Norfolk cohort)

Data are standardized coefficient (β) with 95% confidence intervals (CI).
Unadjusted model; univariate linear regression analysis of plasma LDL-C level (mmol/L) and leukocyte number (10^9/L), percentage monocytes, granulocytes or lymphocytes. Model 1; adjusted for age and sex. Model 2; adjusted for Model 1, total white blood cell count, body mass index, smoking, and C-reactive protein. Betas are reported for each standard deviation increase in LDL-C. The variance inflation factors of all predictor variables were < 1.3. R^2 monocyte percentage model 2: 0.0225. R^2 granulocyte percentage model 2: 0.06384 R^2 lymphocyte percentage model 2: 0.06557



Summary Figure.

DISCUSSION

Here, we report epidemiological and mechanistic evidence for a causal role of LDL-C in driving the production of proinflammatory monocytes at the BM level in hypercholesterolemic patients. Multivariable regression analysis of LDL-C to leucocyte differential count in over 12 000 individuals of the EPIC-Norfolk study showed a positive association with monocyte percentage, and a negative association with granulocyte percentage. Ex vivo BM analyses demonstrated that HSPCs of untreated FH patients are hallmarked by myelomonocytic skewing and a promigratory phenotype, coinciding with perturbed intracellular lipid homeostasis. Twelve weeks of cholesterol-lowering treatment largely reverted these HSPC alterations. However, despite normalization of plasma LDL-C levels, gene expression involved in monocyte and macrophage-mediated inflammation and migration remained upregulated in HSPCs of treated FH patients compared to healthy controls (Graphical abstract).

LDL-C is epidemiologically and mechanistically linked to enhanced monocyte production

Our findings in the EPIC-Norfolk cohort revealed no association of LDL-C with leucocyte count and opposing associations with monocyte and granulocyte percentage, independently of CRP levels. These results suggest that the association of LDL-C with specifically monocyte count is not merely a reflection of the low-grade inflammatory state hallmarking patients with increased CV risk, but could imply an LDL-C-specific biological effect on leucocyte subset formation. These findings were validated mechanistically, where we showed that the HSPC transcriptomic profile was characterized by myelomonocytic skewing in untreated FH patients. Notably, we demonstrated that the myelomonocytic skewed transcriptomic profile largely normalized after cholesterol-lowering treatment, including up-regulation of master regulator and promotor of granulopoiesis CEPBA[38]. Our results are in line with previous preclinical findings demonstrating that hypercholesterolaemia-induced myeloid skewing was in part the result of transcriptional reprogramming of the bipotential GMPs[18]. Combined, our epidemiological and mechanistic results support that LDL-C promotes monocyte production in the BM compartment at least in part via modulated GMP fate, thereby impeding granulocytic differentiation. Of note, in the absence of a lymphoid skewed transcriptomic pattern in BM HSPCs of untreated FH patients, the significant positive association between LDL-C and lymphocyte number in the EPIC-Norfolk study may imply a positive effect of hypercholesterolaemia on extramedullary lymphopoiesis.

LDL-C promotes promigratory phenotype of monocytes and their progenitors

Besides increased monocyte number, also the promigratory phenotype of monocytes and their progenitors contribute to accelerated atherosclerosis. In this respect, we observed an up-regulation of promigratory genes in HSPCs of untreated FH patients, including FLT1. This is of interest, since blocking of Flt1 in a hypercholesterolemic mouse model, abrogated myeloid progenitor egress from the BM compartment into the circulation, which coincided with decreased macrophage content in atherosclerotic lesions[27]. Furthermore, we observed decreased cell surface expression of the BM homing receptor CXCR4 on HSPCs of untreated FH patients of which its down-regulation has been described to promote HSPC mobilization[45]. Indeed, previous human studies have linked hypercholesterolaemia to HSPC migration, evidenced by an association between total cholesterol levels and circulating CD34+ HSPCs[46,47]. In line, we previously showed that circulating monocytes of FH patients have increased CCR2 cell surface expression[5,48], facilitating monocyte migration into the atherosclerotic plaque[49]. Interestingly, we here show that CD14+ monocytes in the BM compartment of FH patients also have persistent increased CCR2 cell surface expression after cholesterol-lowering treatment. The persistent CCR2 expression on both BM as circulating monocytes and concomitant persistent promigratory HSPC transcriptomic profile in treated FH patients implies hypercholesterolaemia-induced HSPC priming in vivo.

Disrupted lipid homeostasis linked to altered HSPC behaviour

Preclinical work has established that hypercholesterolaemia directly impacts HSPCs and their behaviour[10]. Here, we observed that increased plasma LDL-C levels lead to a profound increase in LD number in HSPCs of untreated FH patients, despite compensatory up-regulation of cholesterol efflux transporter genes ABCA1 and ABCG1. Interestingly, in parallel of the increased LD number in HSPCs, we found at both transcriptional as functional level decreased mitochondrial OXPHOS in HSPCs of untreated FH patients and increased PDK4 expression, marking fatty acid oxidation. Inhibition of OXPHOS has been described in the setting of excess intracellular lipid accumulation[50] and could have driven the observed differentiation impairment of HSPCs in untreated FH patients[51]. Corroborating these findings, normalization of plasma LDL-C levels following cholesterol-lowering treatment coincided with reduced LD number in HSPCs, in addition to an increase in OXPHOS and alleviation of the reduced differentiation. However, the reduction in OXPHOS could also be a passive reflection of the observed increased percentage of upstream HS(P)Cs in untreated FH patients. Future studies on whether these aforementioned metabolic changes drive monocytic biased differentiation of HSPCs, and via which

mechanisms the different cholesterol-lowering treatment modalities impact these changes, are therefore warranted.

Clinical implications

Lessons from the CANTOS trial that targeting inflammation alongside cholesterol-lowering therapy is able to further reduce CVD risk[52], has fuelled the search for other anti-inflammatory therapies to combat (residual) inflammatory CVD risk. Our study suggests that even after cholesterol-lowering treatment modulated haematopoiesis contributes to the pro-atherogenic monocyte response in hypercholesterolemic patients, highlighting that BM HSPCs could serve as a new therapeutic target. For example, targeting mobilization of monocytes and their precursors has already been suggested to mitigate accelerated atherosclerosis post-myocardial infarction, whereas our findings emphasize a potentially beneficial effect in the chronic inflammatory setting as well[27,53]. However, targeting HSPCs is probably more complex, since several preclinical studies have established that the changes in HSPCs contributing to prolonged monocyte activation are multifaceted, with metabolic, transcriptomic, and epigenetic alterations[18,43,54]. Especially in the context of trained immunity, further research in hypercholesterolemic patients is warranted, to further investigate whether the observed transcriptomic (and metabolic) HSPC reprogramming is accompanied by epigenetic alterations in these cells. In addition, other CV risk factors including sleep deprivation, diabetes mellitus, and lack of exercise contribute to proatherogenic changes in haematopoiesis[55–57]. It would be of interest to further investigate how a combination of these risk factors and the patient's genetic background impact haematopoiesis and ultimately atherogenesis, both in the acute setting of an ischaemic event and in the setting of chronic inflammation.

CONCLUSION

In conclusion, this study provides epidemiological and mechanistic evidence that hypercholesterolaemia modulates HSPC behaviour in the BM compartment, thereby enhancing proinflammatory monocyte production in patients. Moreover, persistent promigratory and proinflammatory gene expression in HSPCs despite normalization of plasma LDL-C levels suggests that prolonged monocyte activation originates in the BM compartment of hypercholesterolemic patients.

Supplementary material

Supplementary material is available at European Heart Journal online.

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Conflict of interest
L.C.A.S., L.W., Y.K., K.H.M.P., N.J.W., S.M.B., C.K., C.V., M.P.J.d.W., and J.K. have nothing to disclose. M.N. received consulting fees from Verseau Therapeutics, Gimv and IFM Therapeutics. E.S.G.S. reports that his institution has received lecturing fees and advisory board fees from Amgen Inc., Regeneron, Sanofi, Akcea, Novartis, and Esperion.

Data availability
The data underlying this article will be shared on reasonable request to the corresponding author.

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SUPPLEMENTAL INFORMATION

1. SUPPLEMENTAL MATERIALS AND METHODS

1.1 Sternal bone marrow aspirations - Study subjects were in fasting state (≥9 hours) before aspiration. The same physician performed all sternal bone marrow aspirations to avoid inter-physician variability, and were performed in the morning between 8.00-10.00 am to avoid bias in HSPC behavior due to circadian rhythm[67]. All sternal bone marrow aspirations were performed using local anesthesia (5-10mL of lidocaine 2%). A maximum of 10mL bone marrow aspirate was obtained per aspiration and collected in EDTA tubes.

1.2 Biochemical measurements - Study subjects were in fasting state for at least 9 hours before fasting lipid samples were obtained (at the same day as bone marrow aspiration) via venepuncture. Plasma total cholesterol, apolipoprotein B (apoB), high-density lipoprotein cholesterol (HDL-C), and triglycerides were measured with commercially available enzymatic assays. LDL-C was calculated using the Friedewald formula.

1.3 Bone marrow and peripheral blood sample handling - To limit inter- and intra-operator variability, the sample handling was performed following standard operational procedures (protocols) by dedicated members of our study team. All samples were handled in the same laboratory and measured on the same dedicated machinery.

1.4 Bone marrow mononuclear cell (BMMC) isolation - Within 2 hours after bone marrow aspiration, bone marrow mononuclear cells (BMMCs) were isolated using a Ficoll Density Gradient separation technique. In short, the bone marrow aspirate was dissolved in Phosphate Buffered Saline (PBS) and layered on 12.5mL of Ficoll-Paque™ PLUS (density 1.077 g/mL; GE Healthcare Bio-Sciences AB, Uppsala, Sweden). After centrifugation (2000RPM, 15 minutes, room temperature, no brake), BMMCs were collected from the interphase layer and counted using CASY ® Cell Counter (Coulter Isoton II Diluent, Beckman Coulter, 8448011). $5{\cdot}10^6$ BMMCs were used directly for flow cytometry, and $2{\cdot}10^6$ BMMCs for the CFU-GM and BFU-e assays (see below). The remaining BMMCs were further processed for $CD34^+$ isolation (see below).

1.5 Flow cytometry - Bone marrow. For flow cytometry analysis of the bone marrow samples, the following antibodies were used: CD34-PC7 clone 581 (Beckman Coulter,

A21691), CD45-PacO, clone HI30 (LifeTechnologies, MHCD4530), CD14-APC H7, clone MΦP9 (BD, 641394), CD14-PERCP-Cy5.5, clone MΦP9 (BD, 345786), CD16-APC H7, clone 3G8 (BD, 560195), CD16-FITC, clone 5D2(CLB-FcR gran/1) (Sanquin, M1604), CD36-V450, clone CB38 (also known as NL07) (BD, 561535), IgG2a-PE, clone X29 (BD, 349053), IgG2b-AF647, clone 27-35 (BD, 557903), 7-AAD, clone (BD, 559925), CD192-AF647, clone 48607 (BD, 558406), CD11b-PE, clone D12 (BD, 333142), CD18-FITC, clone MEM-148 (AbD Serotec), CD184-PE, clone 12G5 (BD, 555974), CD300e-PE, clone UP-H2 (BioLegend, 339704).

Peripheral blood. For flow cytometry of the peripheral blood samples, the following antibodies were used: CD14-PE-Cy7, clone M5E2 (BD, 557742), CD16-APC-H7, clone 3G8 (BD, 560195), HLA-DR-PerCPCy5.5, clone G46-6 (BD, 552764), CCR2-AF647, clone 48607 (BD, 561744).

Both the bone marrow as peripheral blood samples were measured the same day on a CANTO II flow cytometer. Data were analyzed using BD FACSDiva software (v.8.0). $CD34^+$ HSPCs and $CD14^+$ mature monocytes in the bone marrow samples were characterized using a gating strategy based on the Euroflow protocol[68] (Figure S2). For monocyte characterization in peripheral blood a standardized gating strategy was used, as described previously[69].

1.6 CFU-GM and BFU-e assay - BMMCs were plated in duplicate in 35 mm tissue culture plates at concentrations of 1.0, 0.5, and $0.25 \cdot 10^5$ cells/mL, respectively, in MethoCult GF 4534 (StemCell Technologies, Vancouver, BC, Canada) for CFU-GM, and for BFU-e Methocult H4330 + SCF 50 ng/mL at a concentration of $0.25 \cdot 10^5$/mL was used. Cultures were incubated for 12–14 days for CFU-GM and 14-15 days for BFU-e at 37°C at 5% CO_2. Colony-forming units-granulocyte/monocyte colonies and BFU-e were scored in duplo by microscopy (Leica, Solms, Germany). The CFU/BFU scoring and analyses were performed by two blinded technicians.

1.7 $CD34^+$ HSPC isolation - After BMMC isolation and taking cells for flow cytometry and colony forming assays (see previous paragraphs), remaining BMMCs were used for $CD34^+$ MACS® magnetic bead isolation following manufacturers' protocol (Miltenyi Biotec). After counting, $3 \cdot 10^5$ $CD34^+$ HSPCs were stored in 1mL TriPure Reagent (Roche) at -80˚C, until further processing for RNA isolation (see below). $1 \cdot 10^5$ $CD34^+$ HSPCs were directly used for lipid droplet staining. Remaining $CD34^+$ HSPCs were stored in a biobank (in DMSO 10%, liquid nitrogen), until further processing (Seahorse analysis, see below).

1.8 Lipid droplet staining and quantification - For lipid droplet staining, 50.000 $CD34^+$ HSPCs were mounted on fibronectin coated glass in duplicate. In short, $5{\cdot}10^5$ $CD34^+$ HSPCs (concentration $2.5{\cdot}10^6$ cells/mL) were added on fibronectin (30µl/mL; Sanquin Amsterdam, the Netherlands) coated glass microscope slides in duplicate. After an incubation period of 1 hour at 37˚C and 5% CO_2, cells were fixed with 4% formaldehyde, washed with PBS, permeabilized with 0.1% Triton X-100 in PBS and subsequently incubated with the lipid dye Nile Red (1 µg/mL; N3013-100MG, Sigma Aldrich, Zwijndrecht, the Netherlands). Lastly, cells were washed with PBS and mounted using fluorescent mounting medium (Dako, Heverlee, Belgium). One blinded member of our study team performed imaging of all samples on a Leica TCS SP8 Confocal laser scanning microscope, and quantification of lipid droplets was performed by using Image-J software (http://rsb.info.nih.gov/nih-image). Lipid droplet count was defined as the number of lipid droplets per positive $CD34^+$ HSPC in 6-10 fields of view (FOV).

1.9 RNA isolation - CD34+ cells were lysed using TriPure (Sigma Aldrich, 11667165001) and stored at -80 ºC until further processing. All samples were thawed and processed on the same day by one technician. The quality of the nucleic acids was assessed using a Fragment Analyzer (Agilent Technologies, Santa Clara, California, USA). The NEBNext Ultra II Directional RNA Library Prep Kit for Illumina was used to process the RNA. rRNA was depleted from total RNA using a rRNA depletion kit (NEBNext). RNA amplification, cDNA generation, and adaptor ligation were performed following the manufacturer's instructions. Poly-A containing transcripts were sequenced on an Illumina Novaseq 6000 instrument to a depth of ±20 million reads by GenomeScan (Leiden, The Netherlands).

1.10 Seahorse analysis - Cryopreserved $CD34^+$ HSPCs (of a subset of study subjects of whom we obtained enough cells) were thawed and rested for 24 hours at 37°C and 5% CO_2 in 10mL of CellGenix® medium (20802-0500, Freiburg, Germany), supplemented with stem cell factor (SCF) 1:1000, and fms like tyrosine kinase 3 (Flt3) 1:200. After 24 hours, $CD34^+$ HSPCs were plated ($2.5{\cdot}10^5$ cells/well) on XFe96 microplate (Agilent Seahorse; 101085-004) which were pre-coated with Cell-Tak Cell and Tissue Adhesive (Corning, Cat # 354240). The plate was incubated in unbuffered DMEM assay medium (Merck) for 1h in a non-CO_2 incubator at 37°C. OXPHOS was determined by measuring oxygen consumption rate (OCR). OCR changes were measured in response to oligomycin (1.5µM), FCCP (1.5µM) and rotenone (1.25µM) + antimycin A (2.5µM) injection. Values were corrected for cell count.

1.11 Bioinformatics analysis RNAseq data - Reads were aligned to the human genome (hg38) reference sequence using a short-read aligner based on Burrows-Wheeler Transform with default settings[70]. Binary alignment map (BAM) files were sorted on coordinates and indexed with the samtools v1.3 package[71]. Additionally, RPKM/FPKM (reads/fragments per kilobase of exon per million reads mapped) values were calculated. Differential expression was assessed using the DESeq2 Bioconductor package in an R V.3.6.3 programming environment with gene expression called differential with a false discovery rate (FDR) <0.05 and a median RPKM>1 in at least one group[72]. Presented RPKM values were tested using one-way analysis of variance (ANOVA) followed by Bonferroni's comparisons test. Pathway enrichment analysis was performed using Metascape[73]. Gene Ontology biological processes were selected for the analysis. Gene Set Enrichment Analysis (GSEA) was performed using the GSEA software[74].

2. SUPPLEMENTAL TABLES

Table S1. Baseline characteristics FH patients and healthy controls

	FH patients (n=10)	Healthy controls (n=9)	P-value
Age, years	42.7 (10.2)	41.2 (11.7)	0.772
Sex, n male (%)	8 (72)	7 (78)	0.906
BMI, kg/m²	25.2 (1.9)	24.3 (2.5)	0.403
Smoking, n never/past (%)	8/2 (80/20)	6/3 (67/33)	0.156
SBP, mmHg	120 (11)	123 (5)	0.470
DBP, mmHg	80 (8)	82 (6)	0.563
hs-CRP, mg/L, median [IQR]	0.9 [0.6-1.6]	0.5 [0.3-0.6]	0.022
Total cholesterol, mmol/L*	8.1 (2.7)	5.2 (0.7)	0.009
LDL-cholesterol, mmol/L*	6.0 (2.5)	3.3 (0.6)	0.007
HDL-cholesterol, mmol/L*	1.3 (0.4)	1.5 (0.3)	0.253
Triglycerides, mmol/L†, median [IQR]	1.34 [0.77-2.74]	0.94 [0.80-1.18]	0.243
ApoB, g/L, median [IQR]	1.52 [1.24-1.97]	0.80 [0.75-1.02]	<0.001
Leukocytes, 10^9/L	5.7 (1.6)	5.3 (0.6)	0.552
Neutrophils, 10^9/L	3.2 (1.7)	2.8 (0.4)	0.534
Lymphocytes, 10^9/L	1.8 (0.4)	1.8 (0.2)	0.876
Monocytes, 10^9/L	0.46 (0.07)	0.48 (0.12)	0.721
Hemoglobin, mmol/L	9.4 (0.6)	9.0 (0.7)	0.179
Thrombocytes, 10^9/L	246 (62)	267 (51)	0.453
Glucose, mmol/L	5.2 (0.5)	5.2 (0.4)	0.812

Data are mean (SD), median [interquartile range], or n (%). *ApoB, apolipoprotein B; BMI, body mass index; DBP, diastolic blood pressure; HDL, high-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; SBP, systolic blood pressure*

* To convert to mg/dL, multiply by 38.7; † To convert to mg/dL, multiply by 88.6

Table S2. LDL-C lowering effect per individual FH patient

Subject	Sex	Age	FH type	BMI	Treatment	*ΔLDL-C, mmol/L**	*ΔLDL-C, %*	*Post-treatment LDL-C, mmol/L**
I	F	35	LDLR	24.4	P, E	-1.93	-45	2.35
II	M	24	Clin	23.0	S	-2.67	-66	1.40
III	M	47	Clin	25.5	P	-4.24	-96	0.19
IV	M	56	LDLR	27.4	P, E	-5.31	-57	4.05
V	M	39	LDLR	23.2	S, P, E	-9.72	-87	1.42
VI	M	38	LDLR	23.0	S, E	-3.84	-56	2.98
VII	M	52	APOB	25.2	S, E	-0.94	-24	2.91
VIII	M	43	Clin	25.1	S, E	-3.64	-70	1.57
IX	F	56	Clin	28.7	P, E	-4.97	-85	0.88
X	M	37	Clin	26.0	P, E	-3.57	-76	1.10
					Overall mean (SD)	-4.08 (2.38)	-66 (22)	1.89 (1.16)

APOB indicates mutation in apolipoprotein B gene; Clin, clinical diagnosis of familial hypercholesterolemia (i.e. no FH mutation was found by genetic testing); E, ezetimibe; F, female; LDL-C, low density lipoprotein cholesterol; LDLR, mutation in low density lipoprotein receptor gene; M, male; P, proprotein convertase subtilisin/kexin type 9 antibody; S, statin

* To convert to mg/dL, multiply by 38.7

Table S3. Baseline characteristics EPIC-Norfolk cohort

	EPIC-Norfolk cohort *(n=12,304)*
Age, years	59.0 (9.2)
Sex, n female (%)	6942 (56)
BMI, kg/m²	26.1 (3.7)
Smoking, % yes/never/past	11/48/41
SBP, mmHg	135 (18)
DBP, mmHg	82 (11)
hs-CRP, mg/L, median [IQR]	1.4 [0.7-2.8]
Total cholesterol, mmol/L*	6.2 (1.1)
LDL-cholesterol, mmol/L*	3.9 (1.0)
HDL-cholesterol, mmol/L*	1.5 (0.4)
Triglycerides, mmol/L†, median [IQR]	1.50 [1.10-2.20]
ApoB, g/L	0.94 (0.26)
Leukocytes, 10^9/L	6.4 (1.7)
Neutrophils, 10^9/L	3.9 (1.3)
Lymphocytes, 10^9/L	2.0 (0.8)
Monocytes, 10^9/L	0.52 (0.35)
Hemoglobin, mmol/L	8.6 (0.8)
Thrombocytes, 10^9/L	252 (62)

Data are mean (SD), median [interquartile range], or n (%). *ApoB, apolipoprotein B; BMI, body mass index; DBP, diastolic blood pressure; HDL, high-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; SBP, systolic blood pressure*
* To convert to mg/dL, multiply by 38.7; †To convert to mg/dL, multiply by 88.6

Table S4. Relationship between LDL-C and absolute monocyte/granulocyte/lymphocyte count

	Monocytes, #		**Granulocytes, #**		**Lymphocytes, #**	
Unadjusted model	0.0125 (0.007 – 0.186)	<0.001	-0.059 (-0.082 – -0.036)	<0.001	0.045 (0.032 –0.058)	< 0.001
Model 1	0.008 (0.002 – 0.014)	0.009	-0.054 (-0.077 – -0.030)	<0.001	0.048 (0.034 – 0.062)	<0.001
Model 2	0.009 (0.002 – 0.015)	0.006	-0.056 (-0.069 – -0.043)	<0.001	0.048 (0.037 – 0.058)	<0.001

N=12,304 individuals (general population EPIC Norfolk cohort)

Data are standardized coefficient (β) with 95% confidence intervals (CI), reported per standard deviation increase in LDL-C.
Unadjusted model; univariate linear regression analysis of plasma LDL-C level (mmol/L) and monocyte, granulocyte or lymphocyte number (10^9/L). Model 1; adjusted for age and sex. Model 2; adjusted for Model 1, total white blood cell count, body mass index, smoking, and C-reactive protein.

Table S5. Multivariable regression models LDL-C and leukocyte differential count

	Monocytes, %		**Granulocytes, %**		**Lymphocytes, %**	
	β (95% CI)	p-value	**β** (95% CI)	p-value	**β** (95% CI)	p-value
Intercept	6.18 (5.55, 6.81)	<0.001	60.46 (59.32, 61.61)	<0.001	33.36 (32.49 34.23)	<0.001
Leukocytes	-0.68 (-0.78,-0.58)	<0.001	1.95 (1.77, 2.12)	<0.001	-1.27 (-1.41, -1.14)	<0.001
LDL-C	0.13 (0.04, 0.23)	0.007	-0.88 (-1.05,-0.70)	<0.001	0.75 (0.62, 0.88)	<0.001
Age	0.37 (0.27, 0.48)	<0.001	-0.27 (-0.46,-0.08)	0.006	-0.10 (-0.24, 0.04)	0.176
Female	-0.17 (-0.36, 0.02)	0.078	-0.56 (-0.91,-0.22)	0.001	0.74 (0.48, 1.00)	<0.001
BMI	-0.02 (-0.12, 0.08)	0.712	-0.70 (-0.88,-0.52)	<0.001	0.73 (0.59, 0.86)	<0.001
Current smoker	-0.01 (-0.33, 0.30)	0.933	-1.01 (-1.58,-0.43)	<0.001	1.01 (0.57, 1.44)	<0.001
Former smoker	-0.22 (-0.42,-0.02)	0.032	0.10 (-0.27, 0.46)	0.599	0.11 (-0.16, 0.39)	0.420
CRP	-0.07 (-0.12, 0.26)	0.468	1.64 (1.30, 1.98)	<0.001	-1.72 (-1.98,-1.47)	<0.001

N=12.304 individuals (general population EPIC Norfolk cohort)

Data are standardized coefficient (β) with 95% confidence intervals (CI). Betas are reported per standard deviation increase in leukocyte count, low-density lipoprotein, and body mass index. Age is reported per 10-year increase. C-reactive protein is reported per log increase.

Table S6. Multivariable regression models ApoB/ApoAI and leukocyte differential count

	Monocytes, %		**Granulocytes, %**		**Lymphocytes, %**	
	β (95% CI)	p-value	**β** (95% CI)	p-value	**β** (95% CI)	p-value
Intercept	6.49 (5.85, 7.13)	<0.001	60.37 (59.21, 61.53)	<0.001	33.13 (32.25, 34.01)	<0.001
Leukocytes	-0.69 (-0.78,-0.58)	<0.001	1.95 (1.77, 2.12)	<0.001	-1.27 (-1.41,-1.14)	<0.001
ApoB	0.33 (0.23, 0.44)	< 0.001	-1.05 (-1.24,-0.87)	<0.001	0.72 (0.58, 0.86)	<0.001
ApoAI	-0.03 (-0.13, 0.07)	0.582	0.24 (0.06, 0.43)	0.011	-0.23 (-0.37,-0.08)	0.002
Age	0.33 (0.23, 0.44)	<0.001	-0.28 (-0.47,-0.09)	0.004	-0.05 (-0.19, 0.10)	0.514
Female	-0.15 (-0.35, 0.05)	0.129	-0.78 (-1.14,-0.41)	0.001	0.95 (0.68, 1.23)	<0.001
BMI	-0.02 (-0.12, 0.08)	0.696	-0.70 (-0.89,-0.52)	<0.001	0.73 (0.59, 0.87)	<0.001
Current smoker	-0.03 (-0.35, 0.29)	0.853	-0.98 (-1.56,-0.40)	<0.001	0.99 (0.56, 1.43)	<0.001
Former smoker	-0.21 (-0.41,-0.01)	0.041	0.09 (-0.28, 0.46)	0.627	0.12 (-0.16, 0.39)	0.417
CRP	-0.03 (-0.22, 0.17)	0.792	1.91 (1.56, 2.26)	<0.001	-1.90 (-2.17,-1.64)	<0.001

N=12.128 individuals (general population EPIC Norfolk cohort)

Data are standardized coefficient (β) with 95% confidence intervals (CI). Betas are reported per standard deviation increase in leukocyte count, apolipoprotein-B, apolipoprotein-AI, and body mass index. Age is reported per 10-year increase and C-reactive protein per log increase.

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3. SUPPLEMENTAL FIGURES

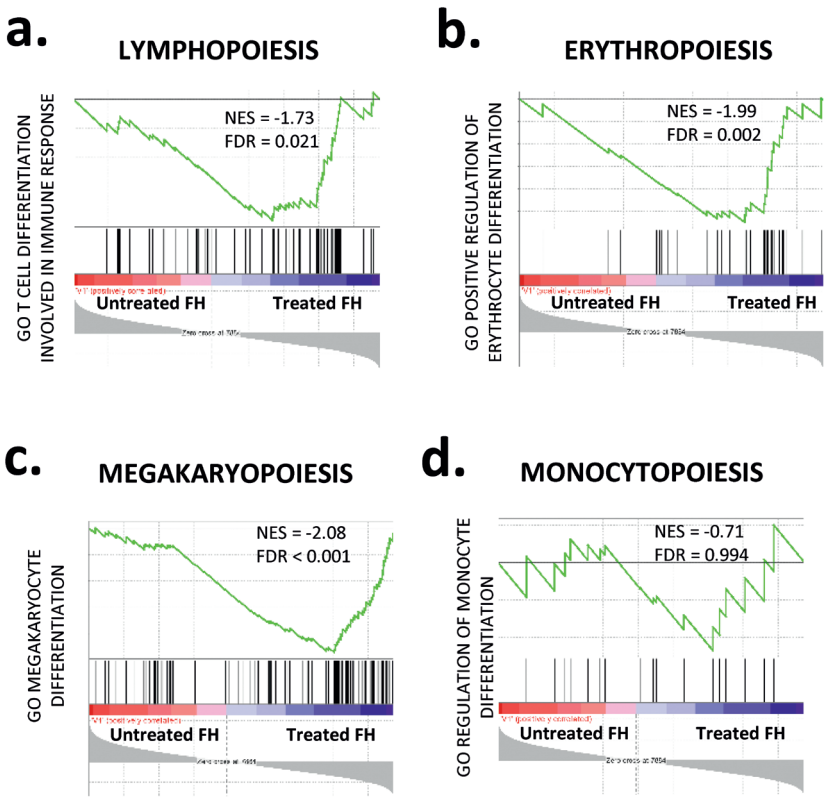

Figure S1. Gene set enrichment analysis of HSPCs before versus after cholesterol-lowering treatment

(A-D) Enrichment of gene sets 'T cell differentiation involved in immune response', 'positive regulation of erythrocyte differentiation', 'megakaryocyte differentiation', and 'regulation of monocyte differentiation'.

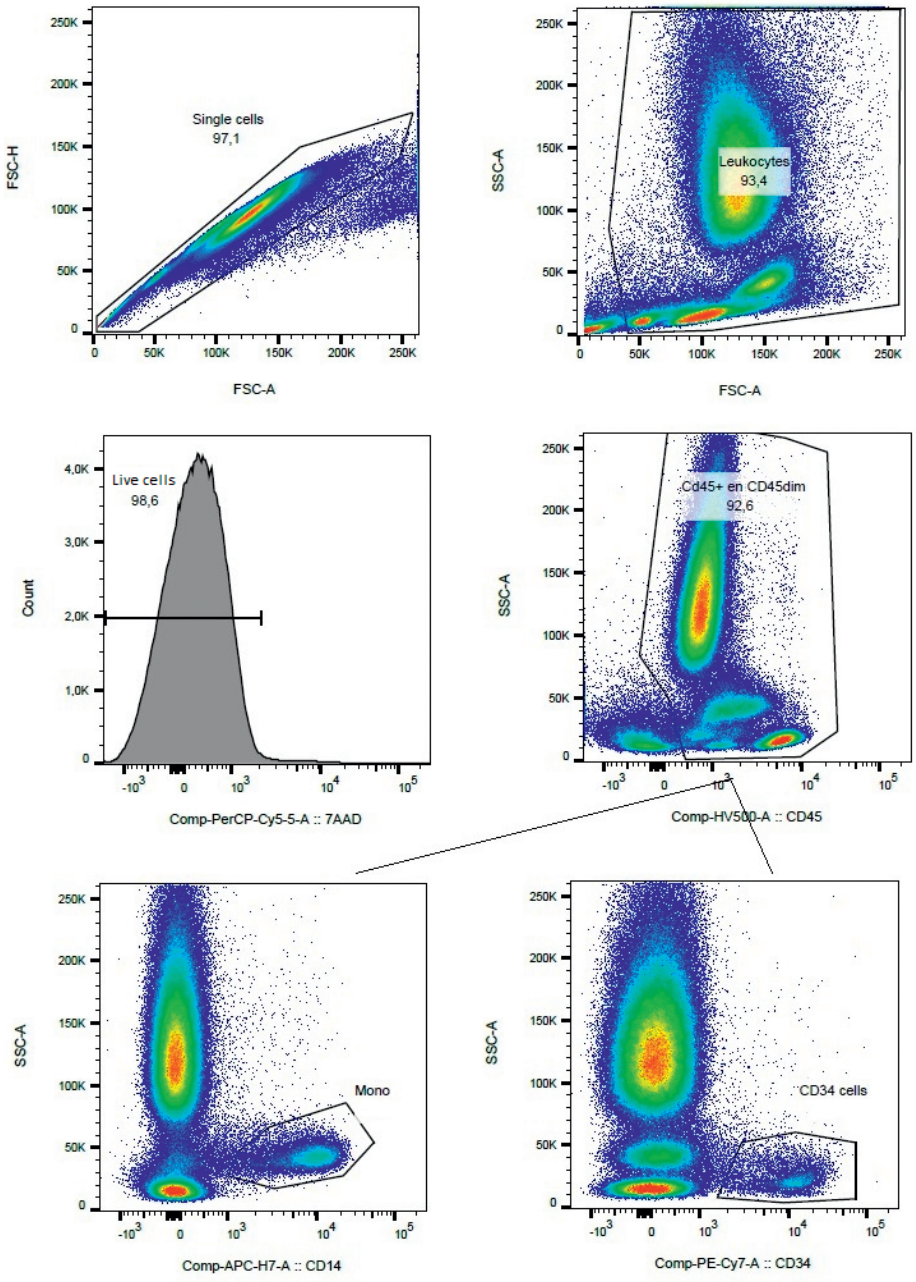

Figure S2. Gating Strategy Gating strategy to identify bone marrow CD14+ and CD34+ cells

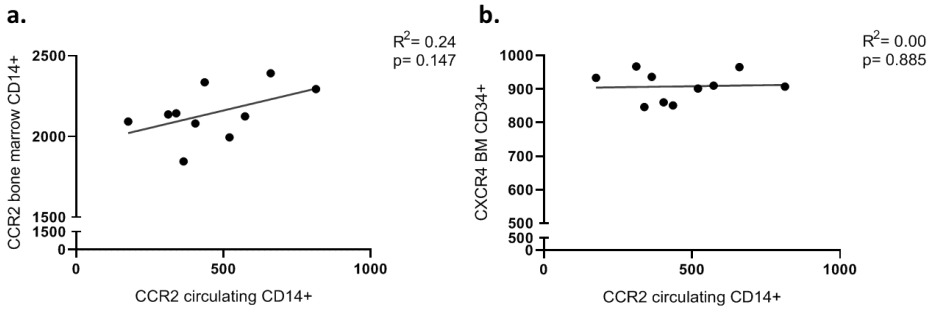

Figure S3. Correlation between cell surface markers on circulating monocytes and BM cells
(A) Correlation between CCR2 expression on circulating monocytes and bone marrow monocytes. (B) Correlation between CCR2 expression on circulating monocytes and CXCR4 expression on CD34+ BM HSPCs.

4

Increased haematopoietic activity in patients with atherosclerosis

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ABSTRACT

Aims

Experimental work posits that acute ischemic events trigger hematopoietic activity, driving monocytosis and atherogenesis. Considering the chronic low-grade inflammatory state in atherosclerosis, we hypothesized that hematopoietic hyperactivity is a persistent feature in cardiovascular disease (CVD). Therefore, we aimed to assess the activity of hematopoietic organs and hematopoietic stem and progenitor cells (HSPCs) in humans.

Methods and Results

First, we performed ^{18}fluorodeoxyglucose positron emission tomographic (^{18}F-FDG PET) imaging in 26 patients with stable atherosclerotic CVD (ischemic event >12 months ago), and 25 matched controls. In splenic tissue, ^{18}F-FDG uptake was 2.68±0.65 in CVD patients versus 1.75±0.54 in controls (1.6-fold higher; p<0.001), and in bone marrow 3.20±0.76 versus 2.72±0.46 (1.2-fold higher; p=0.003), closely related to low-density-lipoprotein cholesterol levels (LDL-c, r=0.72). Subsequently, we determined progenitor potential of HSPCs harvested from 18 patients with known atherosclerotic CVD, and 30 matched controls; both groups were selected from a cohort of cancer patients undergoing autologous stem cell transplantation. In CVD patients, the normalized progenitor potential, expressed as the number of colony forming units-granulocyte/monocyte (CFU-GM) colonies/CD34$^+$ cell, was 1.6-fold higher compared with matched controls (p<0.001). Finally, we assessed the effects of native and oxidized lipoproteins on HSPCs harvested from healthy donors *in vitro.* HSPCs displayed a 1.5-fold increased CFU-GM capacity in co-culture with oxidized LDL *in vitro* (p=0.002), which was inhibited by blocking oxidized phospholipids via E06 (p=0.001).

Conclusion

Collectively, these findings strengthen the case for a chronically affected hematopoietic system, potentially driving the low-grade inflammatory state in patients with atherosclerosis.

Keywords

Atherosclerosis, hematopoietic stem and progenitor cells, inflammation, imaging, oxidized lipoproteins

INTRODUCTION

Atherosclerosis is a chronic disease of the arterial wall, in which lipoproteins and inflammation are closely intertwined[1]. Early lesions arise when, amongst other atherogenic risk factors, elevated low-density-lipoprotein (LDL) particles induce endothelial dysfunction at susceptible arterial sites. The latter allows for LDL accumulation in the intimal space followed by modification into its oxidized forms (oxLDL). In parallel, inflammatory cells, of which monocytes/macrophages are the most abundant, are recruited to the intima and form foam cells after taking up oxLDL[1]. Ultimately, these fatty streaks advance into atherosclerotic plaques, predominantly driven by low-grade inflammatory responses[1].

A unifying view posits the bone marrow as the primary site of monocyte production, however, in an inflammatory disorder, other lymphoid tissues, such as the spleen, can also produce monocytes[2,3]. In the context of atherosclerosis, murine studies showed that an acute ischemic event, such as myocardial infarction (MI) or stroke, induced the release of hematopoietic stem and progenitor cells (HSPCs) from bone marrow niches via sympathetic nervous signaling[4]. A large number of HSPCs seeded the spleen, to subsequently serve as an extramedullary production site of monocytes[5,6]. Moreover, the systemic oversupply of inflammatory monocytes aggravated pre-existing atherosclerotic lesions[4].

Recent clinical studies validated the enhanced hematopoietic activity in MI patients, proposing the acute ischemic event as its trigger[7–9]. However, while signs and symptoms of an ischemic event fade within hours to days, the low-grade inflammatory state might be chronically present in cardiovascular disease (CVD) patients, as suggested by both experimental studies showing the high turn-over of inflammatory lesional cells[6,10], and clinical imaging corroborating continuous influx of immune cells into advanced lesions[11]. Therefore, we hypothesized that hematopoietic activity is chronically enhanced in patients with atherosclerosis.

To this end, we first performed ^{18}fluorodeoxyglucose positron emission tomographic with computed tomography (^{18}F-FDG PET/CT) imaging in patients with stable atherosclerotic CVD and matched control subjects to assess the metabolic activity of both the arterial wall and hematopoietic tissues. Next, we evaluated the progenitor potential of HSPCs that had been harvested from atherosclerotic CVD patients and matched control patients, both identified from a cohort of cancer patients. Finally, we co-incubated healthy donor derived HSPCs with native and oxidized lipoproteins *in vitro* and assessed changes in progenitor potential and phenotype.

METHODS

Study subjects

All subjects provided informed consent, and studies were conducted according to the principles of the International Conference on Harmonization–Good Clinical Practice guidelines. Patients in the PET study were characterized by stable atherosclerotic CVD, which was defined as a positive history of an ischemic event >1 year ago (myocardial or cerebral infarction due to significant coronary or carotid artery stenosis, respectively) and stable medication use for >3 months prior to inclusion. In addition, patients had been diagnosed with elevated LDL cholesterol levels due to familial hypercholesterolemia (FH). For comparison, subjects matched for age, gender and BMI though without a history for CVD underwent PET imaging. In a cohort of hemato-oncological patients (n=953), we identified patients with documented atherosclerotic CVD >1 year prior to (i) the diagnosis of a malignancy and (ii) to the harvesting of HSPCs in the context of an autologous stem cell transplantation (autoSCT), and selected control patients without known CVD, matched for age, gender, BMI, and indication for autoSCT. In general, subjects with (pre)diabetes were excluded.

^{18}F-FDG PET/CT imaging

PET/CT scans were performed on a dedicated scanner (Philips, Best, the Netherlands). Subjects fasted for at least 6 hours prior to infusion of 200 MBq of ^{18}F-FDG (5.5 mCi). 90 min after ^{18}F-FDG infusion, PET imaging was initiated with a low-dose, non-contrast enhanced CT for attenuation correction and anatomic co-registration (slice thickness 3 mm). PET/CT images were analysed with dedicated analysis software (OsiriX, Geneva, Switzerland; http://www.osirix-viewer.com/). Two readers (FM, YK) analysed all images to assess inter-observer variability. One reader (FM) analysed all images twice to assess intra-observer variability. Readers were blinded for patient data.

The maximal target-to-background-ratios (TBR_{max}) in the carotids were determined as described previously[12]. The carotid TBR_{max} represents the average of the left and right carotid arteries. The mean and maximal standard uptake values (SUV_{max} and SUV_{mean}) in the BM and spleen was determined as previously described[9]. In brief, SUV_{max} for each vertebra (T1 to L5) was assessed, and BM activity was calculated as the mean of SUV_{max} of all vertebrae. For the spleen, SUV_{max} was assessed in axial, sagittal, and coronal planes, and splenic activity was calculated as the mean of SUV_{max} values of the 3 planes. A similar approach was used for the SUV_{mean}.

Coronary artery calcium score. CT scans were used to determine the Agatston score, the coronary artery calcium (CAC) score. In a manually set volume of interest, all pixels with intensity higher than 130 HU were selected. Connected areas of these threshold pixels were constructed. All areas smaller than 1 mm^2 were excluded. The score was determined by combining all selected connected areas with a weight. The weight was determined by the highest intensity value of a pixel in the connected areas: 1 for 130–199 HU, 2 for 200–299 HU, 3 for 300–399 HU, and 4 for 400 HU and greater. Because of the difference in slice thickness of the images in his study (5 mm) compared to default Agatston score images (3mm), the sum CAC score was multiplied with 5/3.

CFU-GM assay of patient derived HSPCs

HPSC from patients were mobilized by chemotherapy and G-CSF. Cells were plated in duplicate in 35 mm tissue culture plates at concentrations of 1.0, 0.5 and 0.25*10 5 cells/ml, respectively, in MethoCult GF 4534 (StemCell Technologies, Vancouver, BC, Canada). Cultures were incubated for 12-14 days at 37°C at 5% CO2. CFU-GM colonies, defined as containing at least 40 translucent myelocytic cells, were scored in triplo by microscopy (Leica, Solms, Germany).

In vitro model using healthy HSPCs

Leukapheresis material of healthy donors treated with G-CSF (5-10 mg/kg/day) was used to obtain CD34+ cells. These cells were enriched by magnetic cell sorting according to the manufacturer instructions (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany), resulting in a purity of more than 90 %, as determined by flow cytometry. CD34+ cells were plated in 96 well plates (Thermo Scientific, Waltham, MA, USA) at 30.000 cells/well and kept in maintenance cocktail of 100 µl StemSpan (Stemcell Technologies, Vancouver, British Columbia, Canada) per well, supplemented with 100 U/ml pencillin, 100 µg/ml streptomycin, 50 ng/ml n-plate, 100 ng/ml SCF and 100 ng/ml Flt3. At day 0, CD34+ cells were stimulated with control medium, native LDL (nLDL) or oxLDL (Alfa Aesar, for both 10 µg/ml). In inhibition experiments using E06 (1 nM), the above 3 stimuli were co-incubated with the inhibitor. LAL assay showed negligible endotoxin levels (<0.05 EU/mL) in the stimuli. All cells were cultured at 37°C at 5% CO2. At day 7, cells were counted and flow cytometric and CFU-GM assays were performed.

The cultured cells were counted at day 7 by flow cytometric assays with Cyto-Cal fluorescent beads (Thermo scientific, Fremont, CA, USA), using a CANTO ll + HTS flow cytometer (BD) and were characterized for surface expression of various receptors by flow cytometry. Cells were washed and resuspended in PBS containing 0.2% BSA

prior to incubation (20 minutes at RT) with the following monoclonal antibodies. Antibodies purchases from BD, San Jose CA: CD13-PE (clone WM15), CD14-PerCP-Cy5.5 (clone M5E2), CD33-PE (clone WM53), CD34-PECy7 (clone 8G12), CD38-APC (clone HB7), CD45-PB (clone H130) and CD192 (CCR2)-Alexa-Fluor 647 (clone 48607). Purchased from Sanquin, Amsterdam, The Netherlands: CD16-FITC (clone CLB-Fc-gran/1, 5D2) and CD36-FITC (clone M1613). Purchased from Dako, Cambridgeshire, UK: CD41-FITC (clone 5B12). A minimum of 10.000 cells was recorded, using a CANTO ll + HTS flow cytometer (BD).

For CFU-GM assays, cultured cells were plated in duplicate 0.5 ml in 24 well plates (Corning, NY, USA) at a concentration of 150 cells/well, respectively, in MethoCult H4434 (StemCell Technologies, Vancouver, BC, Canada). Cultures were incubated for 12-14 days at 37°C at 5% CO2. CFU-GM, colonies were scored in triplo by microscopy (Olympus CK2, Shinjuku, Tokyo, Japan).

Statistical Analysis

Continuous variables are expressed as mean ± standard deviation (SD) or median and range (either interquartile range [IQR] or 25th to 75th percentile), and categorical variables as percentage and number (n). Differences in PET imaging parameters between groups were assessed with a multivariable model to account for cardiovascular risk factors. Correlations were assessed using Spearman's tests. The agreement between ^{18}F-FDG PET/CT analyses was assessed using intraclass correlation coefficients (ICC, r). Differences in G-SCF harvested cells between patients were assessed using nonparametric Mann-Whitney U tests. *In vitro* stimulation assays were repeated at least 5 times, and the differences between control, nLDL and oxLDL stimulations were assessed using a Kruskal Wallis analysis. P values were tested two-sided, and <0.05 was defined as statistically significant. All data were analysed using SPSS version 21.0 (SPSS Inc., Chicago, Illinois).

RESULTS

Enhanced hematopoietic activity in CVD patients

PET imaging was performed in 26 patients (aged 54 ± 7) with atherosclerosis disease, as evidenced by increased CAC scores 688 [IQR 356]. The median time post event was 18 months (IQR 3 months), comprising 70% MI and 30% ischemic stroke. In addition, 25 control subjects (aged 52 ± 5) without known atherosclerosis (confirmed by the CAC scores 0 [0]) matched for age, gender and BMI were included. The clinical characteristics are listed in Table 1. In line with secondary prevention, patients were on lipid lowering therapy, ACE-inhibition, acetylsalicylic acid and B-blockage, and

C-reactive protein (CRP) levels were higher in patients. Other characteristics were comparable between groups (Table 1).

Table 1. Clinical characteristics of subjects in imaging study

Characteristic	**Controls** *(n=25)*	**CVD patients** *(n=26)*	**P-value**
Age, y	52 ± 5	54 ± 7	0.115
Gender, %male	72 (18)	80 (20)	0.493
BMI, kg/m^2	25 ± 3	26 ± 3	0.168
SBP, mmHg	131 ± 14	132 ± 6	0.752
DBP, mmHg	80 ± 9	80 ± 6	0.748
Smoking, %active	0 (0)	8 (2)	0.092
MI, %yes	-	70 (18)	
Ischemic stroke, %yes	-	30 (8)	
Time post-event, *months*	-	18 [3]	
Lipid lowering drugs, %yes	-	100 (26)	
Statin use (%)[§]	-	81 (21)	
Ezetimibe use (%)[§]	-	19 (5)	
ACE-inhibitor use (%)[§]	-	100 (26)	
Acetylsalicylic acid use (%)[§]	-	100 (26)	
B-blocker use, %yes[§]	-	100 (26)	
CT-CAC-score*	0 (0)	688 (356)	<0.001
TChol, mmol/L	5.29 ± 0.94	5.92 ± 2.74	0.302
LDL-c, mmol/L	3.14 ± 1.01	3.91 ± 2.67	0.233
Glucose, mmol/L	5.03 ± 0.31	5.29 ± 0.83	0.168
Creatinin, µmol/L	78 [23]	85 [16]	0.075
CRP, mg/L	1.30 [1.35]	2.45 [3.60]	0.029
Leukocytes, 10^9/L	6.09 ± 1.44	6.05 ± 1.39	0.911
Lymphocytes, 10^9/L	1.90 ± 0.49	1.82 ± 0.40	0.568
Monocytes, 10^9/L	0.46 ± 0.14	0.54 ± 0.19	0.054
Neutrophils, 10^9/L	3.58 ± 1.44	3.50 ± 1.13	0.835

Continuous data are shown as mean ± SD, or median [IQR], and categorical data as percentage and (n). *BMI, body mass index; CAC, coronary calcium score, CVD, cardiovascular disease; CRP, C-reactive protein; DBP, diastolic blood pressure; LDL-c, low density lipoprotein cholesterol; MI, myocardial infarction; SBP, systolic blood pressure; Tchol, total cholesterol.* * Agatston score. [§] Drug name and dosages for statin (simvastatin 40mg/day), ezetimibe (10mg/day), ace-inhibitor (lisinopril or perindopril 5-10mg), ASA (ascal 100mg/day) and B-blocker (metoprolol retard 100-150mg/day).

Table 2. 18F-FDG uptake in arterial wall and hematopoietic tissues

Characteristic	**Controls** *(n=25)*	**CVD patients** *(n=26)*	**P-value**
Carotid, TBR_{max}	1.58 ± 0.20	1.95 ± 0.33	<0.001
Spleen, SUV_{mean}	1.13 ± 0.40	1.85 ± 0.44	<0.001
Spleen, SUV_{max}	1.75 ± 0.54	2.68 ± 0.65	<0.001
Bone marrow, SUV_{mean}	1.80 ± 0.21	2.15 ± 0.52	0.004
Bone marrow, SUV_{max}	2.72 ± 0.46	3.20 ± 0.76	0.003

Continuous data are shown as mean ± SD. *[18]F-FDG, [18]Fluorodeoxyglucose; CVD, cardiovascular disease; SUV, standardized uptake values; TBR, target to background ratio.*

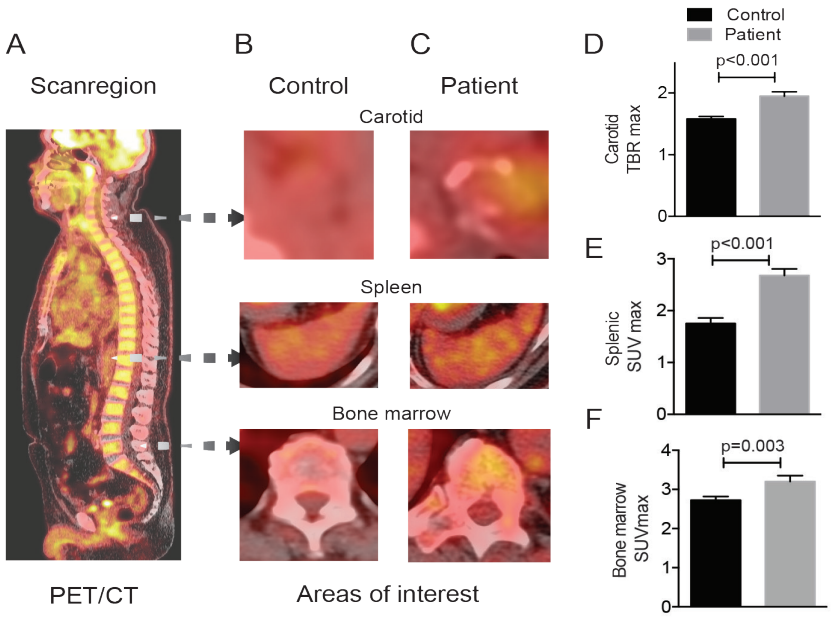

Figure 1. Persistent increased hematopoietic activity in CVD patients
Representative [18]F-FDG PET/CT images showing (A) an exemplary PET/CT scan region with (B-C) an enlarged inlay of the 3 areas of interest (carotids, spleen and bone marrow) for a (B) control subject and (C) patient, (D-F) with the corresponding [18]F-FDG uptake values depicted in bar graphs for (D) carotid TBR_{max} (p<0.001), (E) splenic (p<0.001) and (F) bone marrow SUV_{max} (p=0.003), for both controls (n=25, black bars) and patients (n=26, grey bars). [18]F-FDG, [18]Fluorodeoxyglucose; PET/CT, positron emission tomographic with computed tomography; SUV, standardized uptake values; TBR, target to background ratio.

Figure 1A-C illustrates the multiple levels at which ^{18}F-FDG uptake was assessed in both patients and matched controls. Carotid ^{18}F-FDG uptake was increased in patients compared with controls (1.95 ± 0.33 versus 1.58 ± 0.20, p<0.001, Figure 1D). In addition, ^{18}F-FDG uptake in the spleen and bone marrow was also higher in patients compared with controls (Figure 1E-F, Table 2); the mean difference in splenic SUV_{max} was 0.93 ± 0.17 (p<0.001) between patients and controls, and 0.48 ± 0.18 (p=0.003) for the bone marrow. SUV_{mean} values for spleen and bone marrow showed a similar increase in patients (Table 2).

In patients, splenic and bone marrow ^{18}F-FDG uptake strongly correlated with each other (r=0.76, p<0.001). Additional correlations for hematopoietic organ activity were determined (supplementary Table 1) in patients, revealing significant correlations with LDL-c and CRP levels, and monocyte count. No correlation between ^{18}F-FDG uptake and inflammatory biomarkers (MCP-1, TNFα, IL-6) or traditional cardiovascular risk factors was observed. Lastly, the intra- and inter-observer agreement was excellent for SUV values in bone marrow or spleen, as indicated by the ICCs of >0.94 with narrow 95% confidence intervals (supplementary Table 2).

Increased progenitor potential of HSPCs in CVD patients

Provoked by the enhanced bone marrow ^{18}F-FDG uptake in CVD patients *in vivo*, we screened a cohort of 953 cancer patients in whom HSPCs had been harvested via G-CSF. We identified 18 subjects with documented atherosclerosis, consisting of MI with significant coronary artery stenosis or ischemic stroke with significant carotid artery stenosis, having occurred 17 months (IQR 5 months) prior to HSPCs harvesting. The latter was performed in the context of an autoSCT due to the diagnosis of a multiple myeloma (61%) or relapse of non-Hodgkin lymphoma (39%) in disease stage I-III. For comparison, we selected 30 control patients without known CVD, matched for age, gender, BMI and indication for autoSCT (53% multiple myeloma and 47% relapse of non-Hodgkin lymphoma, in matched disease stages, p=0.886). Clinical characteristics at the time of HSPCs harvesting are listed in Table 3. Conform secondary prevention management all CVD patients used lipid lowering, ACE-inhibition, acetylsalicylic acid and B-blockage, whereas control subjects did not. Glucose, kidney and liver function were comparable between groups. Lipid profiles were not available. In addition, Supplementary Table 3 shows the agreement between both CVD patient cohorts.

Table 3. Clinical characteristics of autoSCT patients

Characteristic*	**Control patients** *(n=30)*	**CVD patients** *(n=18)*	**P-value**
Age, years	54 ± 5	56 ± 10	0.403
Gender, m%	70 (21)	72 (12)	0.870
BMI, kg/m^2	25 ± 3	26 ± 4	0.382
Indication autoSCT - Multiple myeloma - Relapse NHL	 53 (16) 47 (14)	 61 (11) 39 (7)	0.148
Disease stage I-II-III, %	31-19-50%	38-15-46%	0.886
Smoking, *%active*	7 (2)	17 (3)	0.272
Ischemic event - MI, *%yes*	-	50 (9)	
- Ischemic stroke, *%yes*	-	50 (9)	
Time post-event[#], *months*	-	17 [5]	
Statin use (%)[§]	-	100 (18)	
ACE-inhibitor use (%)[§]	-	100 (18)	
Acetylsalicylic acid use (%)[§]	-	100 (18)	
B-blocker use (%)[§]	-	100 (18)	
Glucose, *mmol/L*	5.0 ± 0.4	5.3 ± 0.7	0.333
Creatinin, µmol/L	72 [51]	75 [191]	0.451
ALT, *U/L*	21 ± 9	24 ± 9	0.183

*Characteristics of the subject prior to G-CSF harvesting of HSPCs. # time between ischemic event and G-CSF harvesting of HSPCs. Continuous data are shown as mean ± SD, or median [IQR], and categorical data as percentage and (n). *ALT, alanine aminotransferase; autoSCT, autologous stem cell transplantation; BMI, body mass index; CVD, cardiovascular disease; MI, myocardial infarction; NHL, non-Hodgkin lymphoma*. § Drug name and dosages for statin (simvastatin 40mg/day), ace-inhibitor (lisinopril or perindopril 5-10mg), ASA (ascal 100mg/day) and B-blocker (metoprolol retard 100-150mg/day).

Whereas the percentage of CD34$^+$ cells was comparable between groups (Figure 2A; p=0.744), the CFU-GM capacity of the G-CSF HSPCs harvested from CVD patients was 1.6-fold higher compared with control patients (0.31 ± 0.18 versus 0.19 ± 0.07, p<0.001; Figure 2B).

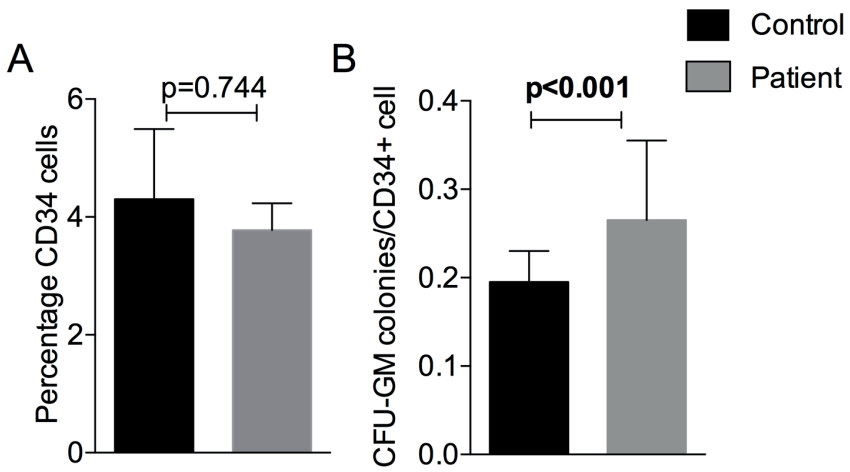

Figure 2. Increased progenitor potential in CVD patients
Bar graphs showing (A) no change in the percentage of CD34+ cells of the mononuclear cells mobilized by G-CSF (p=0.744), whereas (B) an increased progenitor capacity (expressed as the CFU-GM/CD34+ cell) in patients (n=18, grey bars) compared with controls (n=30, black bars, p<0.001). CFU-GM, colony forming units-granulocyte/monocyte.

Lipid risk factors change function and phenotype of HSPCs in vitro

Considering the correlation between bone marrow ^{18}F-FDG uptake and LDL-c, we cultured unrelated healthy donor G-CSF harvested HSPCs (CD34+ cells) with either native LDL (nLDL) or oxidized LDL (oxLDL). After 7 days of co-incubation, the normalized cell count was not different between stimuli (p=0.444; Figure 3A). However, the normalized progenitor capacity, assessed with CFU-GM (Figure 3B), was 1.5-fold higher after co-incubation with oxLDL compared with control (p=0.002; Figure 3C). In addition, we applied the monoclonal antibody E06, which blocks the phosphocholine (PC) headgroup of oxidized phospholipids, and not native phospholipids[13–15]. Blockage of the oxidized phospholipid load on oxLDL via E06 robustly inhibited the effect of oxLDL on normalized progenitor potential (p=0.001; Figure 3D).

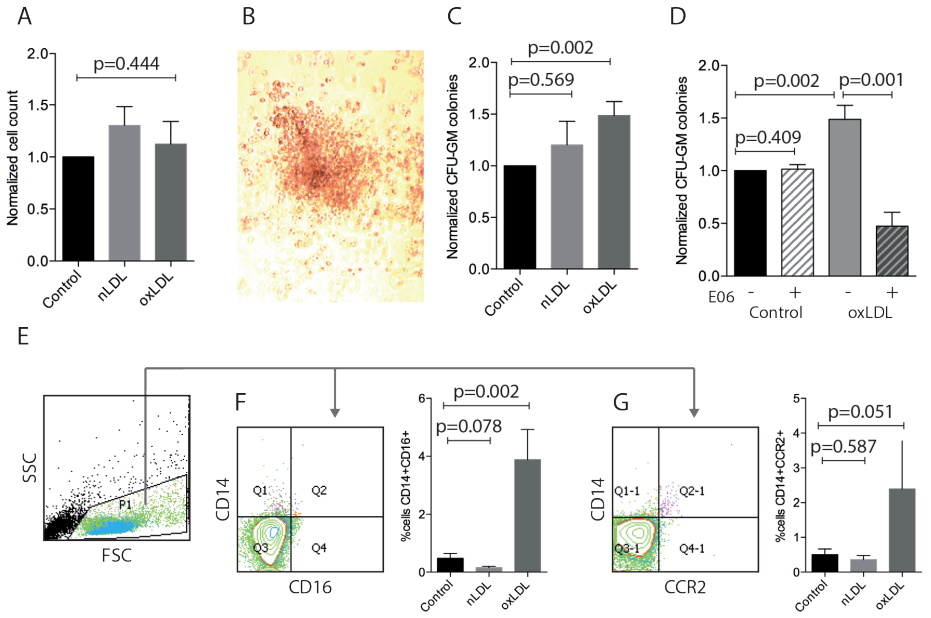

Figure 3. Increased progenitor potential and myeloid differentiation bias induced by oxLDL
Bar graphs showing (A) no differences (p=0.444) in the cell counts after healthy HSPCs were co-incubated for 7 days with either control medium (black bars), nLDL (10 ug/mL, light grey bars) or oxLDL (10 ug/mL, dark grey bars), whereas (B-D) the CFU-GM capacity, as illustrated by the microscopy image (B), increased upon co-incubation with oxLDL (p=0.002) (C), which is inhibited by the antibody E06 (p=0.001) (D), and (E-G) gating strategy with accompanying results showing that the expression of CD14, CD16 and CCR2 was increased after 7 days of HSPCs co-incubation with oxLDL (p=0.002 and p=0.051, respectively). Assays were performed at least 8 times. CFU-GM, colony forming units-granulocyte/monocyte; CCR2, C-C chemokine receptor type 2; HSPCs, hematopoietic stem and progenitor cells; nLDL, native low-density-lipoprotein; oxLDL, oxidized LDL.

Next to CFU-GM capacity, flow cytometric assays were performed to determine the phenotypic changes after nLDL or oxLDL co-incubation. No changes in the expression of CD41, CD13/33 or CD36 were observed (Supplementary Figure 1), whereas the cells showed a myeloid bias after 7 days of oxLDL co-incubation as indicated by the significant increased expression of CD14/CD16 (p=0.002) and borderline significant increased C-C chemokine receptor type 2 (CCR2; p=0.051) (Figure 3E-G).

DISCUSSION

Here, we report that patients with stable atherosclerotic CVD have a high metabolic activity of the carotid arterial wall, as well as hematopoietic tissues *in vivo*. In addition, we show that HSPCs have a higher proliferative capacity *ex vivo* when obtained from patients with atherosclerotic CVD, compared with those without known CVD. *In vitro*, this enhanced proliferative potential and myeloid differentiation bias was substantiated in healthy HSPCs by co-incubation with oxLDL; the oxidized phospholipid antibody E06 could inhibit this adverse effect. Collectively, these findings support the hypothesis that hematopoietic activity is chronically enhanced in patients with table atherosclerotic CVD.

HSPC number and function in CVD patients

Previously, PET imaging studies demonstrated an increased bone marrow and splenic activity in patients with CVD[7–9]. Whereas these studies all focused on the (semi)-acute phase following a MI[7–9], we now show that more than 12 months after the CVD event patients still exhibit higher activity of hematopoietic tissues despite CVD guideline-based management.

What does the chronically increased ^{18}F-FDG uptake in hematopoietic tissues imply for the patient? In general, cellular accumulation of ^{18}F-FDG is increased in proinflammatory, hypoxic as well as rapidly proliferating cells[16]. In the present stable condition, we can speculate that the ^{18}F-FDG in hematopoietic tissues reflects an increased proliferative rate, since HSPCs harvested from patients with CVD also showed an increased proliferative capacity of the GM-precursor cells. A prospective study combining PET imaging with HSPC harvesting is warranted, however, to corroborate this concept.

Besides the increased metabolic activity, we also show an enhanced functional capacity of HSPCs harvested from CVD patients compared with controls. During the last decade, differences in both number and function of circulating CD34+ cells have been reported. Thus, in patients following an acute myocardial infarction, the number of endogenous circulating progenitors was significantly increased compared with controls[17]. This phasic increase in number fell significantly within 60 days after the acute event[17]. Such a transient response of hematopoietic cells following an acute ischemic event has subsequently been corroborated in patients following either a myocardial[18–20] or a cerebral[21,22] infarction. Interestingly, despite normalization of cell number, we observe functional changes of CD34+ cells persisting more than 12 months after the acute event. Whereas the clinical relevance

of a tonic hematopoietic activity warrants further validation, animal studies have indicated that the increased activity of HSPCs can promote features characteristic of vulnerable atherosclerotic lesions [23]. In addition, whether repeating acute events or long-lasting chronic stimulation could also exhaust the bone marrow[24] warrants further studies.

Causes of chronic hematopoietic activity in CVD

Murine ligation studies evidenced that in the acute phase following a MI, anxiety, pain and impaired left ventricular function promote sympathetic nervous signalling, proposing an important role for the β3-adrenoceptor[4] in mediating the increased HSPC response. In view of the chronic, stable phase of the CVD, the absence of left ventricular failure and the use of beta-blockers in the patients included in the present study, these acute stimuli are less likely to be major contributors in our patients. Alternative causal pathways comprise persistent effects of an ischemic event on senescent stem cells[25], or a potential role of atherosclerotic risk factors on HSPCs[26]. In this respect, we observed a significant correlation between plasma LDLc concentration and metabolic activity in hematopoietic tissues lending further support to a direct role of elevated LDL-c in mediating bone marrow hyperresponsiveness.

Several studies detailed an interaction between lipoproteins and the myeloid lineage, both of upstream as well as downstream, differentiated cells[26]. Regarding the differentiated cells, mice with high-fat diet induced hypercholesterolemia display a marked expansion of proinflammatory monocytes[27]. In parallel, patients with significantly elevated LDL levels are also characterized by an increased number of proatherogenic monocytes[28] as well as arterial wall inflammation[29] compared with subjects with normal cholesterol levels.

With respect to the myeloid progenitors, murine studies have showed that elevated cholesterol levels increased the number of HSPCs in the blood[30,31] as well as the bone marrow[32]. In a large community-based study the number of circulating CD34+ cells in humans correlated also positively with total cholesterol levels[33]. Conversely, a reduction in cholesterol levels via statin therapy was followed by a reduction in circulating HSPCs in patients[34].

Oxidized lipoproteins affect HSPCs function and phenotype

Besides the relation between lipids and HSPC number, we also addressed the potential impact on HSPC functionality. First, oxLDL was found to increase the CFU-GM capacity of the HSPCs, whereas nLDL had no effect. The monoclonal

antibody E06, which blocks the phosphocholine (PC) headgroup of oxidized phospholipids[13–15], abolished this effect of oxLDL. Oxidized phospholipids represent danger-associated molecular patterns (DAMPs) which can be recognized by multiple innate pattern recognition receptors (PRRs)[35], igniting a variety of pro-inflammatory and plaque destabilizing processes[35–37]. Secondly, oxLDL also promoted the myeloid differentiation bias of HSPCs of healthy donors, evidenced by the increased expression of the monocytic cell markers CD14 and CD16. This is of specific interest, since HSPC-derived monocytes and macrophages are key cellular effectors in atherosclerosis[38]. In addition, we also observed an increased expression of CCR2 on progenitor cells, which is elementary on upstream hematopoietic precursors contributing to myelopoiesis following ischemic organ injury[39]. Interestingly, a CCR2 positive subset was also present in patients with CVD, whereas this subset had a profound higher proliferative rate and displayed a myeloid differentiation bias[39]. Whereas the latter study examined $CCR2^{+}$ progenitors in the setting of an acute ischemic event[39], our findings underscore the need to evaluate these pathways also in the setting of chronic atherosclerosis.

Study limitations

Several limitations need to be acknowledged. First, due to ethical constraints for clinical research, PET imaging and HSPCs studies were not performed in the same patients. The clinical characteristics, including age, gender, BMI, time post event and medication use, however, were comparable between the CVD patients in both studies (Supplementary Table 3). Nevertheless, extrapolation and generalizability of our findings are limited. In addition, the relative small sample sizes restrain correlation analysis, with amongst others inflammatory biomarkers. Secondly, regarding the HSPCs *ex vivo* assays, we acknowledge that the manner of harvesting (G-CSF), the retrospective nature as well as the type, stage and (pre)treatment of the cancer and statin treatment in the CVD group are confounding variables. Also, a potential causal or accelerating effect of the presence of cancer on ischemic events cannot be excluded. In order to minimize the impact of these effects, however, we carefully matched patients and controls for disease and therapy specifications and only included patients with significant atherosclerotic disease (elevated CAC scores and/or significant carotid artery stenosis). Thirdly, the design of the studies, i.e. harvested HSPCs, precludes repetitive assessments of hematopoietic activity within patients over time. Finally, although we observed a significant interaction between oxidized lipoproteins and HSPC function and phenotype, the present study cannot address causality or consequence of this mechanism in vivo.

CONCLUSIONS

Whereas during the past decade downstream myeloid cells, mostly monocytes and macrophages, have been studied intensively in atherogenesis, the interest in the more upstream HSPCs and their involvement in atherogenesis is expanding[40]. The present experimental work describes that (i) hematopoietic tissues are more active in patients with atherosclerotic disease, (ii) HSPCs are functionally different in these patients, and (iii) potentially affected by oxidized lipoproteins. Further translational research is needed to dissect the interactions between up- and downstream cells and the drivers of chronic low-grade inflammation in human atherogenesis.

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Author contributions
F.M.v.d.V., C.K. performed statistical analysis. M.N., C.V., and E.S.G.S. handled funding and supervision. F.M.v.d.V., C.K., S.L.V., Y.K., L.C.A.S. acquired the data. F.M.v.d.V., C.V., and E.S.G.S. conceived and designed the research. F.M.v.d.V., M.N., C.V., and E.S.G.S. drafted the manuscript. F.M.v.d.V., C.K., S.L.V., L.C.A.S., Y.K., S.Z., M.N., C.V., and E.S.G.S. made critical revision of the manuscript for key intellectual content. S.Z. handling of the cancer patient database.

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SUPPLEMENTARY DATA

Table S1. Correlations with hematopoietic activity

Characteristic	Bone marrow ^{18}F-FDG uptake	Splenic ^{18}F-FDG uptake	P-value
Age	0.25	0.16	0.243
Gender	0.10	0.11	0.681
BMI	0.11	0.03	0.799
MAP	0.37	0.25	0.563
Smoking	0.36	0.28	0.613
Lipid lowering drugs	0.14	0.13	0.776
ACE-inhibitor	0.09	0.14	0.344
Acetylsalicylic acid	0.03	0.08	0.180
B-blocker	0.11	0.04	0.309
Carotid TBR	**0.21***	**0.34***	**0.004**
TChol	0.12	0.15	0.431
LDL-c	**0.72*****	**0.55*****	**<0.001**
HDL-c	0.19	0.18	0.783
CRP	**0.72*****	**0.49*****	**<0.001**
MCP-1	0.20	0.22	0.456
TNFα	0.21	0.19	0.738
IL-6	0.17	0.16	0.651
Leukocytes	0.10	0.01	0.702
Monocytes	**0.18***	**0.25***	**0.003**

Data show Spearman correlation coefficients, and p-values. *^{18}F-FDG, ^{18}fluorodeoxyglucose; BMI, body mass index; CACscores, coronary artery calcium score; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; IL-6, interleukin 6; MAP, mean arterial pressure; MCP-1, monocyte chemoattractant protein-1; TBR, target to background ratio; TChol, total cholesterol, TNFα, tumor necrosis factor α.*

Table S2. Excellent agreement of image analysis

	Bone Marrow SUV_{max}	**Spleen SUV_{max}**
Intra-observer		
Paired difference	0.01 ± 0.02	0.00 ± 0.15
Intra ICC [CI]	0.98 [0.93 – 0.99]	0.99 [0.96 – 0.99]
Inter-observer		
Paired difference	0.03 ± 0.13	0.02 ± 0.19
Inter ICC [CI]	0.98 [0.94 – 0.99]	0.94 [0.85 – 0.97]

Intra-observer reflects the variability within one reader (FM) and inter-observer reflects the variability between two readers (FM and YK). *ICC, intraclass correlation coefficient; CI, 95% confidence interval; SUV, standardized uptake values.*

Table S3. Additional patient characteristics

	PET study		**HSPC study**		
Characteristic	**Controls**	**CVD patients**	**Control patients**	**CVD patients**	**P value**
Number	*25*	*26*	*30*	*18*	
Age, y	52 ± 5	54 ± 7	54 ± 5	56 ± 10	0.361
Gender, %male	72 (18)	80 (20)	70 (21)	72 (12)	0.568
BMI, kg/m^2	25 ± 3	26 ± 3	25 ± 3	26 ± 4	0.784
Smoking, %active	0 (0)	8 (2)	7 (2)	17 (3)	0.732
Time post-event, *months*	-	18 [3]	-	17 [5]	-
Lipid lowering use (%)	-	100 (26)	-	100 (18)	-
ACE-inhibitor use (%)	-	100 (26)	-	100 (18)	-
Acetylsalicylic acid use (%)	-	100 (26)	-	100 (18)	-
B-blocker use, %yes	-	100 (26)	-	100 (18)	-
Glucose, mmol/L	5.03±0.31	5.29 ± 0.83	5.0 ± 0.4	5.3 ± 0.7	0.383
Creatinin, µmol/L	78 [23]	85 [16]	72 [51]	75 [191]	0.462

Continuous data are shown as mean ± SD, or median [IQR], and categorical data as percentage and (n). *ACE-inhibitor, angiotensin-converting-enzyme inhibitor; CVD, cardiovascular disease; BMI, body mass index; HSPC, hematopoietic stem-/progenitor cell; ns, non-significant; PET, positron emission tomography.*

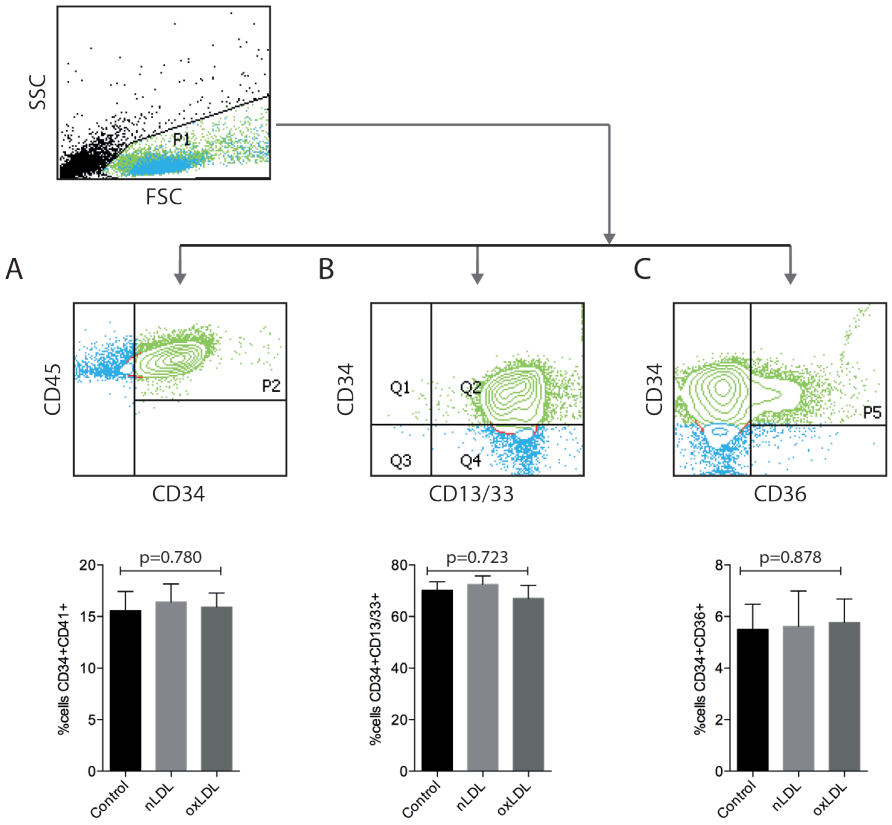

Figure S1. Additional expression markers after oxLDL
Representative gating strategy and bar graphs showing no differences in the expression of CD34, CD41, CD13/33 or CD36 after 7 days of HSPCs co-incubation with oxLDL. Assays were performed at least 5 times. *HSPCs, hematopoietic stem and progenitor cells; nLDL, native low-density-lipoprotein; oxLDL, oxidized LDL.*

5

Prolonged hematopoietic and myeloid cellular response in patients after an acute coronary syndrome measured with ^{18}F-DPA-714 PET/CT

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ABSTRACT

Purpose

An acute coronary syndrome (ACS) is characterized by a multi-level inflammatory response, comprising activation of bone marrow and spleen accompanied by augmented release of leukocytes into the circulation. The duration of this response after an ACS remains unclear. Here, we assessed the effect of an ACS on the multi-level inflammatory response in patients both acutely and after 3 months.

Methods

We performed ^{18}F-DPA-714 PET/CT acutely and 3 months post-ACS in 8 patients and 8 matched healthy controls. DPA-714, a PET tracer binding the TSPO receptor, highly expressed in myeloid cells, was used to assess hematopoietic activity. We also characterized circulating monocytes and hematopoietic stem and progenitor cells (HSPCs) by flow cytometry in 20 patients acutely and 3 months post-ACS and in 19 healthy controls.

Results

In the acute phase, patients displayed a 1.4 fold and 1.3 fold higher ^{18}F-DPA-714 uptake in respectively bone marrow ($p=0.012$) and spleen ($p=0.039$) compared with healthy controls. This coincided with a 2.4 fold higher number of circulating HSPCs ($p=0.001$). Three months post-ACS, ^{18}F-DPA-714 uptake in bone marrow decreased significantly ($p=0.002$), but no decrease was observed for ^{18}F-DPA-714 uptake in the spleen ($p=0.67$) nor for the number of circulating HSPCs ($p=0.75$).

Conclusions

^{18}F-DPA-714 PET/CT reveals an ACS- triggered hematopoietic organ activation as initiator of a prolonged cellular inflammatory response beyond 3 months, characterized by a higher number of circulating leukocytes and their precursors. This multi-level inflammatory response may provide an attractive target for novel treatment options aimed at reducing the high recurrence rate post-ACS.

Keywords

Acute coronary syndrome, ^{18}F-DPA-714 PET/CT, hematopoietic organs, monocytes, hematopoietic stem and progenitor cells

INTRODUCTION

In the first 6 to 9 months following an acute coronary syndrome (ACS), patients face a disproportionally increased risk of re-infarction[1]. Recent experimental studies suggest a crucial role for a multi-level inflammatory response post-ACS[2]. In murine models, ligation of the coronary artery results in increased bone marrow and spleen activation, leading to augmented release of leukocytes into the circulation[2]. These mobilized leukocytes, predominantly myeloid cells, facilitate the progression of atherosclerosis, contributing to growth as well as destabilization of atherosclerotic plaques[3]. Following an ACS in patients, studies have substantiated that ^{18}F-fluordeoxyglucose (^{18}F-FDG) uptake in bone-marrow and spleen is increased, visualized with positron emission tomography/ computed tomography (PET/CT). This increased uptake coincides with increased inflammatory parameters in plasma and arterial wall inflammation[4,5]. Whether this multi-level inflammatory response persists after the acute phase, thereby potentially contributing to the increased re-infarction risk post-ACS, remains unclear.

Although ^{18}F-FDG PET/CT imaging is a validated technique for quantifying inflammation in atherosclerotic plaques[6], FDG is a non-specific glucose analogue reflecting a change in overall metabolic activity. In this respect, FDG uptake in bone marrow and spleen may reflect a wide array of cells and processes, ranging from enhanced cellular activation to increased proliferative activity. Thus, more specific imaging tracers are required to further delineate the role of myeloid cells in the multi-level inflammatory response in patients post-ACS. The tracer N,N-diethyl-2-(2-(4-(2-fluoroethoxy)phenyl) 5,7dimethylpyrazolo [1,5a] pyrimidin-3-yl)acetamide (DPA-714) has been widely used for imaging of neuro-inflammatory processes[7-9]. DPA-714 is a second generation tracer for the translocator protein (TSPO) receptor[10], which is expressed predominantly on cells from the myeloid lineage[11], comprising macrophages, monocytes and microglial cells. Since expression of the TSPO receptor does not change in human myeloid cells following inflammatory stimuli[12], TSPO-targeting tracers for PET/CT are more likely to reflect changes in myeloid cell number rather than in activation phenotype. In support, TSPO-PET studies in patients revealed a higher target to background in symptomatic carotid plaques, whereas TSPO-binding was also found to co-localize with the macrophage CD68 marker in excised atherosclerotic plaques[13]. This suggests that the TSPO receptor could be a valuable target for quantifying the number of myeloid cells in bone marrow and spleen using ^{18}F-DPA-714 PET/CT.

In the present study, we use ^{18}F-DPA-714 PET/CT combined with flow cytometry to investigate the acute and prolonged (3 months and up to 2 years) effects of an ACS on myeloid cellular responses in both hematopoietic organs (bone marrow and spleen) as well as in the plasma compartment (progenitor cell numbers and monocyte phenotypes).

MATERIAL AND METHODS

Study population and design

We performed a case-control study in 20 ACS patients (50 years or older, ACS documented with electrocardiogram and/or cardiac enzymes). Exclusion criteria were previous cardiovascular events, diabetes mellitus, chronic kidney disease, peripheral artery disease and any chronic inflammatory disease. Blood withdrawal for flow cytometry and the measurement of lipid levels and inflammatory parameters was performed within 3 days and 3 months post-ACS. ^{18}F-DPA-714 PET/CT was performed within 10-18 days and also 3 months post-ACS. As controls, 19 healthy subjects matched for age and gender were included. Vital parameters, including weight, height and blood pressure were measured at baseline. Hypertension was defined as the use of antihypertensive medication prior to the event.

The study protocol was approved by the institutional review board of the Academic Medical Center in Amsterdam, the Netherlands and conducted according to the principles of the declaration of Helsinki. Written informed consent was obtained from each participant.

18F-DPA-714 PET/CT

^{18}F-DPA-714 PET/CT was performed on a dedicated scanner (Philips, Best, the Netherlands). DPA-714 (100 MBq) was injected as a bolus lasting 1 minute, followed by a redistribution phase of approximately 60 minutes[8], after which a PET/CT was performed in combination with a low-dose, non-contrast-enhanced CT for attenuation correction and anatomic co-registration[6]. Images were analyzed by experienced readers blinded for patient data, using dedicated software (Hybrid viewer, HERMES medical solutions AB, Stockholm, Sweden). In patients, ^{18}F-DPA-714 uptake was assessed in bone marrow and spleen 10-18 days after the event, and repeated after 3 months. Healthy controls underwent an ^{18}F-DPA-714 PET/CT scan once. ^{18}F-DPA-714 uptake in the bone marrow was assessed by drawing regions of interest (ROIs) in the lumbar vertebrae, uptake in the spleen was assessed by drawing 5 ROIs in the axial plane[4]. ^{18}F-DPA-714 uptake was determined as the mean of maximal standard uptake value (SUV_{max}). We also assessed the corrected

SUV of bone marrow and spleen, using DPA-714 uptake in the pectoral muscle as background (DPA-714 uptake in bone marrow or spleen minus DPA-714 uptake in pectoral muscle). Additional information on the DPA-714 tracer can be found in the supplemental methods.

A polymorphism of the TSPO gene is responsible for three different binding affinities of ^{18}F-DPA-714 to the TSPO receptor: a high, intermediate and low binding affinity[14]. We only scanned patients with high and intermediate binding affinity and matched the healthy controls for similar binding affinity. Genotyping is described in the supplemental methods.

Flow cytometry of HSPCs and monocytes

Flow cytometry of circulating HSPCs and monocytes was performed within 3 days post-ACS and repeated 3 and 6 to 24 months post-ACS. A mononuclear cell fraction (MNC) was isolated using Ficoll; MNCs were stored in medium (RPMI 1640-medium + 20% Fetal calf serum + 1% penicillin-streptomycine). Cells were incubated with fluorochrome labelled antibodies (supplemental table S1). Samples were analyzed using a FACS Canto-B Tube Loader. HSPCs were classified as $CD34^{+}CD45_{dim}$ cells (supplemental figure S1A-D)[15]. Monocytes were classified according to CD45, CD14 and CD16 expression (supplemental figure S1E-I), with subsequent determination of surface markers involved in monocyte adhesion and migration (supplemental table S1). Samples were analyzed using FlowJo software (version 10.0 FlowJO, LLC, Ashland, OR). Delta median fluorescence intensity (MFI) was obtained by subtracting the MFI from an unstained control from the MFI of the marker. Flow cytometry analysis of TSPO receptor expression is provided in the supplemental methods.

Statistical analysis

Data were analyzed using Prism version 6.0 (GraphPad software, LaJolla, California) and SPSS version 23.0 (SPSS Inc., Chicago, Illinois). Data are presented as the mean ± standard deviation (SD) and median with interquartile range (IQR) for respectively normally and non-normally distributed data or as a number (percentage) for categorical variables. Differences in clinical characteristics, number of circulating HSPCs, monocyte phenotype and ^{18}F-DPA-714 uptake between patients and healthy controls were assessed using a Student's T-tests or Mann Whitney U-tests for respectively normally and non-normally distributed variables. To assess differences between baseline measurements and the repeated measurements, paired Student's T-tests or Wilcoxon signed rank tests for respectively normally and non-normally data were performed. A 2-sided p-value<0.05 was considered statistically significant.

RESULTS

Study population

We included 20 patients with an ACS (63±8 years, 80% male), with troponin levels of 1.3[0.2-5.5] µg/L, comprising 16 patients with a ST-Elevation Myocardial Infarction (STEMI) and 4 patients with a Non-ST Elevation Myocardial Infarction (NSTEMI). Nineteen healthy controls (62±9 years, 63% male) matched for age and gender were also included. Baseline characteristics are listed in table 1. Additional baseline clinical characteristics of the ACS patients are provided in the supplements (table S3). Patients with an ACS had an adverse cardiovascular risk profile compared with healthy controls, including a higher body mass index (BMI), a higher prevalence of hypertension and a trend towards more active smokers. Patients had higher HDL levels; other lipid levels were comparable between patients and controls. Patients in the acute phase were characterized by elevated inflammatory parameters (CRP and leukocyte count) compared with healthy controls. After the event, acetyl-salicylic acid and ticagrelor treatment was initiated in all patients; if not used yet, anti-hypertensive medication and/or statin treatment was provided in line with current guidelines. At baseline, troponin levels were associated with plasma monocyte count (r=0.517, p=0.012) with a trend for association with CRP levels (r=0.385, p=0.094).

Three months post-ACS, lipid levels were significantly lower, corresponding to the start of statin treatment. CRP levels and leukocyte counts decreased significantly, as shown in table 1; although these inflammatory parameters remained elevated compared with healthy controls (data not shown). No serious adverse events occurred in the participating ACS patients during the 3-month study period.

Elevated DPA-714 uptake in bone marrow and spleen

In 8 patients and 8 matched controls, we assessed hematopoietic activity using ^{18}F-DPA-714 uptake in bone marrow and spleen (for one patient, splenic data was insufficient) (figure 1A). In the acute phase, the scan was performed 14±2 days post-ACS. Baseline characteristics of this subgroup were comparable to the whole cohort (table S4).

In the acute phase, patients with an ACS showed a 1.4 fold higher uptake of ^{18}F-DPA-714 in the bone marrow (p=0.012) as well as a 1.3 fold higher uptake in the spleen (p=0.039) compared with healthy controls (figure 1B-C). Three months post-ACS, ^{18}F-DPA-714 uptake in bone marrow decreased significantly (SUV-BM$_{max}$ acute phase 6.3±1.4 versus 3 months 4.4±.8, p=0.002), which no longer differed from healthy controls (p=0.813). In contrast, ^{18}F-DPA-714 uptake in the spleen remained elevated

Table 1. Baseline characteristics

Baseline characteristics	**ACS patients acute phase** *(n=20)*	**Healthy controls** *(n=19)*	**P-value** acute phase vs healthy controls	**Patients 3 months post-ACS** *(n=16)*	**P-value** acute phase vs 3 months post-ACS	**P-value** 3 months post-ACS vs healthy controls
Age, years	63±8	62±9	0.503	n/a	n/a	n/a
Sex, men/women	16/4	12/7	0.412	n/a	n/a	n/a
BMI, kg/m^2	28.5±5	25±3	0.026	28.7±5	0.351	0.014
Systolic blood pressure, mmHg	135±18	127±16	0.189	n/a	n/a	n/a
Diastolic blood pressure, mmHg	79±13	80±11	0.696	n/a	n/a	n/a
Smoking, yes/past/no	6/7/7	1/7/11	0.137	n/a	n/a	n/a
Hypertension, yes/no	9/11	0/19	0.001	n/a	n/a	n/a
CRP, mg/dl	13.2[5.2-18]	0.8[0.4-1.2]	<0.001	1.7[0.4-3.7]	0.001	0.042
Total cholesterol, mmol/L	5.1±0.7	5.6±1.0	0.087	3.9±0.7	<0.001	<0.001
HDL cholesterol, mmol/L	1.3±0.5	1.7±0.4	0.014	1.4±0.5	0.667	0.030
LDL cholesterol, mmol/L	3.2±0.8	3.5±0.8	0.295	2.0±0.5	<0.001	<0.001
Triglycerides, mmol/L	1.2[0.8-1.7]	0.8[0.5-1.3]	0.065	1.1[0.8-1.3]	0.887	0.059
Leukocytes, *10^9/L	9.7±2.6	4.9±0.8	<0.001	7.3±1.9	0.012	<0.001
Neutrophils, *10^9/L	6.0±3.1	2.7±0.6	<0.001	4.2±1.1	0.061	<0.001
Lymphocytes, *10^9/L	2.3±0.6	1.55±0.5	<0.001	2.1±0.9	0.603	0.021
Monocytes, *10^9/L	0.9±0.3	0.4±0.1	<0.001	0.7±0.3	0.035	<0.001

Values are n, mean ± SD or median [IQR] for respectively normally and non-normally distributed data. *BMI: body mass index; CRP: C-reactive protein; HDL: high-density cholesterol; LDL: low-density cholesterol*

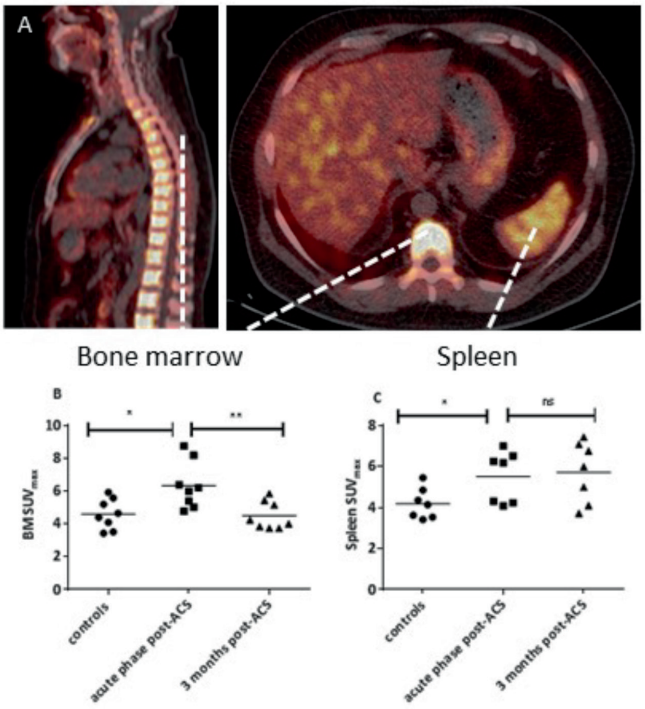

Figure 1. Elevated ^{18}F-DPA-714 uptake in bone marrow and spleen post-ACS
^{18}F-DPA-714 uptake in the bone marrow and spleen (yellow color in A) was quantified as maximal standardized uptake value (SUV_{max}). ^{18}F-DPA-714 uptake in the bone marrow was assessed by drawing regions of interest (ROIs) in the lumbar vertebrae (visible on both sagittal and transversal views), uptake in the spleen was assessed by drawing 5 ROIs in the axial plane (visible on the transversal view). Patients in the acute phase post-ACS showed elevated DPA-714 uptake in the bone marrow and spleen compared with healthy controls. Three months post-ACS, ^{18}F-DPA-714 uptake in bone marrow decreased (B), which no longer differed from healthy controls, while ^{18}F-DPA-714 uptake in the spleen remained elevated (C), which is still significant higher compared with healthy controls. Data are represented as mean with single values of the subjects, *p<0.05, **p<0.01. *BM: bone marrow; ns: non-significant; SUV: standardized uptake value*

(acute phase: 5.5±1.2 versus 3 months: 5.7±1.5, p=0.671), which is still significant higher compared with healthy controls (p=0.032). In the supplements, figure S2 shows individual lines connecting individual data points (acute phase and after 3 months) per ACS patient. When applying background correction, corrected SUV values show similar results substantiating significant differences between controls and ACS patients in the acute phase (bone marrow: p=0.009; spleen: p=0.028), followed by a significant decrease of bone marrow corrected SUV after 3 months (p=0.0018), at which time point spleen corrected SUV remained elevated (p=0.352) (supplemental figure S3).

To assess potential cell specific DPA-714 uptake in bone marrow and spleen, we determined the expression of the TSPO receptor on HSPCs and myeloid lineage cells. As shown in supplemental figure S4, the TSPO receptor is highly expressed on monocytes and less on lymphocytes. Furthermore, the TSPO receptor is also highly expressed on HSPCs.

Higher number of circulating HSPCs

Following the elevated ^{18}F-DPA-714 uptake in bone marrow and spleen, we assessed the number of circulating HSPCs in the acute phase post-ACS and after 3 months. Using flow cytometry, we observed that patients in the acute phase had a 2.5 fold higher number of circulating HSPCs compared with healthy controls (patients 12[7-16] cells/µl versus controls 5[4-7] cells/µl, p=0.001; figure 2). This coincided with a trend towards myeloid skewing of circulating inflammatory cells; there was a significantly lower percentage of lymphoid cells and a trend towards elevated percentages of monocytes (supplemental table S5).

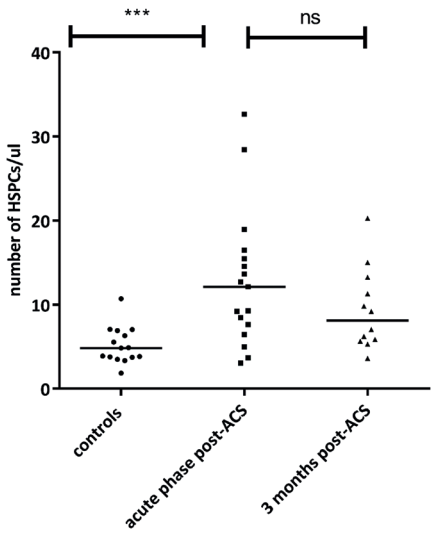

Figure 2. Elevated number of circulating HSPCs post-ACS

The number of circulating HSPCs is assessed using flow cytometry, classifying HSPCs as $CD34^{+}CD45_{dim}$ cells. Patients in the acute phase showed significantly elevated numbers of circulating HSPCs compared with healthy controls. Three months post-ACS, there is a numerical decrease of number of circulating HSPCs, however the number of circulating HSPCs is still significant higher compared with healthy controls. Data are represented as median with single values of the subjects, *p<0.05, **p<0.01, ***p<0.001. *HSPCs: hematopoietic stem and progenitor cells; ns: non-significant*

After 3 months, the number of circulating HSPCs of patients showed a numerical decrease (acute phase 12[7-16] cells/μl versus 3 months 8[6-13] cells/μl, p=0.754; figure 2). At this point in time, the number of circulating HSPCs was still higher compared with healthy controls (p=0.009). Myeloid skewing of inflammatory cells disappeared (supplemental table S5).

Elevated CCR2 expression on monocytes

Previous data suggests that in addition to increased cell counts, leukocytes/monocytes may show a more pro-inflammatory phenotype post-ACS[2]. Using flow cytometry, we observed that the distribution of the monocyte subsets (divided in classical($CD14^{+}CD16^{-}$), intermediate ($CD14^{+}CD16^{+}$) and non-classical monocytes ($CD14^{+}CD16^{++}$)) in the circulation were similar in both the acute phase and 3 months post-ACS, comparable with the distribution of monocyte subsets in healthy controls (figure 3A). Interestingly, monocyte expression of the chemokine receptor CCR2, involved in migration of monocytes into the arterial wall, was significantly elevated directly post-ACS compared with healthy controls (CCR2 expression acute phase: 755[570-860] MFI versus controls 528[414-729] MFI, p=0.006). Three months post-ACS, the expression of CCR2 showed a numerical decrease (CCR2 expression 636[393-951] MFI, p=0.36; figure 3B), which now no longer differed from healthy controls (p=0.44).

Long-term cellular response post-ACS

In view of the prolonged inflammatory response after 3 months, we explored persistence up to 6 to 24 months post-ACS. Flow cytometry on HSPCs and monocytes was repeated in 12 patients, on average 19[10-21] months post-ACS, and compared with the 3 month time point. Inflammatory parameters are listed in supplemental table S6. Our data showed a trend towards a decrease of leukocytes 6 to 24 months post-ACS. Other inflammatory parameters as well as the number of circulating HSPCs were comparable to the level of the 3 month time point (p=0.4; supplemental figure S5A), despite the use of guideline-based CV-therapy in these patients. However, the activation of circulating monocytes 6 to 24 months post-ACS, based on their CCR2 expression, showed a trend towards a decrease compared with the level of the 3 month time point (p=0.06), back to levels observed in healthy controls (supplemental figure S5B). No serious adverse events occurred in the subgroup of 12 patients with a follow-up of 6 to 24 months.

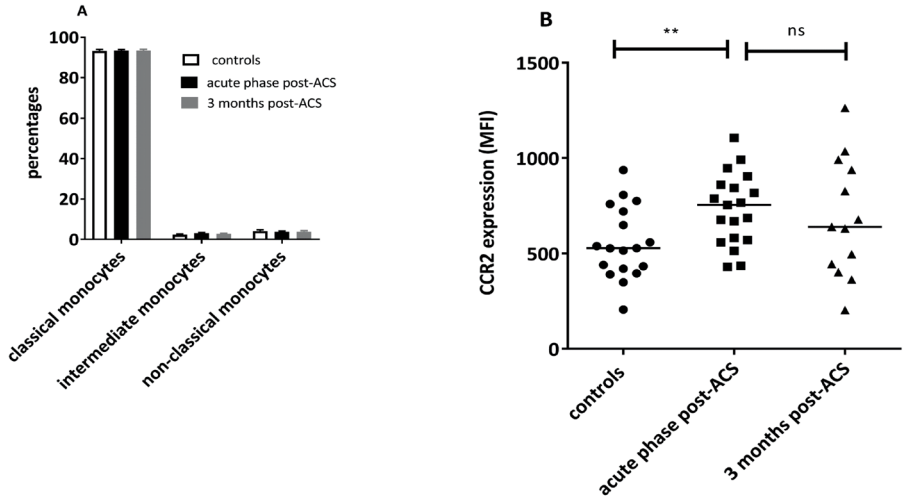

Figure 3. Elevated CCR2 expression on monocytes post-ACS
Monocyte subset distribution, classified according to CD14 and CD16 expression, and CCR2 expression on monocytes were assessed using flow cytometry. Monocyte subset distribution was similar in both the acute phase and 3 months post-ACS, the highest percentage of monocytes consisted of classical monocytes ($CD14^{+}CD16^{-}$; 93%), followed by both intermediate ($CD14^{+}CD16^{+}$; 3%) and non-classical monocytes ($CD14^{+}CD16^{++}$; 4%) in almost equal percentages (A). However, in the acute phase we showed an elevated expression of CCR2 on monocytes, with a non-significant decrease after 3 months post-ACS (B), which now no longer differed from healthy controls. Data are presented for A as mean±sem and as median with single values of the subjects for B, *p<0.05, **p<0.01. *ACS indicated acute coronary syndrome*

DISCUSSION

In the present study, we observe elevated ^{18}F-DPA-714 uptake in bone marrow and spleen compared with controls in the acute phase post-ACS, coinciding with a higher number of circulating HSPCs as well as an increased monocyte count showing a pro-inflammatory phenotype. Three months post-ACS, ^{18}F-DPA-714 uptake remained elevated in the spleen only, with a concomitant plasma monocytosis and persistent elevation of circulating HSPCs. Collectively, these data support the presence of a prolonged, multi-level inflammatory response post-ACS.

Prolonged multi-level inflammatory response post-ACS

Following an ACS, monocytes are important for repair of the damaged heart. In this study, we support the presence of an acute hematopoietic response post-ACS[16], resulting in multi-level inflammatory activation. In the acute phase we observe a

leukocytosis and monocytosis, with concomitant elevation of circulating HSPCs[17]. This increased myeloid cellular response in plasma corresponds with an elevated ^{18}F-DPA-714 uptake in the bone marrow and spleen compared with healthy controls. Using flow cytometry, we substantiated the expression of the TSPO receptor on particularly monocytes and HSPCs, supporting the concept that DPA-714 uptake occurs predominantly in myeloid cells and HSPCs in bone marrow and spleen. Therefore, we used the ^{18}F-DPA-714 signal as a reflection of the number of myeloid cells and HSPCs in the hematopoietic organs[12].

Three months post-ACS, ^{18}F-DPA-714 uptake in bone marrow was no longer significantly different from healthy controls, which implies a transient increase of cellular density in the bone marrow. In contrast, we recently reported a persistently elevated metabolic activity in bone marrow in patients ≥ 1 year after a CV-event, using ^{18}F-FDG PET/CT[18]. This apparent discrepancy may relate to the different molecular targets of DPA-714 and FDG. Whereas DPA-714 reflects predominantly myeloid cell number[12], FDG reflects overall metabolic activity. Combined, these data imply normalization of myeloid cell count in the bone marrow 3 months post-ACS, whereas overall metabolic activity of the bone marrow may persist for a longer time.

In contrast to the transient elevation of ^{18}F-DPA-714 uptake in bone marrow, uptake in the spleen remained elevated 3 months post-ACS, with a concomitant monocytosis and persistent elevation of circulating HSPCs. Whereas the bone marrow is the main organ for hematopoiesis in humans, it has been shown previously that the spleen can facilitate extramedullary hematopoiesis[19]. The latter corresponds with observations in experimental models, showing that a CV-event results in a marked release of HSPCs from the bone marrow to the spleen followed by prolonged extramedullary hematopoiesis in this organ[2,20]. In mice, increased numbers of circulating HSPCs and monocytes in the spleen were also still present 3 months after the acute event[2], which corresponds to our finding in post-ACS patients.

Transient monocyte activation post-ACS

Even after 6 to 24 months post-ACS, HSPCs and monocyte counts in the circulation remained elevated compared with matched controls. In the acute phase, the increased monocyte count was found to be accompanied by monocyte activation, illustrated by increased expression of CCR2. CCR2 is the major chemokine receptor contributing to migration of monocytes into the arterial wall. We recently reported a close correlation between CCR2 expression on circulating monocytes and transendothelial migration *ex vivo*[21]. More recently, we also observed a correlation between monocyte CCR2 expression and arterial wall inflammation in patients at

increased CV-risk[22]. Collectively, these findings support the concept that increased monocyte count, showing an activated phenotype, may partly contribute to the elevated recurrence risk in the first 6 months post-ACS.

Study limitations

This study has several limitations. First, PET/CT could only be performed in a relatively limited number of subjects. The latter precludes adjustment for potential confounders and correlation with infarct size. Notwithstanding, we did observe significant differences in ^{18}F-DPA-714 uptake, comparable to studies using ^{18}F-FDG PET/CT in the acute phase post-ACS. Second, we compared ACS patients in the acute phase with healthy controls, followed by a repeat scan after 3 months. In absence of a scan preceding the event, we cannot exclude that the ACS patients may already have elevated hematopoietic activity prior to the acute event[4]. However, DPA-714 uptake in bone marrow was no longer different in ACS patients compared with matched controls 3 months post-ACS, which suggests an event-related 'transient' increased uptake. Third, TSPO binding is significantly affected by polymorphisms of the TSPO gene. To minimize potential impact of these polymorphisms, we excluded the low-binding affinity genotype and matched patients and controls for intermediate or high binding affinity. Finally, the TSPO receptor is also expressed in various peripheral tissues in humans[10]. In the heart and arterial wall, we observed a continuous high signal for DPA-714 in controls as well as patients with or without a recent event. Since the signal in these organs were comparably high directly following an ischemic event and after 3-months, this most likely represents non-specific binding of DPA-714 to smooth muscle cells[10]. This data shows that DPA-714 performs worse than the more traditional FDG as a tracer to measure inflammatory activity in cardiovascular organ[6].

CONCLUSIONS

^{18}F-DPA-714 PET/CT showed an ACS-triggered hematopoietic organ activation as initiator of a prolonged leukocyte oversupply, characterized by an elevated number of circulating leukocytes and their precursors. Further studies are required to determine whether strategies aimed at reducing this prolonged hematopoietic activation may translate into a reduced re-infarction rate in the vulnerable post-ACS period.

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SUPPLEMENTALS

METHODS

Additional information on the tracer DPA-714

A putative antagonist of the TSPO receptor, DPA-713, has been described previously[1, 2]. Concurrently, the fluoro-ethoxy derivative DPA-714, a TSPO agonist, was developed and labeled with fluorine-18[3]. Both DPA-713 and DPA-714 exhibit higher affinity for the TSPO in vitro (Ki=4.7 nM and Ki=7.0 nM, respectively) than PK11195 (Ki=9.3 nM)[1, 3].

The effective dose of ^{18}F-DPA-714 estimated from biodistribution in mice was 17.2 μSv/MBq. Modeling of regional brain and plasma data showed good in vivo stability of ^{18}F-DPA-714 in humans, with only 20% of blood metabolites 20 min post-injection. Whole-body images demonstrate uptake in the gallbladder, heart, spleen and kidneys[4].

Genotyping polymorphism rs6971

All individuals were genotyped for the rs6971 polymorphism within the TSPO gene. First, DNA was isolated using the QIAamp DNA mini kit 50 (Qiagen, Valencia, California; cat no 51303).Then a qPCR was performed. Per well, 10 ul mastermix (5 ul Taqman Genotyping mastermix (Applies Biosystems, Foster City, USA), 0.1ul primer (rs6971; TSPO gene, thermofisher scientific) and 4.9 ul milliQ) and 10-20 ng DNA was added. Samples were analyzed using Biorad CFX manager. The genetic analysis showed the binding affinity (high, intermediate or low) for the TSPO receptor per patient.

TSPO receptor analysis on leukocytes and HSPCs

The expression of the TSPO receptor on leukocytes and HSPCs was assessed using flow cytometry. For the leukocytes, red blood cells were lysed with red blood cell lysis buffer (Affymetrix, eBioscience, San Diego, USA). Leukocytes were incubated with fluorochrome labelled antibodies (supplemental table S2) for 15 minutes and washed with phosphate buffered saline (PBS). Samples were analyzed using BD FACS Canto II (Becton, Dickinson, Fanklin Lakes, New Jersey). Monocytes were classified according to HLA-DR, CD14 and CD16 expression, B-cells according to CD19 expression, NK cells according to CD56 expression and T cells according to CD3 and CD8 expression. Subsequently, the expression of the TSPO receptor was determined (supplemental table S2). Samples were analyzed using FlowJo software (version 10.0 FlowJO, LLC, Ashland, OR). Delta median fluorescence intensity (MFI)

was obtained by subtracting the MFI from an unstained control from the MFI of the marker.

For the HSPCs, sorted CD34+ cells were used. These cells were incubated with a fluorochrome labelled antibody for the TSPO receptor (supplemental table S2). Samples were analyzed using FlowJo software (version 10.0 FlowJO, LLC, Ashland, OR). Delta median fluorescence intensity (MFI) was obtained by subtracting the MFI from an unstained control from the MFI of the marker.

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TABLES

Table S1. Markers used for flow cytometry of circulating HSPCs and monocytes

	Surface markers	Color	Company
HSPCs	CD34	PeCy7	Beckman Coulter
	CD45	PacB	BD Horizon
Monocytes	CD45	PacB	DAKO
	CD14	PerCP	BD Pharmingen
	CD16	APC H7	BD Pharmingen
	CCR2	APC	BD Pharmingen

APC indicates allophycocyanin; Cy: CyChrome ; FITC: fluorescein isiothiocyanate ; PE: phycoerythrin; PerCP: peridinin-chlorophyll-protein

Table S2. Markers used for flow cytometry of the TSPO receptor on circulating leukocytes and HSPCs

	Surface markers	Color	Company
TSPO receptor	Ab199779 (anti-PBR antibody)	FITC	Abcam
Monocytes	HLA-DR	Percp-C5.5	BD Pharmingen
	CD14	PEcy7	BD Pharmingen
	CD16	APC-Cy7	BD Pharmingen
B-cells	CD19	APC	BD Pharmingen
NK-cells	CD56	APC	BD Pharmingen
T-cells	CD3	Percp	BD Pharmingen
	CD8	APC	BD Pharmingen

APC indicates allophycocyanin; Cy: CyChrome ; FITC: fluorescein isiothiocyanate ; PE: phycoerythrin; PerCP: peridinin-chlorophyll-protein

Table S3. Additional baseline characteristics ACS patient

Characteristics	**ACS patients** *(n=20)*
Troponin levels, ug/L	1.340 [0.215 – 5.245]
Infarct region	
- anterior infarction, n (%)	14 (70%)
- inferior infarction, n (%)	3 (15%)
- lateral infarction, n (%)	3 (15%)
Type of infarction (STEMI/non-STEMI)	16/4
LVEF after myocardial infarction	
- Good LVEF (≥55%), n (%)	7 (35%)
- Moderate LVEF (35-55%), n (%)	2 (10%)
- Unknown	11 (55%)
ACS treatment	
- PCI with stent	19 (95%)
- Drug therapy	1 (5%)
Medication use before ACS	
- Diuretics, n (%)	4 (20%)
- ACE inhibitor, n (%)	2 (10%)
- CCB, n (%)	3 (15%)
- β-blocker, n (%)	4 (20%)
- ARB, n (%)	3 (15%)
- Statins, n (%)	3 (15%)
- Anti-inflammatory compounds, n (%)	0 (0%)
Medication use after ACS	
- Diuretics, n (%)	3 (15%)
- ACE inhibitor, n (%)	12 (60%)
- CCB, n (%)	1 (5%)
- β-blocker, n (%)	15 (75%)
- ARB, n (%)	3 (15%)
- Statins, n (%)	19 (95%)
- Anti-inflammatory compounds, n (%)	0 (0%)

Values are n (%) or median [IQR]. The normal range for the troponin assay in our institution is 0 – 0,05ug/L.

ACE indicated Angiotensin Converting Enzyme inhibitor; ACS: Acute Coronary Syndrome; ARB: Angiotensin II Receptor Blocker; CCB: Calcium Channel Blockers; LVEF: Left Ventricular Ejection Fraction; non-STEMI: non-ST Elevated Myocardial Infarction; PCI: Percutaneous Coronary Intervention; STEMI: ST Elevated Myocardial Infarction

Table S4. Baseline characteristics of the groups who underwent DPA-714 PET/CT

Baseline characteristics	**ACS patients; acute phase** *(n=8)*	**Healthy controls** *(n=8)*	**p value** acute phase vs healthy controls
Age, years	62±5	61±7	0.832
Sex, men/women	7/1	5/3	0.281
BMI, kg/m²	31±6	24±2	0.011
Systolic blood pressure, mmHg	127±21	122±19	0.613
Diastolic blood pressure, mmHg	71±14	76±9	0.397
Smoking, yes/past/no	4/2/2	1/4/3	0.264
Hypertension, yes/no	3/5	0/8	0.055
Statin use, yes/no	0/8	0/8	1.0
TSPO receptor mutation, homozygotes / heterozygotes	3/5	3/5	1.0

Values are n or mean ± SD. *BMI indicates body mass index.*

Table S5. Myeloid skewing of circulating inflammatory cells post-ACS

Inflammatory cells	**ACS patients; acute phase** *(n=20)*	**Healthy controls** *(n=19)*	**p-value** acute phase vs healthy controls	**ACS patients; 3 months post-ACS** *(n=16)*	**p-value** acute phase versus 3 months post-ACS
Neutrophils, percentages	60±18%	56±8%	0.360	58±7%	0.243
Lymphocytes, percentages	25±8%	32±8%	0.023	30±6%	0.042
Monocytes, percentages	10±4%	8±1%	0.059	9%±2	0.093

Values are percentages, mean ± SD

Table S6. Inflammatory parameters 6 to 24 months post-ACS

Characteristics	ACS patients; 3 months post-ACS *(n=12)*	ACS patients; 6 to 24 months post-ACS *(n=12)*	p-value
Total cholesterol, mmol/L	3.9±0.7	3.8±0.6	0.351
HDL cholesterol, mmol/L	1.5±0.4	1.6±0.4	0.088
LDL cholesterol, mmol/L	2.0±0.5	1.8±0.4	0.066
Triglycerides, mmol/L	1.1[0.8-1.3]	0.9[0.7-1.3]	0.433
CRP, mg/dl	1.4[0.3-2.6]	1.6[0.5-3.3]	0.721
Leukocytes, *10^9/L	6.9±1.6	6.4±1.2	0.080
Neutrophils, *10^9/L	3.9±1.0	3.4±0.8	0.010
Lymphocytes, *10^9/L	2.0±0.8	2.0±0.6	0.982
Monocytes, *109/L	0.6±0.1	0.6±0.2	0.357

Values are n, mean ± SD or median [IQR] for respectively normally and non-normally distributed data.
CRP indicates C-reactive protein, HDL; high-density cholesterol, LDL; low-density cholesterol

FIGURES

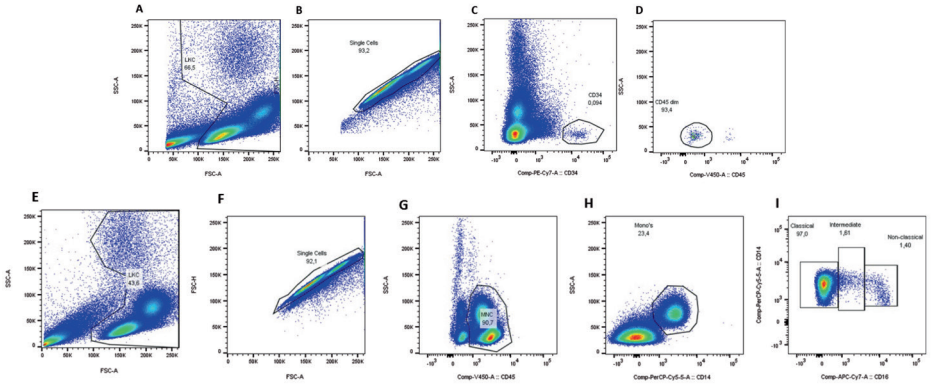

Figure S1. Gating strategy for HSPCs and monocytes using flow cytometry
Flow cytometry on whole blood was performed to study HSPC's and monocyte subsets and specific surface expression markers. First, leukocytes were separated using forward/sideward scatter (A), followed by single cell analysis (B), HSPCs were classified as $CD34^{+}$ cells (C) and $CD45_{dim}$ cells (D). Regarding the monocyte gating strategy, first single cell leukocytes were separated using forward/sideward scatter (E, F). Next, monocytes were classified according to CD45 (G) followed by CD14 expression (H). Monocyte subsets were divided according to CD14 and CD16 expression, identifying classical ($CD14^{+}CD16^{-}$), intermediate ($CD14^{+}CD16^{+}$) and non-classical ($CD14^{+}CD16^{++}$) monocytes (I).

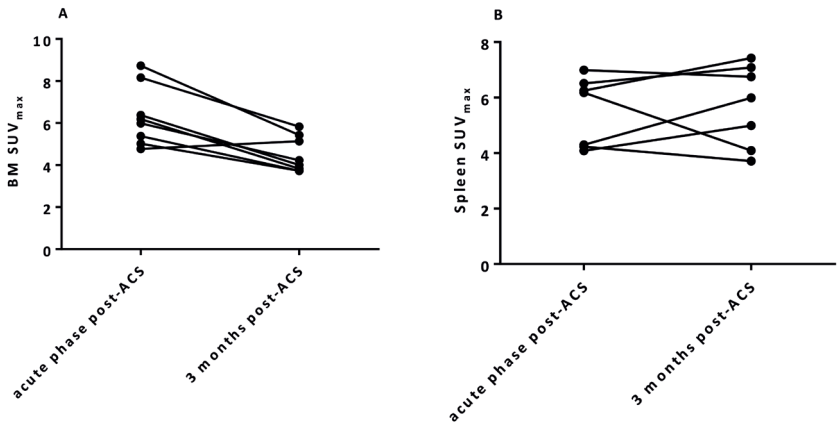

Figure S2. DPA-714 uptake in the acute phase and after 3 months in ACS patients
This figure shows lines connecting individual ^{18}F-DPA-714 uptake in bone marrow (A) and spleen (B) (acute phase and after 3 months) per ACS patient. *BM indicates bone marrow*

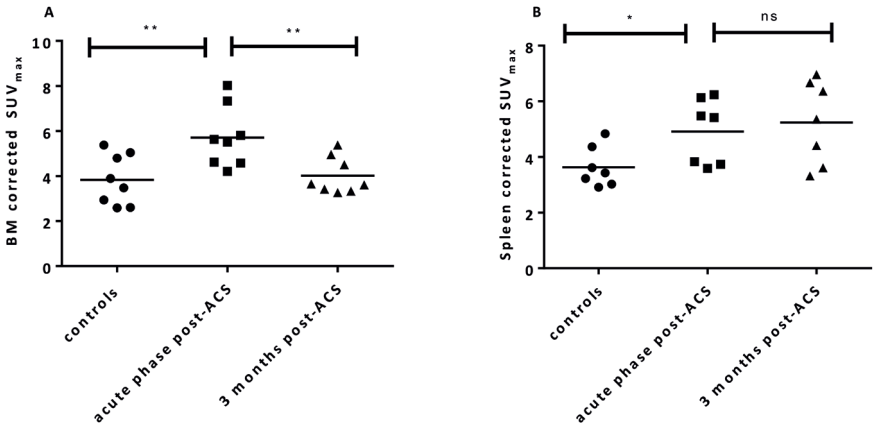

Figure S3. Elevated ^{18}F-DPA-714 uptake in bone marrow and spleen post-ACS shown as corrected SUV

^{18}F-DPA-714 uptake in bone marrow and spleen is shown as corrected SUV. Patients in the acute phase post-ACS showed elevated DPA-714 uptake in the bone marrow and spleen compared with healthy controls. Three months post-ACS, ^{18}F-DPA-714 uptake in bone marrow decreased (A), while ^{18}F-DPA-714 uptake in the spleen remained elevated (B). Data are represented as mean with single values of the subjects, *p<0.05, **p<0.01 *BM: bone marrow; ns: non-significant*

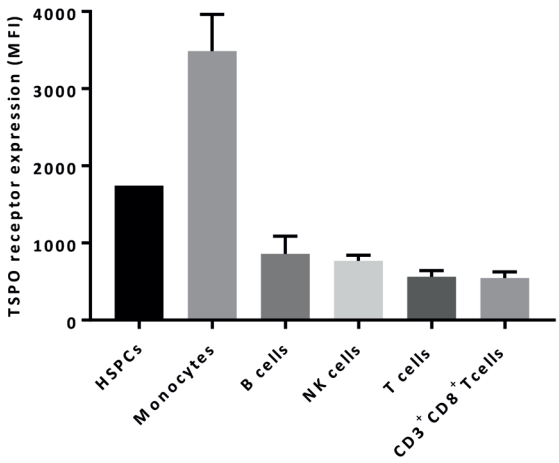

Figure S4. Expression of the TSPO receptor on HSPCs and leukocytes

Flow cytometry was performed to study the expression of the TSPO receptor on leukocytes (whole blood) and HSPCs (sorted CD34$^+$ cells). As shown in this figure, the TSPO receptor is highly expressed on monocytes and less expressed on the lymphocytes (B-cells, T-cells and NK cells), the TSPO receptor is also relatively high expressed on HSPCs.

HSPCs indicated hematopoietic stem and progenitor cells; NK: natural killer cells

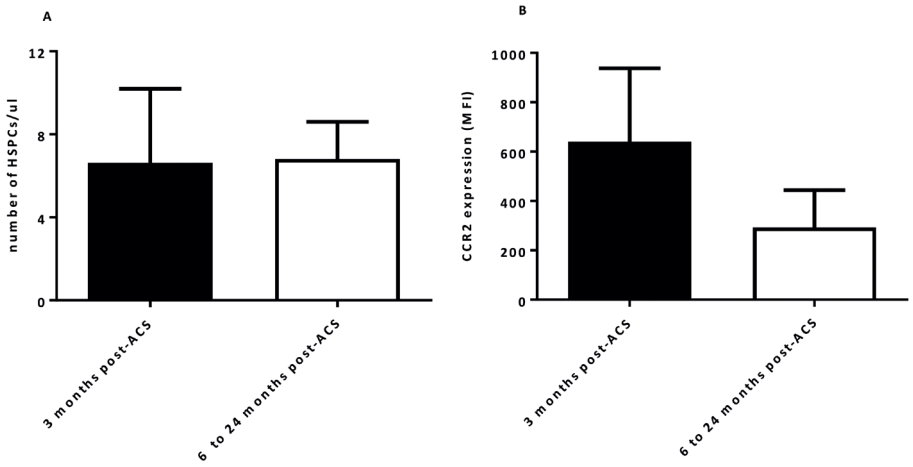

Figure S5. HSPCs and monocytes 6 to 24 months post-ACS
The number of circulating HSPCs and the expression of CCR2 on monocytes (showed as MFI) were assessed using flow-cytometry, comparing the level of the 3 month time point versus 6 to 24 months post-ACS. 6 to 24 months post-ACS, the number of circulating HSPCs were comparable to the numbers of the level of the 3 month time point (A; p=0.43), while a trend towards a decrease of CCR2 expression on monocytes 6 to 24 months post-ACS was observed (B; p=0.06) using Wilcoxon signed-rank test.

Data are represented as median±IQR. *ACS indicates acute coronary syndrome; HSPCs: hematopoietic stem and progenitor cells*

PART 2.

BREAKING THE VICIOUS CIRCLE OF LIPOPROTEIN(A)-MEDIATED INFLAMMATION

6

Short-term regulation of hematopoiesis by lipoprotein(a) results in the production of pro-inflammatory monocytes

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ABSTRACT

Background

Lipoproteins are important regulators of hematopoietic stem and progenitor cell (HSPC) biology, predominantly affecting myelopoiesis. Since myeloid cells, including monocytes and macrophages, promote the inflammatory response that propagates atherosclerosis, it is of interest whether the atherogenic lowdensity lipoprotein (LDL)-like particle lipoprotein(a) [Lp(a)] contributes to atherogenesis via stimulating myelopoiesis.

Methods and results

To assess the effects of Lp(a)-priming on long-term HSPC behavior we transplanted BM of Lp (a) transgenic mice, that had been exposed to elevated levels of Lp(a), into lethally-irradiated C57Bl6 mice and hematopoietic reconstitution was analyzed. No differences in HSPC populations or circulating myeloid cells were detected ten weeks after transplantation. Likewise, in vitro stimulation of C57Bl6 BM cells for 24 h with Lp(a) did not affect colony formation, total cell numbers or myeloid populations 7 days later. To assess the effects of elevated levels of Lp(a) on myelopoiesis, C57Bl6 bone marrow (BM) cells were stimulated with lp(a) for 24 h, and a marked increase in granulocyte-monocyte progenitors, pro-inflammatory Ly6high monocytes and macrophages was observed. Seven days of continuous exposure to Lp(a) increased colony formation and enhanced the formation of pro-inflammatory monocytes and macrophages. Antibody-mediated neutralization of oxidized phospholipids abolished the Lp(a)-induced effects on myelopoiesis.

Conclusion

Lp(a) enhances the production of inflammatory monocytes at the bone marrow level but does not induce cell-intrinsic long-term priming of HSPCs. Given the short-term and direct nature of this effect, we postulate that Lp(a)-lowering treatment has the capacity to rapidly revert this multi-level inflammatory response.

Keywords

Lipoprotein (a) [Lp(a)], atherosclerosis, inflammation, hematopoiesis, monocytes

INTRODUCTION

Atherosclerosis, a major cause of cardiovascular disease (CVD), is a chronic inflammatory disease of the arterial wall[1]. Myeloid cells, particularly monocytes and macrophages, have a critical role in the local and systemic inflammatory process that drives atherosclerosis[1]. Epidemiological and experimental studies have demonstrated that circulating myeloid cell numbers are associated with an increased cardiovascular risk[2]. Monocytes are derived from hematopoietic stem and progenitor cells (HSPC), which are located in the bone marrow (BM)[2]. During atherogenesis, hematopoiesis, the process that results in the formation of mature immune cells, is skewed towards the myeloid lineages[2,3], thereby increasing circulating inflammatory monocyte number and aggravating atherosclerotic lesion formation[2,3]. Pioneering studies in the last decade have demonstrated that lipoproteins, including low-density lipoprotein cholesterol LDL-C and high-density lipoprotein cholesterol (HDL-C), are important regulators of HSPC biology during atherogenesis[4]. Hypercholesterolemia induces cell intrinsic priming of HSPCs, which enhances stem cell proliferation and the production of pro-inflammatory monocytes, whereas HDL-C promotes HSPC quiescence[2-4].

The LDL-like particle lipoprotein(a) [Lp(a)] is an independent risk factor for CVD, and several mechanisms by which Lp(a) mediates CVD are proposed, including pro-inflammatory and pro-thrombotic effects[5,6]. Most notably, Lp(a) has strong pro-inflammatory properties, predominantly provoked by the pro-inflammatory oxidized phospholipids(OxPLs) present on Lp(a)[5]. Lp(a) not only exerts a local inflammatory response in atherosclerotic plaques, but also has the potential to activate monocytes in the circulation, leading to enhanced monocyte migration into atherosclerotic lesions[7,8]. We hypothesize that Lp(a) also influences myelopoiesis, leading to an enhanced production of pro-atherogenic monocytes, thereby fueling the detrimental innate immune response in atherosclerosis.

MATERIALS AND METHODS

A comprehensive material & methods section can be found in the supplemental material. In brief, one day prior to the bone marrow transplantation (BMT), recipient mice were lethally irradiated. The next day, $1x10^6$ BM cells obtained from transgenic mice expressing both apo(a) and human apoB-100 (apoB), which assemble to form Lp(a), or from C57Bl6 donors were intravenously injected in the recipients[9]. Hematopoietic reconstitution was analyzed in BM and peripheral blood 10 weeks after the BMT. All animal experiments were approved by the local Animal

Experimentation Ethics Committee. Results are shown as mean standard error of the mean. A p-value < 0.05 was considered statistically significant.

RESULTS

Lp(a) does not induce long-term priming of HSPCs

To investigate if Lp(a) induces cell intrinsic priming of HSPC *in vivo*, we transplanted BM cells of Lp(a) transgenic mice with elevated plasma Lp(a) levels or control BM cells obtained from C57Bl6 mice with undetectable levels of plasma Lp(a) in lethally irradiated C57Bl6 recipients. Mice do not contain the LPA gene and in these transgenic mice, Lp(a) is produced by the liver, resulting in high circulating levels of Lp(a) and high levels of OxPLs[9]. The donor BM was exposed for 8 weeks to Lp(a) before harvest, our BMT approach is therefore an appropriate model to study priming of BM by Lp(a). Ten weeks post-BMT, HSPC population counts in the BM and circulating myeloid population counts did not differ between the two groups, reflecting absence of long-term Lp(a)-induced HSPC priming (Fig. 1A, B).

To confirm that Lp(a) has indeed no intrinsic priming effects on HSPCs, or that the potentially weaker priming effect of Lp(a) was abolished by the transplantation itself, *in vitro* stimulation assays with wild type BM cells and Lp(a) were performed. C57Bl6 BM was exposed to Lp(a) (1 mg/ml) for 24 hours and this Lp(a)-primed BM was analyzed in colony forming unit (CFU) assays. After 7 days no differences in colony formation (Fig. 1D) or total myeloid cell numbers (Fig. 1D) were observed between control and Lp(a)-primed BM. Additionally, flow cytometry revealed no differences in granulocytes, monocyte subsets or macrophages (Fig. 1E), indicating absence of Lp(a)-induced HSPC priming.

Lp(a) directly promotes myelopoiesis and enhances the production of inflammatory monocytes

Next, BM cells were analyzed directly after 24 hours of stimulation with Lp(a) *in vitro*, an 8.6% increase in granulocyte-monocyte progenitors was observed, without affecting other progenitor populations, including common lymphoid progenitors (Fig. 1F). Analysis of mature myeloid populations showed a 14.4% increase in pro-inflammatory Ly6^{high} monocytes and 6.7% increase in macrophages (Fig. 1F). Total monocytes slightly decreased, possible due to increased differentiation into macrophages (Fig. 1F). Continuous exposure to Lp(a) for 7 days induced a proliferative BM profile, characterized by a 20.5% increase in BM cells in the S-phase and 10.6% increase in BM cells in the G2/M-phase of the cell cycle (Fig. 1G). Accordingly, colony formation of Lp(a)-stimulated BM was increased by 13.3% (Fig.

1H). The production of mature myeloid cells (Fig. 1I), especially inflammatory Ly6C^{high} monocytes and macrophages (Fig. 1J) increased upon Lp(a) stimulation.

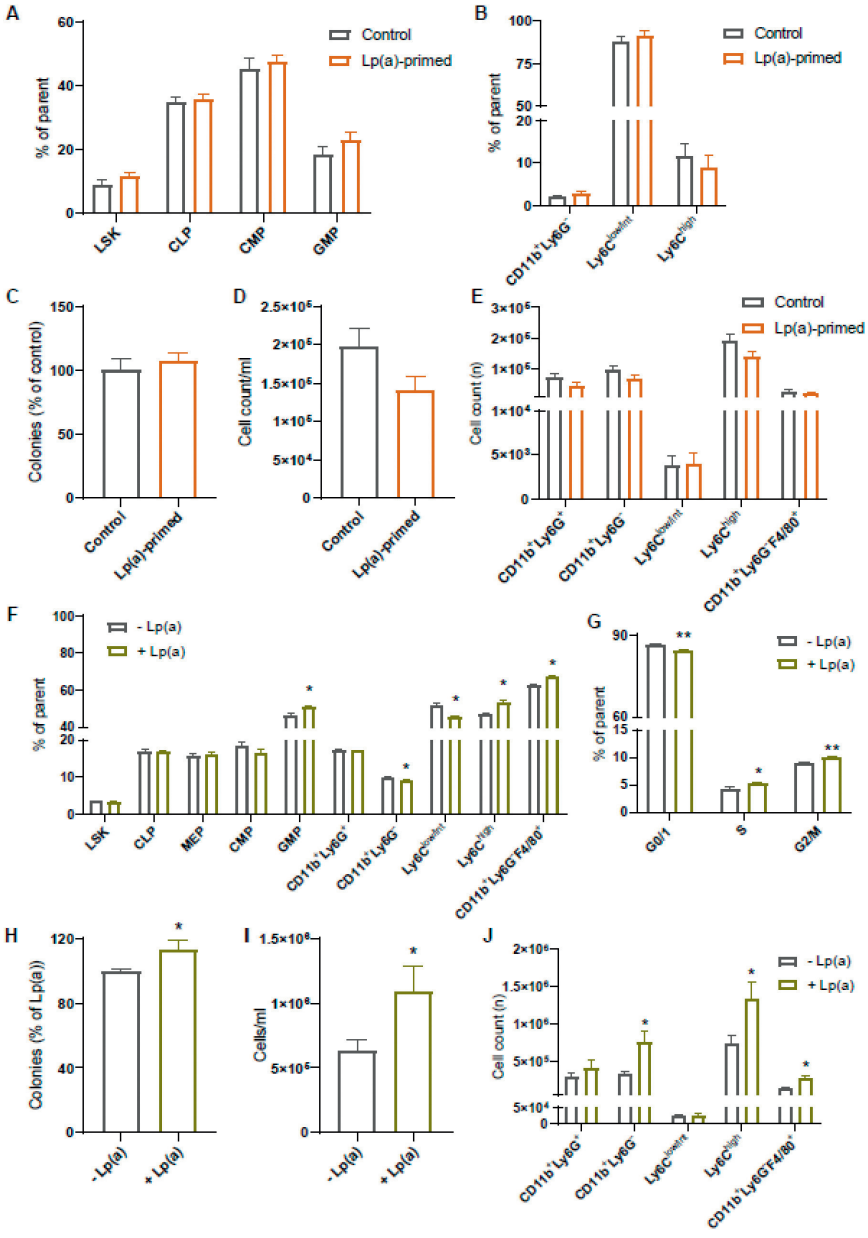

Figure 1. Lp(a) promotes myelopoiesis, but does not induce long-term priming of HSPCs
(A) Lp(a) exposure did not affect the reconstitution of HSPC populations within the BM

of C57Bl6 mice (n = 12), *LSK: Lin$^-$Sca-1$^+$cKit$^+$; CLP: Lin$^-$cKit$^+$Sca-1$^+$CD135$^+$CD127$^+$; CMP: Lin$^-$cKit$^+$CD16/32$^+$CD34$^+$; GMP: Lin$^-$cKit$^+$CD16/32^{int}CD34^{int}.* (**B**) Lp(a) exposure did not affect the reconstitution of circulating monocyte (*CD11b$^+$Ly6G$^-$*) populations at week 10 post-BMT (n = 12). (**C**) Colony forming unit (CFU) assays demonstrated that short-term (24 hours) exposure of BM cells to Lp(a) did not affect colony formation after 7 days. (D) No differences in total cell counts and (**E**) myeloid populations were observed (n = 10), *monocytes*: *CD11b$^+$Ly6G$^+$*, *granulocytes: CD11b$^+$Ly6G$^+$*, *macrophages CD11b$^+$Ly6G$^-$F4/80$^+$*. (**F**) After 24 hours of stimulation with Lp(a), increased granulocyte-monocyte progenitor, Ly6C^{high} monocytes and macrophages was observed. The relative number of total monocytes decreased and the number of Ly6C$^{low/int}$ monocytes decreased due to the shift towards Ly6C^{high} monocytes (n = 10). (**G**) Continuous exposure to Lp(a) for 7 days induced proliferation of BM cells, as shown by PI flow cytometry, which resulted in increased colony formation (**H**) and total cell numbers in CFU assays (**I**) (n = 8). (**J**) Continuous exposure to Lp(a) for 7 days increased pro-inflammatory monocyte and macrophage numbers (n = 8). *Data represented as mean ± SEM. * p < 0.05, ** p < 0.01, *** p < 0.001.*

To investigate if the hematopoietic effects of Lp(a) depended on its oxidized phospholipid content, the monoclonal antibody E06, which binds the phosphocholine moiety of oxidized phospholipid and blocks its inflammatory effects, was included in the BM culture assays. Antibody-mediated blockage of oxidized phospholipids on Lp(a) prevented the Lp(a)-induced increase in colony formation (Fig. 2A), total cell numbers (Fig. 2B), as well as the production of mature myeloid cells, in particular granulocytes and Ly6C^{high} monocytes (Fig. 2C). Together these data demonstrate that Lp(a) enhances the formation of inflammatory myeloid cells *in vitro*, which is mediated by its oxidized phospholipid content.

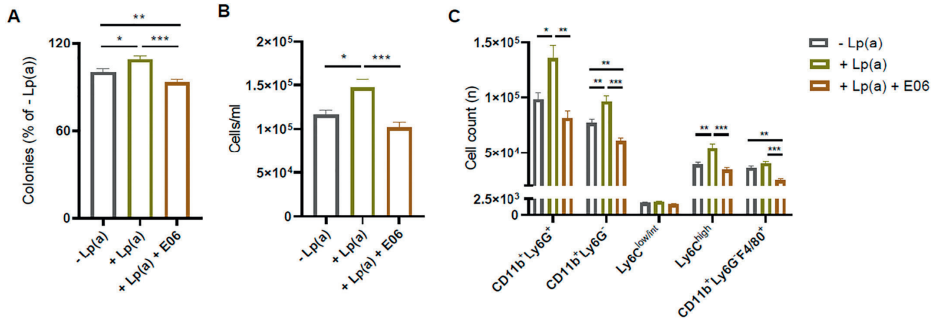

Figure 2. Antibody-mediated blockage of oxidized phospholipids abolishes the Lp(a)-induced effects on myelopoiesis

CFU assays demonstrated that E06 prevented the Lp(a)-induced increase in colony formation (**A**), total cell counts (**B**), and mature myeloid cells, in particular CD11b$^+$Ly6G$^+$, CD11b$^+$Ly6G$^-$ and CD11b$^+$Ly6G$^-$LyC^{high} cells (**C**) (n = 10). *Data represented as mean ± SEM. * p < 0.05, ** p < 0.01, *** p < 0.001.*

DISCUSSION

A few conclusions can be drawn from these studies. First, Lp(a)-stimulated HSPC produce pro-inflammatory monocytes, which indicates that direct interaction of Lp(a) with monocytes in the circulation leads to monocyte activation, but also that Lp(a) affects their production on the marrow level. Second, although LDL-C exerts *long-term* priming effects of HSPC that persist in a normocholesterolemic environment, Lp(a) has a *short-term* effect on HSPC[3]. We hypothesize that this is attributed to the relative low concentrations of Lp(a) compared to LDL concentrations used in other experiments and therefore the lack of Lp(a)-induced priming[5]. This relies on the fact that the inflammatory effects of Lp(a) are mainly attributed to the signaling capacity of its oxidized phospholipid content and not due to NLRP3 inflammasome activation resulting from intracellular cholesterol accumulation, which is the mechanism of LDL-C-induced intrinsic priming of myeloid cells and their progenitors[10,11]. Previous data suggest that besides the LDL-entity of Lp(a), the apo(a) tail of Lp(a) does not induce cellular activation without presence of OxPLs[7,12]. Furthermore, Lp(a) was able to activate HSPCs directly (i.e. 24 h and 7 days), however, once the Lp(a) stimulus was absent (i.e. 24 h stimulation and 6 days without stimulus or BMT experiment) HSPC activity exerted normal function further indicating that Lp(a) does not induce NLRP3 activation but its mode of action is via OxPLs. These oxidized phospholipids represent danger-associated molecular patterns, recognized by a variety of pattern recognition receptors, including Toll-like receptor 4 (TLR4) and scavenger receptor-B1 (SR-B1), which are expressed on HSPC and regulate their biology[13]. For example, TLR4 engagement on HSPCs skews hematopoiesis towards the myeloid lineage, whereas SR-B1 activation limits HSPC proliferation and the production of inflammatory cells, indicating that activation of pattern recognition receptors on HSPCs has diverse effects that may be ligand-dependent[14,15]. Although our data demonstrate that the oxidized phospholipid content of Lp(a) promotes myelopoiesis, the pattern recognition receptor for these oxidized phospholipids on HSPCs has yet to be discovered.

Our data imply that depleting the HSPC microenvironment of Lp(a) will lead to a rapid reversal of the activated monocyte profile that is attributed to elevated Lp(a) levels, while the LDL-induced hematopoietic effects will persist for much longer period following LDL-C reduction. This concept concurs with the attenuation of transendothelial migration of monocytes by an approximately 70% Lp(a) reduction following 12 weeks of apo(a) antisense therapy in Lp(a) patients, whereas an approximately 44% LDL-C reduction following 14 weeks of statin therapy is still

characterized by an unchanged, hyperinflammatory monocyte phenotype in patients with genetic LDL-C elevation[16, 17].

Study limitations: this proof of concept study in mice identifies direct effects of Lp(a) on BM proliferation, myelopoiesis and the formation of inflammatory monocytes, and additional studies are needed to elucidate the underlying mechanisms of these Lp(a)-induced hematopoietic effects. Clinical studies are required to determine if the activated monocyte profile in patients with elevated Lp(a) levels results from aberrations in HSPC biology.

In conclusion, this study proposes that in addition to direct activation of monocytes in the circulation, Lp(a) also enhances the production of activated monocytes on bone marrow level. Given the short-term and direct nature of this effect on hematopoiesis, we postulate that Lp(a)-lowering treatment has the capacity to rapidly revert this multi-level inflammatory response.

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Conflict of interest
E.S.G.S. reports that his institution has received lecturing fees and advisory board fees from Amgen Inc., Regeneron, Sanofi, Akcea, Novartis and Esperion. S.T. is a co-inventor and receives royalties from patents owned by UCSD on oxidation-specific antibodies and of biomarkers related to oxidized lipoproteins, has a dual appointment at UCSD and Ionis Pharmaceuticals, is a co-founder of Oxitope, Inc. and Kleanthi LLC, and is a consultant to Boston Heart Diagnostics. The other authors have nothing to disclose.

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SUPPLEMENTAL MATERIAL

Bone marrow transplantation

All animal experiments were approved by the local Animal Experimentation Ethics Committee. C57Bl6 mice were bred in the local animal facility. All mice were aged-matched males. C57Bl6 recipient mice were housed in filter-top cages and received water containing antibiotics (polymyxine B sulfate, 60,000 U/L, and neomycin, 100 mg/L) for 5 weeks, starting 1 week before the bone marrow transplantation (BMT). One day prior to the BMT, the mice were lethally irradiated (9.5 Gy, 0.5 Gy/min; Philips MU15F/225 kV; Philips). The next day, 1x106 BM cells obtained from transgenic mice expressing both apo(a) and human apoB-100 (apoB) (Schneider, JLR, 2005), which assemble to form Lp(a) or from C57Bl6 donors in the recipients. Hematopoietic reconstitution was analysed in BM and peripheral blood 10 weeks after the BMT.

Flow cytometry

Bone marrow (BM) was harvested in cold phosphate buffered saline (PBS) and a BM cell suspension was prepared and filtered through a 70-µm nylon mesh (BD Falcon, BD Biosciences, Breda, The Netherlands). Lineage depletion by magnetic bead isolation was performed according to the manufacturer's instructions (Lineage Cell Depletion Kit, Miltenyi Biotec, Teterow, Germany). Cell suspensions were treated with red blood cell lysis buffer that contained 8.4 g NH4Cl and 0.84 g NaHCO3 per liter distilled water. Staining was performed with anti-mouse antibodies against the following antigens: lineage cocktail (Miltenyi Biotec, Teterow, Germany); Ly6G (clone 1A8), CD11b (clone M1/70), CD127 (clone HIL-7R-M21) and Gr-1 (clone RB6-8C5) (BD Pharmingen, Breda, The Netherlands); CD117 (clone 2B8), CD34 (clone RAM34, F4/80 (clone BM8) and CD32/16 (clone 93) (Ebioscience, Vienna, Austria); Sca-1 (clone D7) and CD135 (A2F10) (aBiolegend, San Diego, CA, USA); and Ly6C (clone 1G7.G10) (Miltenyi Biotech, Teterow, Germany). Nonspecific binding was prevented by pre-incubation of the cells with an Fc receptor-blocking antibody (Ebioscience, Vienna, Austria). For cell-cycle analysis, bone marrow cells were fixed in 70% ethanol for 24 hours and treated with propidium iodide/RNase buffer according to the manufacture's protocol (BD Biosciences, San Jose, CA, USA). Staining was analysed by flow cytometry (FACS Canto II; BD Biosciences, San Jose, CA, USA) and FlowJo software version 7.6.5 (Treestar, Ashland, OR, USA).

Lp(a) isolation

Lp(a) was isolated from plasma of healthy volunteers. Blood was collected in EDTA (ethylenediaminetetraacetic acid (EDTA)-containing 10 mL Vacutainer tubes.

Lp(a) was obtained after KBr (VWR, Radnor, Pennsylvania, USA)-density gradient ultracentrifugation. Here plasma was adjusted to d=1.25 g/mL with solid KBr solution and a discontinuous gradient was formed by carefully layering 2 mL of d=1.225 KBr solution, followed by 4 mL of d=1.100 KBr solution and a 3 mL of d=1.006 KBr solution and centrifuged at vacuum for 19 hours at 29.000 rpm at 10°C in a SW 41 Ti rotor without brake (Beckman Coulter Inc., CA). Next, Lp(a) was isolated and was filter sterilized (0.2 µm pore size; Sartorius, Göttingen, Germany) and concentrated using Amicon centrifugal filter units filter (10.000 MWCO; Millipore). Lp(a) concentration was measured using commercially available immunoturbidimetric enzymatic assays (Diasys, Holzheim, Germany) on a Selectra system (Sopachem, Ochten, The Netherlands).

Colony-forming unit (CFU) assays

BM was isolated from C57Bl6 mice and a single-cell suspension was prepared as described above. BM cells (1×10^4) were cultured in 2 ml semisolid methylcellulose medium supplemented with growth factors (MethoCult; Stem Cell Technologies, Grenoble, France) at 37°C in 98% humidity and 5% CO2 for 7 days. Total colonies were scored after 7 days using an inverted microscope in a blinded protocol. In selected experiments, BM cells were co-cultured with Lp(a) (1 mg/ml) and/or E06 (100 µg/ml; Avanti Polar Lipids, Inc., Alabaster, AL, USA), as indicated in the figures.

Statistical analysis

Results are shown as mean standard error of the mean. A p-value < 0.05 was considered statistically significant. Data (mean ± SEM) were analysed by an unpaired, 2-tailed Student's t test. Grahphad Prism 5 software (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis.

7

Persistent arterial wall inflammation in patients with elevated lipoprotein(a) despite strong low-density lipoprotein cholesterol reduction by PCSK9 antibody treatment

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ABSTRACT

Aims

Subjects with lipoprotein(a) (Lp[a]) elevation have increased arterial wall inflammation and cardiovascular risk. In patients at increased cardiovascular risk, arterial wall inflammation is reduced following lipid-lowering therapy by statin treatment or lipoprotein apheresis. However, it is unknown whether lipid-lowering treatment in elevated Lp(a) subjects alters arterial wall inflammation. We evaluated whether evolocumab, which lowers both low-density lipoprotein cholesterol (LDL-C) and Lp(a), attenuates arterial wall inflammation in patients with elevated Lp(a).

Methods and Results

In this multicentre, randomized, double-blind, placebo-controlled study, 129 patients (median [IQR]: age 60.0 [54.0-67.0] years, Lp(a) 200.0 [155.5-301.5] nmol/L [80.0 (62.5-121.0) mg/dL]; mean [SD] LDL-C 3.7 [1.0] mmol/L [144.0 (39.7) mg/dL]; National Cholesterol Education Program high risk, 25.6%) were randomized to monthly subcutaneous evolocumab 420 mg or placebo. Compared to placebo, evolocumab reduced LDL-C by 60.7% (95% CI: 65.8-55.5) and Lp(a) by 13.9% (95% CI: 19.3-8.5). Among evolocumab-treated patients, the week 16 mean (SD) LDL-C level was 1.6 (0.7) mmol/L (60.1 [28.1] mg/dL), and the median (IQR) Lp(a) level was 188.0 (140.0-268.0) nmol/L (75.2 [56.0-107.2] mg/dL). Arterial wall inflammation (most diseased segment target-to-background ratio [MDS TBR]) in the index vessel (left carotid, right carotid, thoracic aorta) was assessed by ^{18}F-fluoro-deoxyglucose positron-emission tomography/computed tomography. Week 16 index vessel MDS TBR was not significantly altered with evolocumab (-8.3%) versus placebo (-5.3%) (treatment difference -3.0% [95% CI: -7.4%, 1.4%]; P=0.18).

Conclusion

Evolocumab treatment in patients with median baseline Lp(a) 200.0 nmol/L led to a large reduction in LDL-C and a small reduction in Lp(a), resulting in persistent elevated Lp(a) levels. The latter may have contributed to the unaltered arterial wall inflammation.

Keywords

PCSK9 antibodies, evolocumab, lipoprotein(a), arterial wall inflammation, atherosclerosis

INTRODUCTION

Lipoprotein(a) (Lp[a]) is a potential independent and causal risk factor for cardiovascular disease (CVD)[1, 2]. Lp(a) consists of an apolipoprotein(a) (apo[a]) tail covalently bound to a low-density lipoprotein cholesterol (LDL-C) core by a disulphide bridge. Lp(a)-mediated CVD risk is partly driven by pro-inflammatory oxidized phospholipids (OxPL), which are abundant on the apo(a) tail of Lp(a)[2]. Previously, we reported that Lp(a)-carried OxPL are crucial intermediates in the arterial wall inflammation process among patients with elevated Lp(a)[3].

Contrary to LDL-C, no large outcome studies on dedicated Lp(a) lowering are available, since potent Lp(a)-lowering therapies remain in development[4]. The current European Society of Cardiology/European Atherosclerosis Society guideline therefore proposes altering other modifiable CVD risk factors such as LDL-C to lower CVD risk in patients with elevated Lp(a)[5]. Treatment with monoclonal antibodies directed against proprotein convertase subtilisin/kexin type 9 (PCSK9) may benefit patients with elevated Lp(a), as these agents produce a strong LDL-C reduction combined with a modest Lp(a) reduction. Evolocumab is an anti-PCSK9 monoclonal antibody that reduces LDL-C by a mean of 60% and Lp(a) by approximately 20%-30% in patients without elevated Lp(a) levels[6]. In post-hoc analysis of the phase III FOURIER study, patients with higher baseline Lp(a) levels had a greater absolute CVD risk reduction after evolocumab treatment[7]. It should be noted, however, that PCSK9 inhibition in patients with Lp(a) elevation induces a lesser percent Lp(a) reduction compared to the 20%-30% reduction observed in patients with normal Lp(a) levels[8]. Hence, PCSK9 inhibition fails to establish low Lp(a) levels in patients with baseline Lp(a) elevation. Moreover, agents offering modest Lp(a) reduction without LDL-C reduction have not been shown to reduce CVD risk[9-11].

In ANITSCHKOW, we evaluated whether potent LDL-C lowering, combined with modest Lp(a) lowering with evolocumab, would attenuate arterial inflammation as a surrogate for CVD risk in patients with elevated Lp(a).

METHODS

Study design

This study was a phase 3b, multicentre, randomized, double-blind, placebo-controlled trial. Eligible patients were randomized 1:1 to monthly subcutaneous injections of either evolocumab 420 mg or placebo for 16 weeks. Randomization was performed with an interactive voice or web response system.

The study was conducted according to the principles of the Declaration of Helsinki and the study protocol, including amendments, were approved by the ethic committees at all participating sites. All patients provided written informed consent prior to enrolment. Qualified researchers may request data from Amgen clinical studies. Complete details are available at http://www.amgen.com/datasharing. Clinical trial registration information is accessible at https://clinicaltrials.gov/ct2/show/NCT02729025.

Study population

Eligible patients were ≥50 years of age, had a fasting LDL-C of ≥2.6 mmol/L (100 mg/dL), an Lp(a) level of ≥125 nmol/L (50 mg/dL), and arterial wall inflammation as assessed by a most diseased segment target-to-background ratio (MDS TBR) of ≥1.6 in an index vessel measured with ^{18}F-fluoro-deoxyglucose positron-emission tomography/computed tomography (^{18}F-FDG PET/CT). For patients receiving lipid-lowering therapy, the treatment and dosage had to be stable for ≥8 weeks prior to screening. Key exclusion criteria included diabetes mellitus and a cardiovascular event within 3 months before randomization. The complete eligibility criteria are in the Supplement.

Biochemical measurements

Patients fasted for ≥9 hours before lipid samples were obtained. Total cholesterol, high-density lipoprotein (HDL)-C, triglycerides and apolipoprotein B-100 (ApoB-100) were measured by commercially available kits at the Medpace core lab (Medpace Reference Laboratories; Leuven, Belgium). LDL-C was calculated using the Friedewald formula[12]. For calculated LDL-C values <40 mg/dL or triglycerides >400 mg/dL, ultracentrifugation-determined LDL-C was measured and reported. Lp(a) levels were measured at baseline, week 8, and week 16 using an isoform-independent immunoturbidometric assay (Polymedco, Cortlandt Manor, NY) and reported in nmol/L. A conversion factor of 2.5 was used to provide approximate values in mg/dL.

PET/CT imaging

Arterial inflammation was assessed using ^{18}F-FDG PET/CT. Arterial ^{18}F-FDG uptake is correlated with arterial macrophage content[13], and predicts cardiovascular events[14].

^{18}F-FDG PET/CT scans were performed on dedicated PET/CT scanners. Patients fasted for ≥6 hours prior to infusion of 240 MBq of ^{18}F-FDG; 90 minutes later, an ultra-low dose (20 mAs), non-contrast enhanced CT of the carotid arteries and ascending thoracic aorta for attenuation correction and anatomic co-registration

was performed, followed by PET. Images were analysed using a dedicated US Food and Drug Administration–approved analysis software package (OsiriX MD, Pixmeo SARL, Switzerland). An experienced radiologist blinded to all patient characteristics analysed the PET/CT images; 10% of all datasets were reanalysed by a separate analyst, and for a second time by the primary analyst, to assess inter- and intra-observer reproducibility.

Target-to-background ratio was calculated from the ratio of the standardized uptake value (SUV) of the artery (left carotid, right carotid, or thoracic aorta) and the background venous activity according to previously reported methods[15].

Endpoints

The primary endpoint was percentage change from baseline in MDS TBR of the index vessel (left carotid, right carotid, or thoracic aorta) measured by ^{18}F-FDG PET/CT after 16 weeks of study drug treatment. Secondary endpoints were percentage change in Lp(a), LDL-C, and ApoB from baseline at week 16. Adverse events were assessed during the study.

Statistical analysis

The planned sample size was 120 patients. This sample size, accounting for a 25% drop-out rate, provides >90% power for testing superiority of evolocumab over placebo in the percentage change in MDS TBR at week 16, assuming an effect size of 14% reduction in the evolocumab arm compared to the placebo arm[13] and a common standard deviation of 20%.

Randomization was stratified by background statin therapy and by final screening Lp(a) (Supplement). To estimate the treatment difference in the primary endpoint, a multivariate regression was used and modelled on the primary endpoint as well as three other response variables (percent change in Lp[a] at weeks 8 and 16, baseline MDS TBR, and baseline Lp[a]). The primary endpoint was regressed on the treatment group and statin stratification factor; baseline MDS TBR and Lp(a) were regressed on the statin stratification factor, and percent changes in Lp(a) were regressed on the treatment group, statin stratification factor, visit, and treatment group by visit. Missing data for the primary endpoint were handled using the correlations of the error terms from the response variables in the model. Lp(a) in nmol/L is used for all statistical analyses.

Analysis of the secondary endpoints Lp(a), ApoB, and LDL-C was performed with a repeated measures linear mixed effects model including terms for treatment group,

statin stratification, scheduled visit, and the interaction of treatment with scheduled visit. LDL-C levels corrected for Lp(a)-derived cholesterol were calculated using the Dahlen formula[16]. Summary statistics for continuous variables were reported. For categorical variables, the frequency and percentage were reported. No adjustments for multiplicity were applied.

RESULTS

A total of 240 patients were screened and 129 patients (evolocumab, n=65; placebo, n=64) were enrolled at 14 sites in the Netherlands, Canada, and United States between April 2016 and April 2018 (Supplementary Figure). Baseline characteristics were generally comparable between groups (Table 1). Mean (SD) LDL-C was 3.7 (1.0) mmol/L (144.0 [39.7] mg/dL) and median (IQR) Lp(a) was 200.0 (155.5, 301.5) nmol/L (80.0 [62.5, 121.0] mg/dL) in the total population. Mean (SD) LDL-C corrected for Lp(a)-derived cholesterol was 3.0 (1.1) mmol/L. Baseline statin use was present in 54.3% of patients. Baseline MDS TBR of the index vessel was comparable between groups (median [IQR] 2.2 [2.0, 2.5], evolocumab vs 2.2 [1.9, 2.6], placebo).

Lipid profile

Evolocumab significantly reduced LDL-C at week 16 (mean [95% CI] percent change treatment difference versus placebo: -60.7% [-65.8, -55.5]; P<0.0001) (Table 2; Figure 1); total cholesterol and triglycerides were also reduced (Table 2). Mean (SD) LDL-C was 1.6 (0.7) mmol/L (60.1 [28.1] mg/dL) at week 16 in the evolocumab group. The evolocumab-induced mean (SD) percent reduction in LDL-C corrected for Lp(a)-derived cholesterol was greater than the reduction in LDL-C (-74.9% [23.9%] vs -59.0% [14.8%], respectively; Table 2). Evolocumab resulted in a mean (95% CI) percent change treatment difference in Lp(a) versus placebo of -13.9% (-19.3%, -8.5%; P<0.0001) (Table 2; Figure 1). Median (IQR) absolute changes in Lp(a) were -28.0 (-56.5, 9.0) nmol/L (-11.2 [-22.6, 3.6] mg/dL) for evolocumab vs 1.5 (-19.0, 18.0) nmol/L (0.6 [-7.6, 7.2] mg/dL) for placebo (Table 2). The median (IQR) Lp(a) was 188.0 (140.0, 268.0) nmol/L (75.2 [56.0, 107.2] mg/dL) in the evolocumab group at week 16

Table 1. Baseline characteristics[a]

	Evolocumab (n=65)	Placebo (n=64)
Age, years, median (IQR)	59.0 (55.0, 65.0)	60.5 (54.0, 68.0)
Male, n (%)	26 (40.0)	34 (53.1)
Caucasian, n (%)	58 (89.2)	58 (90.6)
BMI, kg/m^2, median (IQR)	26.7 (24.2, 29.0)	26.6 (24.0, 28.9)
Cardiovascular disease, n (%)		
Coronary artery disease	9 (13.8)	11 (17.2)
Peripheral artery disease	2 (3.1)	1 (1.6)
Cerebrovascular disease	5 (7.7)	0
Hypertension	26 (40.0)	19 (29.7)
Current smoker, n (%)	9 (13.8)	5 (7.8)
NCEP high risk, n (%)	18 (27.7)	15 (23.4)
Statin use, n (%)	36 (55.4)	34 (53.1)
High-intensity[b]	15 (23.1)	14 (21.9)
Moderate-intensity[b]	19 (29.2)	18 (28.1)
Low-intensity[b]	2 (3.1)	2 (3.1)
Ezetimibe, n (%)	10 (15.4)	17 (26.6)
Lipids[c]		
Total cholesterol, mmol/L[d]	5.9 (1.3)	5.8 (1.1)
HDL-cholesterol, mmol/L[d]	1.4 (0.4)	1.5 (0.4)
LDL-cholesterol, mmol/L[d]	3.8 (1.1)	3.7 (0.9)
Triglycerides, mmol/L[e]	1.4 (0.5)	1.5 (1.0)
Lp(a), nmol/L[f]	203.0 (162.5, 301.5)	198.0 (151.3, 300.0)
ApoB, g/L	1.1 (0.2)	1.1 (0.2)
hs-CRP, mg/L, median (IQR)	1.1 (0.6, 2.2)	1.0 (0.6, 1.6)
Glucose, mmol/L, median (IQR)	5.1 (4.8, 5.6)	5.3 (5.2, 5.6)
MDS TBR of index vessel,[g] median (IQR)	2.2 (2.0, 2.5)	2.2 (1.9, 2.6)

ApoB, apolipoprotein B; BMI, body mass index; HDL, high-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; Lp(a), lipoprotein(a); MDS TBR, most diseased segment target-to-background ratio.

[a]Baseline characteristics were generally comparable between groups. Numerical imbalances between groups are likely due to chance after randomization given the small sample size of each treatment group.

[b]Intensity per American College of Cardiology/American Heart Association definition.[39]

[c]Values are mean (SD) with the exception of Lp(a), which is median (IQR).

[d]To convert to mg/dL, multiply by 38.7.

[e]To convert to mg/dL, multiply by 88.6.

[f]To convert to mg/dL, divide by 2.5.[40]

[g]Mean of the maximum TBR in the MDS of the index vessel.

Table 2. Absolute and percent change in plasma lipid levels from baseline at week 16

	Evolocumab (n=65)	Placebo (n=64)
Lipid levels – absolute change[a]		
Total cholesterol, mmol/L[b]	-2.2 (0.8)	0.0 (0.6)
HDL-cholesterol, mmol/L[b]	0.1 (0.2)	0.0 (0.2)
LDL-cholesterol, mmol/L[b]	-2.2 (0.8)	0.0 (0.6)
LDL-cholesterol corrected for Lp(a), mmol/L	-2.1 (0.8)	0.0 (0.5)
Triglycerides, mmol/L[c]	-0.3 (0.4)	-0.0 (0.5)
Lp(a), nmol/L[d]	-28.0 (-56.5, 9.0)	1.5 (-19.0, 18.0)
ApoB, g/L	-0.5 (0.2)	0.0 (0.1)
Lipid levels – LS mean (95% CI) percent change, %		
LDL-cholesterol	-59.0 (-62.6, -55.4)	1.6 (-2.0, 5.3)
Treatment difference[e]	-60.7 (-65.8, -55.5)	
LDL-cholesterol corrected for Lp(a)	-74.53 (-79.69, -69.36)	1.23 (-4.03, 6.50)
Treatment difference[e]	-75.76 (-83.13, -68.39)	
Lp(a)	-12.8 (-16.6, -9.0)	1.1 (-2.8, 4.9)
Treatment difference[e]	-13.9 (-19.3, -8.5)	
ApoB	-48.3 (-51.3, -45.3)	3.3 (0.3, 6.3)
Treatment difference[e]	-51.6 (-55.9, -47.3)	
Total cholesterol	-37.99 (-40.59, -35.38)	0.83 (-1.82, 3.48)
Treatment difference[e]	-38.82 (-42.53, -35.10)	
HDL-cholesterol	9.31 (5.66, 12.95)	0.00 (-3.72, 3.73)
Treatment difference[e]	9.30 (4.09, 14.52)	
Triglycerides	-16.45 (-22.67, -10.22)	-0.06 (-6.43, 6.30)
Treatment difference[e]	-16.38 (-25.29, -7.48)	
hs-CRP, mg/L – absolute change[a]	-1.2 (7.9)	-0.4 (4.5)

[a]Values are mean (SD) with the exception of Lp(a), which is median (IQR). [b]To convert to mg/dL, multiply by 38.7. [c]To convert to mg/dL, multiply by 88.6. [d]To convert to mg/dL, divide by 2.5. [e]P<0.001 for evolocumab vs placebo. *ApoB, apolipoprotein B; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Lp(a), lipoprotein(a); LS, least squares.*

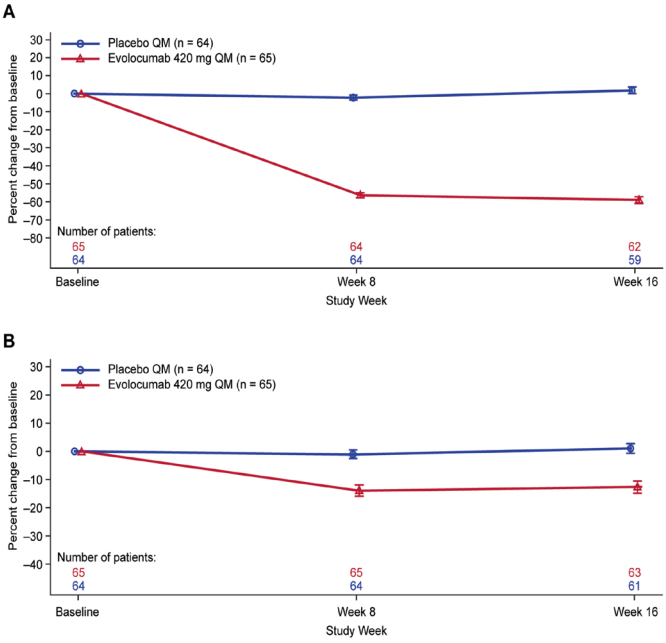


Figure 1. Mean change from baseline in (A) LDL-C and (B) Lp(a) over time
LDL-C, low-density lipoprotein cholesterol; Lp(a), lipoprotein(a); QM, monthly.

Arterial wall inflammation

Least squares (LS) mean (95% CI) percentage change from baseline in MDS TBR of the index vessel was -8.3% (-11.6%, -5.0%) in the evolocumab group and 5.3% (-8.6%, -2.0%) in the placebo group (treatment difference -3.00 [-7.40, 1.39] P=0.18; Figure 2). Among patients receiving evolocumab, percentage change from baseline in MDS TBR of the index vessel was similar regardless of baseline statin use (LS mean [95% CI] percent change -8.7% [-12.7%, -4.6%], statin; -7.7% [-12.9%, -2.5%], no statin). The treatment difference in MDS TBR of the index vessel was -5.62 (95% CI, -11.10, -0.14; P=0.045) in patients who received statins and 0.09 (95% CI, -6.90, 7.08; P=0.98) in those who did not; the interaction P value was 0.21. Also, no correlation was found between baseline Lp(a) and baseline MDS TBR (Pearson correlation coefficient [R]=-0.05; P=0.61) or between baseline LDL-C levels and baseline MDS TBR (R=-0.06; P=0.48). Absolute change in LDL-C or Lp(a) and change in MDS TBR were not correlated in patients receiving evolocumab (R=0.01; P=0.95, LDL-C and R=-0.16; P=0.21, Lp[a]). Exploratory endpoints of percent change from baseline in the mean of the maximum TBR in the MDS of the whole index vessel, active slices of the index vessel, and the non-index vessel at week 16 are shown in Tables S1 and S2.

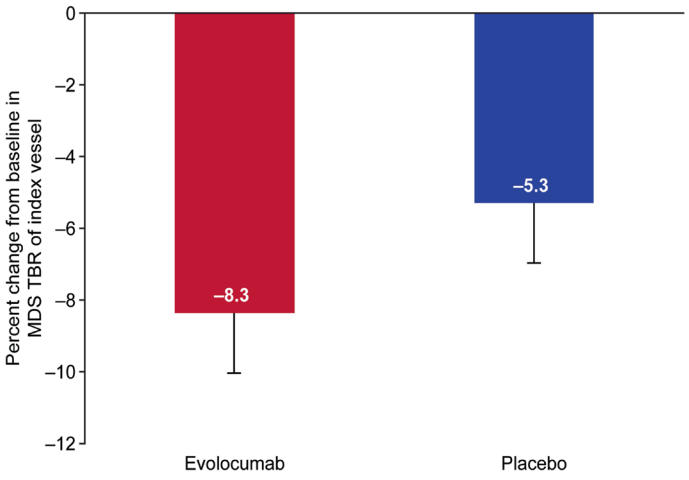
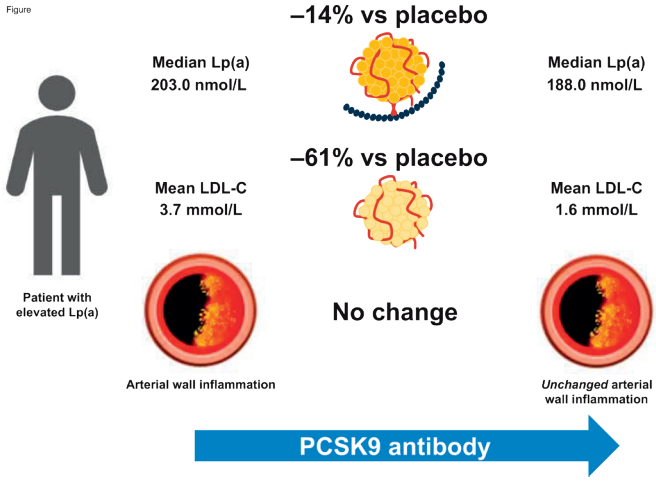


Figure 2. Least squares mean percentage change from baseline in MDS TBR of index vessel P=0.18 for evolocumab vs placebo)
Vertical bar indicates standard error of the mean. MDS TBR, most diseased segment target to background ratio.

Safety

Rates of total adverse events, serious adverse events, or adverse events leading to discontinuation of study drug were similar between the placebo and evolocumab groups (Supplement).



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DISCUSSION

This placebo-controlled study evaluated the effect of evolocumab on lipids and arterial wall inflammation in patients with elevated Lp(a) (median 200 nmol/L [80 mg/dL]). Sixteen weeks of evolocumab resulted in a 61% mean reduction in LDL-C and a 14% mean reduction in Lp(a) versus placebo. However, evolocumab did not significantly alter arterial wall inflammation, assessed as MDS TBR of the index vessel, in patients with elevated Lp(a). The current data imply that in patients with persistently elevated Lp(a) levels, 16 weeks of potent LDL-C reduction is unable to attenuate the pro-inflammatory state of the arterial wall.

Lp(a) lowering by PCSK9 antibody depends on baseline Lp(a) levels

The 61% LDL-C reduction with evolocumab resulted in a mean post-treatment LDL-C of 1.6 mmol/L (61.9 mg/dL), consistent with results reported in other patient groups[17-20]. Notably, this study found only a 14% Lp(a) reduction compared to placebo in patients with elevated Lp(a), instead 20%-30% as reported in previous studies in which the baseline Lp(a) values were much lower[6,18-20]. The pathways contributing to Lp(a) clearance are incompletely understood and may depend on the concentration of circulating Lp(a) and possibly other lipoproteins[21]. In vitro data and meta-analyses of human intervention trials suggest the involvement of the LDL-receptor (LDL-R) in mediating Lp(a) clearance, as Lp(a) was found to bind to the LDL-R and its reduction was closely associated with the degree of LDL-C reduction[6,21,22]. In support, fractional catabolic rate of apo(a) is increased during PCSK9 antibody treatment[23], lending further support to a role for LDL-R in Lp(a) reduction. Conversely, PCSK9 antibody treatment also has LDL-R-independent effects, as it can lower Lp(a) particle production by 36%[24]. The attenuated Lp(a) reduction in the present study may relate to the fact that this is the first study that exclusively included patients with elevated Lp(a), with median levels of 200 nmol/L [80 mg/dL] versus a median Lp(a) below 40 nmol/L (16 mg/dL) in previous studies[6,17,19,20]. Patients with elevated Lp(a) have Lp(a) that is characterized by smaller apo(a) isoforms[25]. The attenuated Lp(a) reduction could reflect less efficient clearance of smaller isoforms by the LDL-R. In support, a post-hoc analysis of FOURIER also reported a -16% Lp(a) change among patients in the upper baseline Lp(a) quartile (median Lp[a] >165 nmol/L [66 mg/dL])[7]. Similarly, LAPLACE demonstrated a -15% Lp(a) change following evolocumab in the highest baseline Lp(a) quartile[8].

No impact of evolocumab on arterial wall inflammation in patients with high Lp(a)

Previous studies substantiated that patients with Lp(a) elevation have marked pro-inflammatory changes in the arterial wall assessed using PET/CT[3], and arterial inflammation is a major risk factor for cardiovascular events[14,26]. Intervention studies targeting LDL-C have reported a reduction in arterial wall inflammation in patients at increased cardiovascular risk, following either statin therapy or lipoprotein apheresis[13, 27-29]. In the absence of available Lp(a)-lowering strategies, intensive LDL-C reduction in patients with elevated Lp(a) appeared promising, as previous studies suggested that Lp(a) confers risk predominantly in conjunction with elevated LDL-C levels[30,31]. In support, we previously substantiated that the risk associated with Lp(a) was attenuated at LDL-C levels lower than 2.5 mmol/L (96.8 mg/dL) in the primary prevention setting[32]. However, despite robust LDL-C reduction combined with a 14% Lp(a) reduction with evolocumab compared to placebo, MDS TBR of the index vessel did not change significantly compared to placebo in the present study. In comparison, previous LDL-C-lowering strategies reported a 2.1%-3.2% reduction in MDS TBR of the arterial wall for every 10% reduction in LDL-C in patients with CVD[13,29]. Several factors may have contributed to this discrepancy. First, we included patients with Lp(a) elevation[33]. A direct consequence is that a small Lp(a) reduction following evolocumab still leads to elevated post-treatment levels (median 188.0 nmol/L [75.2 mg/dL])[5,33]. In a prior study, Lp(a) levels in this range were associated with a pro-inflammatory effect on the arterial wall in untreated patients[3]. Second, the absence of an effect of modest Lp(a) reduction on MDS TBR fits with recent data obtained from Mendelian randomization studies[34]. estimating that an absolute Lp(a) reduction of 100 mg/dL (250 nmol/L) may be required to achieve a meaningful cardiovascular risk reduction. The need for substantial Lp(a) changes is further supported by the absence of clinical benefit by other compounds offering only modest Lp(a)-lowering potential[10, 11]. Third, our findings could relate to the absence of an anti-inflammatory effect specifically when targeting the PCSK9 pathway[35]. Since no prior study has addressed arterial wall inflammation assessed with PET/CT after PCSK9-antibody-induced LDL-C lowering, this is currently being investigated in a separate study evaluating the impact of PCSK9 inhibition on arterial wall inflammation in participants at increased CV-risk but normal Lp(a) levels (EU Clinical Trials register 2016-004794-41). However, since multiple other modes of LDL-C lowering associate with MDS TBR lowering[13, 28, 29] and PCSK9 inhibition was also found to attenuate cellular inflammation[36], it is less likely that the mode of LDL-C lowering is responsible. Prior studies as well as ours show that PCSK9 inhibitors do not reduce the inflammatory marker C-reactive protein (CRP). However, CRP is not a good biomarker for arterial wall inflammation, since CRP level is not correlated

with MDS-TBR in patients with cardiovascular risk factors[37]. Finally, a causal role of other inflammatory pathways, unresponsive to LDL-C lowering, may also contribute.

Clinical implications

In contrast to the anti-inflammatory effect of LDL-C lowering in previous studies, PCSK9 antibody treatment does not reduce arterial wall inflammation in patients with persistent Lp(a) elevation. In this environment, lowering other modifiable risk factors in patients with elevated Lp(a)[5] may be less likely to fully mitigate the increased cardiovascular risk. Highly potent Lp(a)-lowering antisense can be expected to reduce pro-inflammatory changes, as 80% Lp(a) reduction was found to reduce cellular inflammatory responses[4].

A strength of our trial is that it is a randomized, placebo-controlled study, and the largest lipid lowering–PET/CT trial focusing on arterial wall inflammation to date. A potential limitation of our study is that the 16-week timeframe may not be sufficient to observe a change in arterial wall inflammation. However, arterial wall inflammation measured by PET/CT is a dynamic functional parameter and 12 weeks of statin therapy or a single apheresis episode have been associated with significant reductions in arterial wall inflammation[13,29]. A second limitation is the absence of a correlation between baseline Lp(a) and MDS TBR. However, in the present study we deliberately excluded all patients with normal Lp(a) levels. This exclusion minimizes the chance of demonstrating a significant relation between (elevated) Lp(a) and MDS TBR. Finally, an additional limitation is that this study evaluated just one potential mechanism of the effect of Lp(a) on cardiovascular risk. The mechanism by which Lp(a) may mediate cardiovascular disease risk is multifactorial, comprising arterial wall inflammation,[3] pro-thrombogenic effects[38], and other pro-atherogenic effects[2]. Hence, absence of a significant anti-inflammatory effect does not indicate absence of a potential plaque stabilizing effect of the lipid reduction observed in this study.

CONCLUSION

Sixteen weeks of PCSK9 inhibition with evolocumab 420 mg led to large percent reductions in LDL-C and modest percent reductions in Lp(a) plasma levels in patients with median baseline Lp(a) of 200 nmol/L (80 mg/dL), resulting in persistent Lp(a) elevation during evolocumab therapy. This persistent elevation may have contributed to the observation that lipid lowering by evolocumab did not lead to a reduction in arterial wall inflammation. These data support further evaluation of novel therapies with a potent Lp(a) lowering effect on both arterial wall inflammation and, eventually, cardiovascular outcome.

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Conflict of interest
E.S.G.S. reports that his institution has received lecturing fees and advisory board fees from Amgen Inc., Regeneron, Sanofi, Akcea, Novartis and Athera. H.K., L.C., and S.M.W. are employees and stockholders of Amgen Inc. M.S.S. reports that his institution has received research grant support from Amgen Inc.; AstraZeneca; Daiichi-Sankyo; Eisai; GlaxoSmithKline; Intarcia; Janssen Research and Development; Medicines Company; MedImmune; Merck; Novartis; Pfizer; Poxel; Takeda (All >$10 000 per year); and he has consulted for Amgen; AstraZeneca, Bristol-Myers Squibb; CVS Caremark; Dyrnamix; Esperion; Intarcia; Janssen Research and Development; Medicines Company; MedImmune; Merck; Novartis (all ≤$10 000 per year except Amgen and Esperion). V.M. reports that his institution has received funding from Amgen Inc., Daiichi Sankyo, and Medlion Inc. Z.A.F. reports research funding from Amgen Inc. and Daiichi Sankyo. All other authors declared no conflict of interest.

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SUPPLEMENTARY MATERIAL

Persistent arterial wall inflammation in patients with elevated Lp(a) despite strong LDL-C reduction by PCSK9 antibody treatment

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Table of Contents

Participating Sites

Site Name	Address	Country
Clinique des Maladies Lipidiques de Quebec Incorporated	2600 boulevard Laurier,Suite 880 Quebec, QC G1V 4W2	Canada
SKDS Research Incorporated	679 Davis Drive,Suite 221 Newmarket, ON L3Y 5G8	Canada
Ecogene-21	930 Jacques Cartier Est,Bureau B-210, C.P.39 Chicoutimi, QC G7H 7K9	Canada
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Protenium Clinical Research	1725 Chadwick Court,Suite 200 Hurst, TX 76054	United States
Nuren Medical and Research Center	8260 West Flagler Street,Suite 2N Miami, FL 33144	United States
Einstein Healthcare Network	5501 Old York Road Philadelphia, PA 19141	United States
Crossroads Clinical Research Inc	444 Williamson Road,Suite B Mooresville, NC 28117	United States

Patient Eligibility

Inclusion criteria

- Patient has provided written informed consent prior to initiation of any study specific activities/procedures
- Male or female, ≥50 years of age at the time of informed consent
- Fasting Lp(a) ≥125 nmol/L (50 mg/dL)
- Fasting LDL-C ≥2.6mmol/L (100 mg/dL)
- For patients receiving lipid-lowering therapy (not required to participate in this study), lipid-lowering therapy, including statin dose, must be unchanged for ≥ 8 weeks prior to screening TBRmax >1.6 (either right carotid, left carotid or thoracic aorta) on FDG-PET/CT
- Exclusion criteria
- Currently receiving, or <4 weeks since receiving, treatment in another investigational device or drug study(ies), or participating in other investigational procedures
- Known diagnosis of diabetes mellitus or screening fasting serum glucose ≥7 mmol/L or glycated haemoglobin (HbA1c) ≥6.5%
- History of homozygous familial hypercholesterolemia
- Recent cardiovascular event (myocardial infarction, unstable angina, percutaneous coronary intervention [PCI], coronary artery bypass graft, or stroke) within 3 months prior to randomization, or planned cardiac surgery, PCI or carotid stenting, or planned major non-cardiac surgery during the course of the study period
- Currently undergoing lipid apheresis
- Known contraindications or limitations to FDG-PET/CT (eg, scanner weight limit, devices that can cause image artifacts, or carotid/aortic stents/grafts)
- Auto-immune disease/vasculitis, active inflammatory diseases, proven or suspected bacterial infections
- Recent (<1 month prior to screening) or ongoing serious infection requiring intravenous antibiotic therapy
- Recent (<6 weeks prior to screening) or current treatment with medications that may have a significant effect on plaque inflammation as measured by plaque TBR, including: oral, rectal, or injectable corticosteroids or immunosuppressive medications (eg, cyclosporine, methotrexate, tacrolimus, azathioprine, anti-thymocyte globulin, sirolimus, anti-tumour necrosis factor agents such as infliximab, anti-interleukin [IL] 6 therapy such as tocilizumab, or anti-IL1 therapy)

- Recent (<6 weeks prior to screening) or current treatment with aspirin (>325 mg/day) or nonsteroidal anti-inflammatory drugs (NSAIDs) (>1000mg/day)
- Known sensitivity to any of the active substances or excipients (eg, carboxymethylcellulose) to be administered during dosing
- Treatment with a cholesterol ester transfer protein inhibitor (eg, anacetrapib, dalcetrapib, evacetrapib) or mipomersen or lomitapide in the last 12 months prior to screening
- Known systemic disorders such as hepatic, renal, hematologic, and malignant diseases or any clinically significant medical condition that could interfere with the conduct of the study
- History of malignancy (except non-melanoma skin cancers, cervical in-situ carcinoma, breast ductal carcinoma in situ, or stage 1 prostate carcinoma) within the last 5 years-
- Patients likely to not be available to complete all protocol-required study visits or procedures, or unreliability as a study participant (eg, alcohol or other drug abuse in the past year or psychosis), to the best of the patient's and investigator's knowledge
- History or evidence of any other clinically significant disorder, condition or disease that, in the opinion of the investigator or sponsor physician, if consulted, would pose a risk to subject safety or interfere with the study evaluation, procedures, or completion
- Prior treatment with evolocumab or any other therapy to inhibit PCSK9
- Pregnant or breastfeeding or planning to become pregnant or breastfeed during treatment with study drug and for an additional 15 weeks after the last dose of study drug
- Female patient not willing to use an acceptable method(s) of effective birth control during treatment with study drug and for an additional 15 weeks after the end of treatment with study drug. Female patients who have had a hysterectomy, bilateral salpingectomy, bilateral oophorectomy, or who are postmenopausal, are not required to use contraception. Menopause is defined as 12 months of spontaneous and continuous amenorrhoea in a female ≥55 years old; or age <55 years but no spontaneous menses for at least 2 years; or age <55 years and spontaneous menses within the past 1 year, but currently amenorrhoeic (eg, spontaneous or secondary to hysterectomy), and with postmenopausal gonadotropin levels (luteinizing hormone and follicle stimulating hormone levels >40 IU/L) or postmenopausal oestradiol levels (<5 ng/dL) or according to the definition of "postmenopausal range" for the laboratory involved. Acceptable methods of effective birth control include: true sexual abstinence if this is in line with the preferred and usual lifestyle

of the subject (periodic abstinence [eg, calendar, ovulation, symptothermal, post-ovulation methods], declaration of abstinence for the duration of a trial, and withdrawal are not acceptable methods of contraception); surgical contraceptive methods (male partner who has had vasectomy with medical confirmation of surgical success or bilateral tubal ligation), use of hormonal birth control methods (oral, injectable, implantable transdermal, or intravaginal), intrauterine device, intrauterine hormone-releasing system, or 2 barrier methods (each partner must use 1 barrier method- males must use a condom with spermicide and females must choose either a diaphragm with spermicide, OR cervical cap with spermicide, OR contraceptive sponge with spermicide). Note: Additional medications given during treatment with evolocumab may alter the contraceptive requirements. These additional medications may require an increase in the number of contraceptive methods and/or length of time that contraception is to be utilized or length of time breastfeeding is to be avoided after the last dose of protocol-required therapies. The investigator is to discuss these contraceptive changes with the study subject.

Statistical Methods – Lp(a) Stratification Factor

Prior to unblinding, a protocol error was discovered regarding the units for stratification based on final screening Lp(a), where 175 mg/dL was used instead of the correct 175 nmol/L (70 mg/dL). As a result, the Lp(a) stratification factor was not included in the primary model, but the intended stratification (final screening Lp[a] <175 nmol/L and ≥175 nmol/L) was used as a covariate added to the primary analysis model in a sensitivity analysis.

Safety Summary

The patient incidences of adverse events of any severity were 75.4% in patients receiving evolocumab and 73.4% in patients receiving placebo. Two patients receiving evolocumab (3.1%) experienced serious adverse events that were deemed unrelated to study drug: one patient discontinued treatment after being diagnosed with cancer (tumour histology confirmed malignancy after randomization); one patient developed nephrolithiasis and continued study treatment. No serious adverse events occurred in the placebo arm.

SUPPLEMENTARY TABLES

Table S1. Multivariate regression analysis with percent change from baseline in the mean of the maximum TBR in the MDS of the whole index vessel and active slices of the index vessel at week 16.

	Evolocumab (n=65)	**Placebo (n=64)**
Mean (SD) of the maximum TBR in the MDS of the whole index vessel		
LS mean[a,b] (95% CI)	-4.0 (-6.6, -1.4)	-0.7 (-3.3, 1.9)
Treatment difference (evolocumab– placebo) (95% CI)	-3.3 (-6.7, 0.1)	
P-value	0.06	
Mean (SD) of the maximum TBR in the active slices of the index vessel		
LS mean[a,b] (95% CI)	-4.6 (-7.2, -1.9)	-1.2 (-3.9, 1.5)
Treatment difference (evolocumab– placebo) (95% CI)	-3.4 (-7.0, 0.1)	
P-value	0.06	

[a]LSmean is from a multivariate regression model with response variables percent change from baseline in mean of the maximum TBR in the whole or active slices of the index vessel at week 16, baseline mean of the maximum TBR in the whole or active slices of the index vessel, percent change from baseline in Lp(a) at week 8 and week 16, and baseline Lp(a).
[b]Percent change models include treatment group, baseline statin stratification factor, scheduled visit, and the interaction of treatment group with scheduled visit as covariates. Baseline models include the baseline statin stratification factor as a covariate.
Lp(a), lipoprotein(a); LS, least squares; MDS, most diseased segment; TBR, target to background ratio

Table S2. Percent change from baseline in the mean of the maximum TBR in the MDS of non-index vessels at week 16.

Percent Change from Baseline	**Left Common Carotid**		**Right Common Carotid**		**Ascending Thoracic Aorta**	
	Evolocumab (n=60)	**Placebo (n=61)**	**Evolocumab (n=52)**	**Placebo (n=54)**	**Evolocumab (n=14)**	**Placebo (n=9)**
Mean (SD), %	-4.3 (12.5)	-4.2 (15.1)	-4.0 (12.3)	-0.6 (13.2)	-5.5 (7.8)	-2.4 (7.0)

MDS, most diseased segment; TBR, target-to-background ratio

SUPPLEMENTARY FIGURES

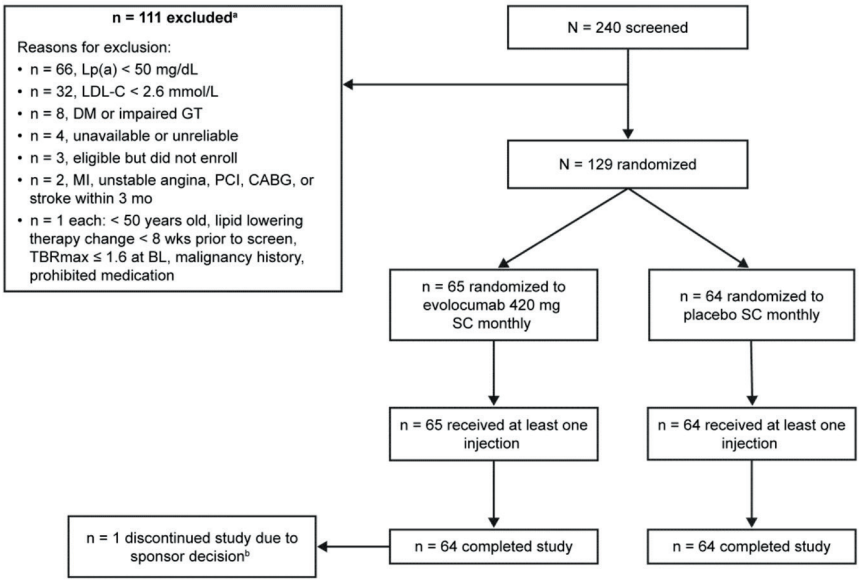

Figure S1. Patient disposition.

BL, baseline; CABG, coronary artery bypass graft; DM, diabetes mellitus; GT, glucose tolerance; LDL-C, low-density lipoprotein cholesterol; Lp(a), lipoprotein(a); MI, myocardial infarction; PCI, percutaneous coronary intervention; SC, subcutaneous; TBRmax, maximum target-to-background ratio.

[a]More than one reason for screen failure may have been documented for a patient.

[b]Patient had a malignancy that resulted in study drug discontinuation and withdrawal from the study; the reason for ending the study was documented as decision by sponsor.

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Potent lipoprotein(a) lowering following apolipoprotein(a) antisense treatment reduces the pro-inflammatory activation of circulating monocytes in patients with elevated lipoprotein(a)

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ABSTRACT

Introduction

Elevated lipoprotein(a) [Lp(a)] is strongly associated with an increased cardiovascular disease (CVD) risk. We previously reported that pro-inflammatory activation of circulating monocytes is a potential mechanism by which Lp(a) mediates CVD. Since potent Lp(a)-lowering therapies are emerging, it is of interest whether patients with elevated Lp(a) experience beneficial anti-inflammatory effects following large reductions in Lp(a).

Methods and results

Using transcriptome analysis, we show that circulating monocytes of healthy individuals with elevated Lp(a), as well as CVD patients with increased Lp(a) levels, both have a pro-inflammatory gene expression profile. The effect of Lp(a)-lowering on gene expression and function of monocytes was addressed in two local sub-studies, including fourteen CVD patients with elevated Lp(a) who received apo(a) antisense (AKCEA-APO(a)-L_{Rx}) (NCT03070782), as well as eighteen patients with elevated Lp(a) who received proprotein convertase subtilisin/kexin type 9 antibody (PCSK9ab) treatment (NCT02729025). AKCEA-APO(a)-L_{Rx} lowered Lp(a) by 47% and reduced the pro-inflammatory gene expression in monocytes of CVD patients with elevated Lp(a), which coincided with a functional reduction in transendothelial migration capacity of monocytes *ex vivo* (-17%, $p<0.001$). In contrast, PCSK9ab treatment lowered Lp(a) by 16% and did not alter transcriptome nor functional properties of monocytes, despite an additional reduction of 65% in low-density lipoprotein cholesterol (LDL-C).

Conclusion

Potent Lp(a)-lowering following AKCEA-APO(a)-L_{Rx}, but not modest Lp(a)-lowering combined with LDL-C reduction following PCSK9ab treatment, reduced the pro-inflammatory state of circulating monocytes in patients with elevated Lp(a). These *ex vivo* data support a beneficial effect of large Lp(a) reductions in patients with elevated Lp(a).

Key words

Lipoprotein(a), apo(a)-antisense, PCSK9ab, inflammation, monocytes, transcriptomics

INTRODUCTION

Lipoprotein(a) [Lp(a)] is a lipoprotein consisting of a low-density lipoprotein (LDL)-like particle, covalently bound to apolipoprotein(a) [apo(a)], and is associated with cardiovascular disease (CVD) risk[1]. Epidemiologic and Mendelian randomization studies suggest an independent and causal relationship between elevated Lp(a) levels (above 50 mg/dL or ~125 nmol/L) and CVD risk[2,3]. Previously we demonstrated that healthy subjects with elevated Lp(a) have an activated innate immune system, characterized by pro-inflammatory circulating monocytes. These findings coincided with an increased white blood cell influx in the arterial wall and a concomitant increase in arterial wall inflammation assessed by positron emission tomography/computed tomography (PET/CT)[4]. We identified oxidized phospholipids (OxPL), predominantly carried by Lp(a) in the plasma, as key intermediates in inducing the pro-inflammatory activation of monocytes[4]. However, the mechanisms underlying monocyte activation in individuals with elevated Lp(a), as well as reversibility of this inflammatory state remains to be established.

Recently we reported that a 14% reduction in Lp(a) following proprotein convertase subtilisin/kexin type 9 antibody (PCSK9ab) was not associated with a reduction of arterial wall inflammation on PET/CT in subjects with elevated Lp(a)[5]. The absence of an anti-inflammatory effect of modest Lp(a)-lowering concurs with negative results of modest Lp(a) reduction on CVD-risk in both randomized clinical trials[6,7] and Mendelian randomization studies[8]. In the advent of antisense strategies targeting apo(a), with the potential to reduce Lp(a) by more than 90%[9], it provides an opportunity to test whether a greater absolute reduction in Lp(a) exerts a favorable biological effect in patients with elevated Lp(a). We hypothesize that potent Lp(a)-lowering conveys an anti-inflammatory effect as opposed to moderate Lp(a)-reducing strategies.

To test this hypothesis, we assessed the gene expression profile of circulating monocytes of healthy individuals and CVD patients, both with elevated Lp(a). Reversibility of an Lp(a) effect on monocyte activation was evaluated by comparing monocyte gene expression and function before and after treatment with either potent Lp(a)-lowering [antisense (AKCEA-APO(a)-L_{Rx})] or moderate Lp(a)-lowering (PCSK9ab (evolocumab)) in two separate clinical intervention trials (NCT03070782 and NCT02729025 respectively).

METHODS AND MATERIALS

Study population and design

This mono-center study comprises four study groups in total: healthy individuals with normal Lp(a), healthy individuals with elevated Lp(a), and two groups of patients with elevated Lp(a) who participated in the AKCEA-APO(a)-L_{Rx} study (NCT03070782) and the ANITSCHKOW study (NCT02729025). An Lp(a) plasma level of ≥ 50mg/dL or 125nmol/L was defined as elevated. The healthy individuals with elevated Lp(a) were matched for age, sex, and BMI to healthy individuals with normal Lp(a). In- and exclusion criteria and study design of the AKCEA-APO(a)-L_{Rx} and the ANITSCHKOW study have been published previously[5,10]. In these intervention trials, blood sampling for the purpose of this monocyte study was performed at baseline and after 26 weeks of AKCEA-APO(a)-L_{Rx} or 16 weeks of PCSK9ab treatment, respectively. The protocol for the current sub study was approved by the ethics committee of the Amsterdam UMC (location Academic Medical Center) and was conducted according to the principles of the Declaration of Helsinki. All participants provided written informed consent prior to enrollment.

Biochemical measurements

Blood sampling was obtained in a fasting state (≥9h). The lipid profiles of the healthy individuals were measured in the local clinical laboratory. Plasma total cholesterol, high-density lipoprotein cholesterol (HDL-C), triglycerides, and apolipoprotein B (apoB) were measured with commercially available enzymatic assays. LDL-C was calculated using the Friedewald formula. Lipoprotein(a) was measured by an isoform-independent immunoturbidometric assay (QUANTIA Lp(a) 7K00-01, Abbott, Wiesbaden, Germany). In the AKCEA-APO(a)-L_{Rx} study, lipid profiles were measured with commercially available kits at Medpace Reference Laboratories (Medpace Reference Laboratories; Leuven, Belgium), and Lp(a) levels were measured by an isoform-independent assay (Northwest Lipid Metabolism and Diabetes Research Laboratories, University of Washington, USA). In the ANITSCHKOW study, lipid profiles were assessed at the Medpace core lab (Medpace Reference Laboratories; Leuven, Belgium), and Lp(a) levels were measured using an isoform-independent immunoturbidometric assay (Polymedco, Cortlandt Manor, NY, USA).

Ex vivo monocyte experiments

All the laboratory experiments and statistical analyses regarding the monocyte characterization are available in detail in the Supplementary material online.

Statistical analyses

All data were analyzed using GraphPad Prism 8 (La Jolla, California), SPSS version 25 (SPSS Inc., Chicago, Illinois), and R version 3.5.3 (R Core Team, Vienna, Austria). Data are presented as mean ± standard deviation (SD) for normally distributed data, median [InterQuartile Range] [IQR] for non-normally distributed data, or as a number (n) with percentage from total (%) for categorical variables. Since this study was not specifically designed to evaluate the effects of both AKCEA-APO(a)-L_{Rx} and PCSK9ab on biochemical measurements, p-values are not provided for the differences in lipid and inflammatory plasma markers, but are stated as mean (SD), or median [IQR] absolute and/or percent change from baseline at week 26 or 16, respectively.

RESULTS

Clinical characteristics

Thirteen healthy individuals with normal Lp(a) [median Lp(a) 7 mg/dL (18 nmol/L)] and twelve age, sex, and BMI matched healthy individuals with elevated Lp(a) [median Lp(a) 87mg/dL (218 nmol/L)] were included (Table 1, S1). In the phase-2b AKCEA-APO(a)-L_{Rx}-trial, informed consent was obtained in fourteen sequential patients who were randomized at the Amsterdam UMC site and were included in this site-specific sub-study (Table 1). According to study protocol, all patients had established CVD, in the vast majority based on coronary artery disease (86%). All patients received standard-of-care preventive therapy, including lipid-lowering therapy (86% statin therapy, 64% ezetimibe, and 14% PCSK9ab). Median Lp(a) at baseline was 82 mg/dL (205 nmol/L), and mean LDL-C 1.9 mmol/L. In the phase-3b ANITSCHKOW trial, informed consent was obtained in eighteen patients who were randomized at the Amsterdam UMC site to evolocumab 420mg once every four weeks (Q4W) and were included in this sub-study (Table 1). Four patients had established CVD based on coronary artery disease (3 subjects) and stroke (1 subject). Nine patients received lipid-lowering therapy in primary prevention setting, of which five patients had the diagnosis of familial hypercholesterolemia. 72% of the subjects used statins at baseline, and 22% ezetimibe. Median Lp(a) at baseline was 102 mg/dL (255 nmol/L), with a mean LDL-C of 3.3 mmol/L.

Table 1. Baseline characteristics in healthy control and patient cohorts

	Healthy individuals normal Lp(a) *(n=13)*	**Healthy individuals elevated Lp(a)** *(n=12)*	**AKCEA-APO(a)-L^{Rx} subjects elevated Lp(a)** *(n=14)*	**PCSK9ab subjects elevated Lp(a)** *(n=18)*
Age, years	44.4 (16.8)	44.3 (13.1)	53.0 (7.5)	60.6 (7.4)
Sex, n male (%)	6 (46)	6 (50)	12 (86)	9 (50)
BMI, kg/m^2	23.6 (2.8)	25.4 (3.3)	29.0 (5.4)	25.8 (3.3)
Smoking, n active (%)	0 (0)	0 (0)	1 (7)	2 (11)
SBP, mmHg	122 (16)	135 (15)	132 (15)	135 (16)
DBP, mmHg	79 (10)	83 (7)	82 (7)	82 (9)
CVD, n (%)	0 (0)	0 (0)	14 (100)	4 (22)
Coronary artery disease	0 (0)	0 (0)	12 (86)	3 (17)
Stroke	0 (0)	0 (0)	1 (7)	1 (6)
Peripheral artery disease	0 (0)	0 (0)	1 (7)	0 (0)
Medication use, n (%)	0 (0)	0 (0)	14 (100)	14 (78)
Antihypertensives	0 (0)	0 (0)	11 (79)	7 (39)
Antidiabetics	0 (0)	0 (0)	1 (7)	0 (0)
Statins	0 (0)	0 (0)	12 (86)	13 (72)
Ezetimibe	0 (0)	0 (0)	9 (64)	4 (22)
PCSK9-ab	0 (0)	0 (0)	2 (14)	0 (0)
Total cholesterol, mmol/L[a]	5.1 (0.9)	5.5 (0.8)	3.8 (0.7)	5.4 (0.9)
LDL-cholesterol, mmol/L[a]	3.0 (0.8)	3.4 (0.8)	1.9 (0.6)	3.3 (0.7)
HDL-cholesterol, mmol/L[a]	1.8 (0.4)	1.6 (0.4)	1.2 (0.3)	1.4 (0.4)
Triglycerides, mmol/L[b]	0.8 (0.3)	1.1 (0.5)	1.3 (0.5)	1.4 (0.3)
ApoB, g/L	0.9 (0.2)	1.0 (0.2)	0.8 (0.2)	1.0 (0.1)
Lipoprotein(a), mg/dL[c]	7 [3-17]	87 [79-114]	82 [62-121]	102 [64-121]
Leukocytes, 10^9/L	5.09 (1.41)	5.55 (0.86)	6.60 (2.19)	5.66 (1.63)

Neutrophils, 10^9/L	2.56 (1.25)	3.00 (0.44)	3.81 (1.44)	3.38 (1.20)
Lymphocytes, 10^9/L	1.92 (0.60)	1.91 (0.48)	1.96 (0.65)	1.66 (0.39)
Monocytes, 10^9/L	0.39 (0.13)	0.43 (0.12)	0.52 (0.187	0.40 (0.15)
hs-CRP, mg/L	0.5 [0.3-1.8]	1.0 [0.5-1.3]	0.5 [0.4-2.3]	0.9 [0.5-1.3]

Data are mean (SD), median [interquartile range], or n (%). *ApoB, apolipoprotein B; BMI, body mass index; CVD, cardiovascular disease; DBP, diastolic blood pressure; HDL, high-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; Lp(a), lipoprotein(a); PCSK9ab, proprotein convertase subtilisin/kexin type 9 antibody; SBP, systolic blood pressure.*

[a] To convert to mg/dL, multiply by 38.7, [b] To convert to mg/dL, multiply by 88.6, [c] To convert to nmol/L, multiply by 2.5

Effect of AKCEA-APO(a)-LRx and PCSK9ab treatment on lipid levels and inflammatory plasma markers

The fourteen CVD patients participating in the AKCEA-APO(a)-L_{Rx} trial were randomized to one of the five dose-regimens with the investigational product AKCEA-APO(a)-L_{Rx} (3 subjects (21%) in 20mg/Q4W group, 2 subjects (14%) in 40mg/Q4W group, 2 subjects (14%) in 20mg/Q2W group, 4 subjects (29%) in 60mg/Q4W group, and 3 subjects (21%) in 20mg/QW group). Compared to baseline, a pooled mean absolute reduction of 51 (53) mg/dL (or 128 (132) nmol/L) and a pooled mean percent reduction of 47 (18) % was achieved after a treatment duration of 26 weeks (Table 2). Minute reductions in apoB and LDL-C were seen (mean percent change of -6.7% (14.4), and -7.1% (20.4) respectively) after AKCEA-APO(a)-L_{Rx} treatment, as well as small absolute changes in CRP (-0.1 [-0.2 – 0.5] mg/L) and monocyte count (-0.02 (0.07) *10^9/L) (Table 2). These results were generally comparable with the main phase-2b trial[10]. The eighteen subjects participating in the ANITSCHKOW trial received monthly subcutaneous injections of evolocumab 420mg, reaching a mean absolute reduction in Lp(a) of 19 (20) mg/dL (or 48 (49) nmol/L) and a mean percent reduction of 16 (19) % (Table 2) after 16 weeks of treatment. LDL-C was reduced by 65 (14) %. Almost no effect on both CRP and monocyte count was seen (-0.2 [-1.3 – 0.5] mg/L, and -0.00 (0.15) *10^9L respectively).

Table 2. Effect of AKCEA-APO(a)-L_{Rx} and PCSK9ab treatment on lipid levels and inflammatory plasma markers

	AKCEA-APO(a)-L_{Rx}	**PCSK9ab**
Lipoprotein(a)		
Mean absolute change Lp(a), (SD), mg/dL[a]	-50.6 (52.6)	-18.9 (19.5)
Mean percent change Lp(a), (SD), %	-46.6 (18.3)	-16.1 (18.7)
Median post-treatment Lp(a), [IQR], mg/dL[a]	35.0 [26.1 – 84.6]	83.3 [59.1 – 105.4]
Change in other lipid levels[b]		
Total cholesterol	-2.2 (13.4)	-40.5 (8.9)
LDL-cholesterol	-7.1 (20.4)	-64.5 (14.4)
HDL-cholesterol	8.8 (13.8)	11.2 (10.6)
Triglycerides	-9.3 (-14.4)	-29.7 (14.1)
ApoB	-6.7 (14.4)	-53.1 (9.8)
Change in inflammatory markers[c]		
hs-CRP, mg/L	-0.1 [-0.2 – 0.5]	-0.2 [-1.3 – 0.5]
Leukocytes, *10^9/L	-0.19 (1.35)	-0.17 (1.05)
Monocytes, *10^9/L	-0.02 (0.07)	-0.00 (0.15)

ApoB, apolipoprotein B; HDL, high-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; Lp(a), lipoprotein(a); PCSK9ab, proprotein convertase subtilisin/kexin type 9 antibody

[a] To convert to nmol/L, multiply by 2.5
[b] Change in other lipid levels is defined as mean percent change (SD) from baseline
[c] Change in inflammatory markers is defined as median [IQR] or mean absolute change (SD) from baseline for non-normally and normally distributed data, respectively

Inflammatory gene expression profile in monocytes of healthy individuals with elevated Lp(a)

To get more insight into the molecular pathways underlying monocyte activation in subjects with elevated Lp(a), we first compared the gene expression profile of circulating monocytes from healthy individuals with normal Lp(a) and healthy individuals with elevated Lp(a). Transcriptome analysis revealed 95 significantly differentially expressed genes (DEG), of which 43 genes were upregulated and 52 genes were downregulated in the healthy individuals with elevated Lp(a) (Figure 1A). Canonical pathway and Hallmark (CP&H) pathway enrichment analysis showed a significant increase in several pathways related to the innate immune response (Figure 1B). The interferon-alpha (IFNα) and interferon-gamma (IFNγ) pathways were the most pronounced amongst the significantly upregulated pathways.

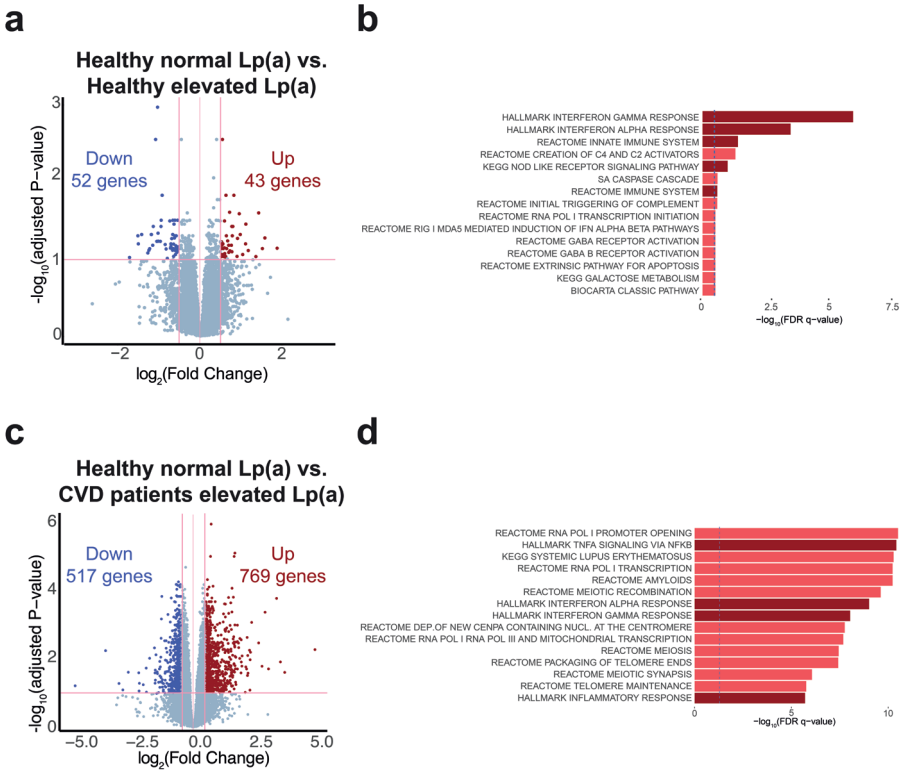


Figure 1. Inflammatory gene expression in circulating monocytes of healthy individuals with elevated Lp(a) and CVD patients with elevated Lp(a)
(A) Volcano plot showing the difference in gene expression between healthy individuals with normal Lp(a) versus healthy individuals with elevated Lp(a). (B) Canonical and Hallmark pathway analysis of top 1000 upregulated genes. Dark red bars indicate inflammatory pathways. Blue dotted line at p = 0.05. (C) Volcano plot showing the difference in gene expression between healthy individuals with normal Lp(a) versus CVD patients with elevated Lp(a). (D) Canonical and Hallmark pathway analysis of top 1000 upregulated genes. Dark red bars indicate inflammatory pathways. Blue dotted line at p = 0.05.

Monocytes of CVD patients with elevated Lp(a) show a robust pro-inflammatory transcriptome profile

Next, we compared the gene expression profile of monocytes of CVD patients with elevated Lp(a) and healthy individuals with normal Lp(a). Whereas healthy individuals with elevated Lp(a) show a modest number of DEG (95), CVD patients with Lp(a) elevation display a larger number of DEG (1286), of which 769 genes are significantly upregulated (Figure 1C). CP&H pathway enrichment analysis revealed a pronounced upregulation of multiple immune response related pathways including the TNFA signaling pathway and, similar to the transcriptome profile of healthy individuals

with Lp(a) elevation, IFNα and IFNγ response pathways (Figure 1D). Collectively, this data implies that elevated Lp(a) contributes to a pro-inflammatory gene expression signature in monocytes of both healthy individuals and CVD patients.

Potent Lp(a)-lowering by AKCEA-APO(a)-L_{Rx} leads to downregulation of inflammatory gene expression in monocytes of CVD patients with elevated Lp(a)

Subsequently, we investigated the effect of potent Lp(a)-lowering by AKCEA-APO(a)-L_{Rx} on the gene expression profile in monocytes of CVD patients with elevated Lp(a) in a paired fashion. Compared to baseline, transcriptome analysis showed 575 significantly DEG, of which 359 genes were upregulated, and 216 genes were downregulated following AKCEA-APO(a)-L_{Rx} treatment (Figure 2A). CP&H pathway enrichment analysis demonstrated a distinct reduction of multiple pathways regulating the immune response in monocytes, including IFNα, IFNγ, and Toll-like receptor (TLR) pathways (Figure 2B). In line with these findings, gene set enrichment analysis (GSEA) showed significant enrichment of genes involved in IFNα/β (NES = 1.7, FDR 0.03), IFNγ (NES = 1.6, FDR = 0.03), and the TLR signaling pathway (NES = 1.5, FDR = 0.01) in CVD patients with elevated Lp(a) compared to healthy individuals with normal Lp(a) (Figure 2C), which were reduced after AKCEA-APO(a)-L_{Rx} treatment (IFNα/β: NES = -1.6, FDR = 0.04; IFNγ: NES = -1.4, FDR = 0.1; TLR: NES = -1.3, FDR = 0.07) (Figure 2C). Several genes of the Interferon Regulatory Factor (IRF) family, including *IRF1*, *IFITM2*, and *GBP2* are amongst the top 15 most significantly upregulated genes in the IFN α/β and γ signaling pathways in CVD patients, which were downregulated after AKCEA-APO(a)-L_{Rx} treatment (Figure 2D, S1A). *JUN*, *MYD88, TLR2, TLR4, and TLR8* were amongst the top 15 most upregulated genes in the TLR pathway in CVD patients before AKCEA-APO(a)-L_{Rx}, and were downregulated after AKCEA-APO(a)-L_{Rx} treatment (Figure 2D, S1B). In contrast, modest Lp(a)-lowering following PCSK9ab did not alter gene expression in monocytes of subjects with elevated Lp(a), despite a concomitant robust LDL-C reduction (Figure 2E).

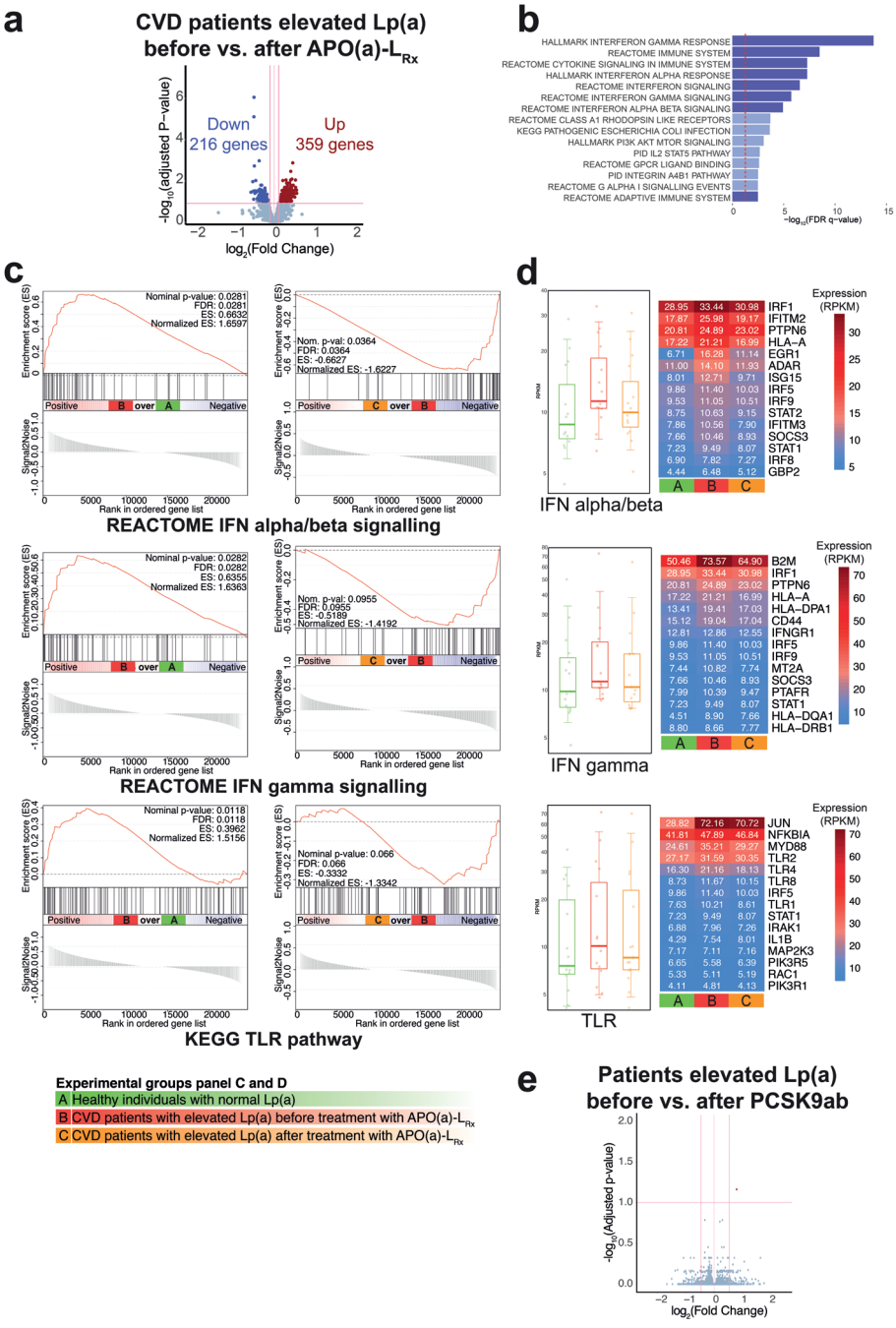


Figure 2. Potent, but not modest, Lp(a)-lowering reduces inflammatory gene expression in circulating monocytes

(A) Volcano plot showing the difference in gene expression between CVD patients before AKCEA-APO(a)-L_{Rx} treatment versus CVD patients after AKCEA-APO(a)-L_{Rx} treatment. (B) Canonical and Hallmark pathway analysis of top 1000 downregulated genes. Dark blue bars indicate inflammatory pathways. Red dotted line at p = 0.05. (C) GSEA enrichment plots of interferon alpha/beta (up), interferon gamma (middle), and TLR pathway (bottom). Left: CVD patients before AKCEA-APO(a)-L_{Rx} (red) versus healthy individuals with normal Lp(a) (green). Right: CVD patients before AKCEA-APO(a)-L_{Rx} (red) versus after AKCEA-APO(a)-L_{Rx} (orange). (D) Boxplot and heatmap showing average expression of the top 15 most expressed genes in CVD patients before AKCEA-APO(a)-L_{Rx} versus healthy individuals with normal Lp(a) for the interferon alpha/beta signaling, gamma signaling, and TLR pathways. Column colors: green, healthy individuals with normal Lp(a), red, CVD patients before AKCEA-APO(a)-L_{Rx} treatment, orange: CVD patients after AKCEA-APO(a)-L_{Rx} treatment. (E) Volcano plot showing the difference in gene expression between patients before PCSK9ab treatment versus patients after PCSK9ab treatment.

Downregulation of inflammatory gene expression following potent Lp(a)-lowering coincides with reduced inflammatory monocyte function

To examine whether the relatively modest effect sizes in pro-inflammatory gene expression after potent Lp(a)-lowering resulted in dampening of pro-inflammatory monocyte function, we performed flow cytometry experiments to functionally assess the expression of chemokine and Toll-like receptors on the monocyte cell surface. In line with the downregulation of their respective genes, the expression of C-C chemokine receptor type 2 (CCR2), CX3C chemokine receptor 1 (CX3CR1), and TLR2 was significantly reduced after Lp(a)-lowering (Figure 3A, S1C, S2). Furthermore, we performed a standardized transendothelial migration (TEM) assay to assess the migratory capacity of monocytes through a layer of arterial endothelial cells *ex vivo*. In accordance with the reduction in inflammatory signature in the transcriptome and flow cytometry data, the trans-endothelial migration activity of monocytes was significantly reduced by 22% ($p<0.0001$) after potent Lp(a)-lowering (Figure 3B). The percent reduction in TEM was positively correlated with the absolute reduction in Lp(a) ($r = 0.38$, $p = 0.02$) (Figure 3C). A trend towards a correlation was observed between percent reduction in TEM and absolute reduction in OxPL-apoB ($r = 0.26$, $p = 0.07$)(Figure S3). We performed the same flow cytometry and TEM experiments in the ANITSCHKOW subjects, and both experiments did not show significant differences before versus after PCSK9ab treatment (Figure 3D-E).

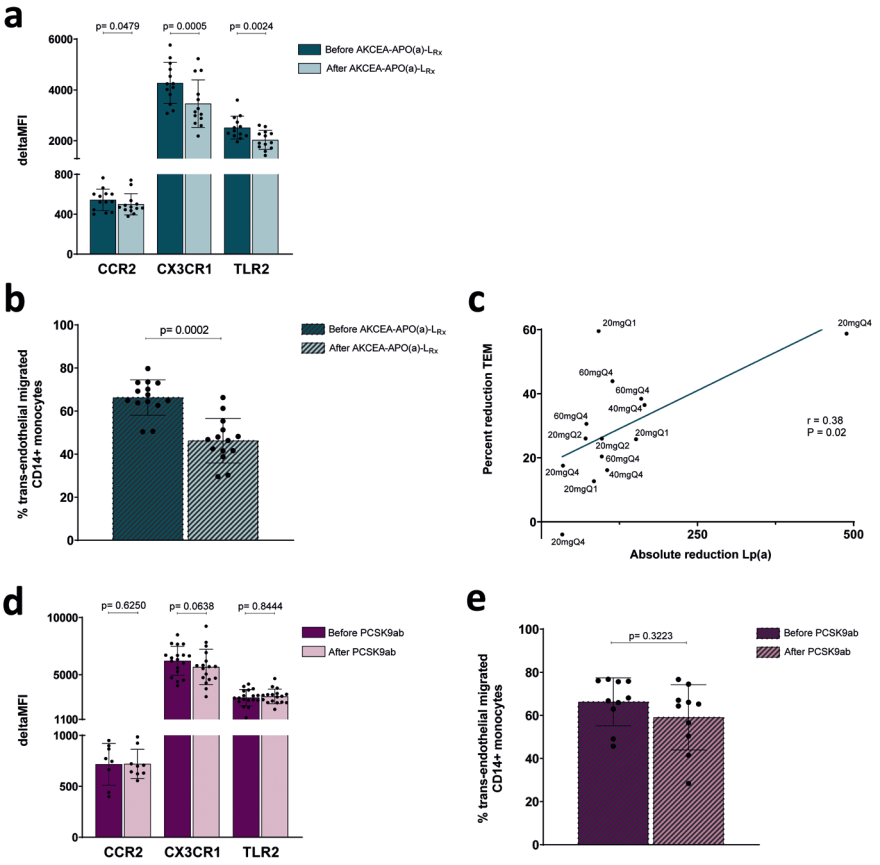
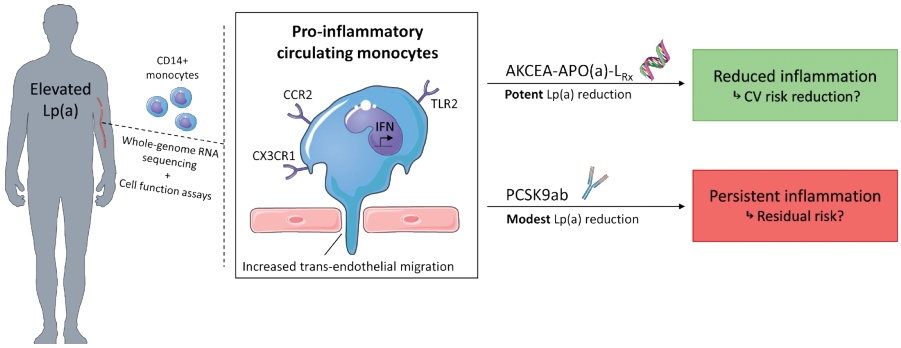


Figure 3. Potent, but not modest, Lp(a)-lowering reduces inflammatory receptor expression, with a concomitant functional improvement

(A) Flow cytometry results of inflammatory markers on circulating monocytes before and after AKCEA-APO(a)-L_{Rx} treatment, expressed as delta Median Fluorescence Intensity (MFI). Data are represented as mean ± SD. (B) Percentage trans-endothelial migrated CD14+ monocytes before and after AKCEA-APO(a)-L_{Rx} treatment. Data are represented as mean ± SD. (C) Correlation between absolute reduction in Lp(a) and percent reduction in trans-endothelial migration. (D) Flow cytometry results of inflammatory markers on circulating monocytes before and after PCSK9ab treatment, expressed as delta MFI. Data are represented as mean ± SD. (E) Percentage trans-endothelial migrated CD14+ monocytes before and after PCSK9ab treatment. Data are represented as mean ± SD, and were analyzed by Wilcoxon signed rank test. (P-values < 0.05 were considered statistically significant)



Take home figure
Unbiased whole-genome RNA sequencing and functional analyses of circulating monocytes of individuals with elevated Lp(a) show a strong pro-inflammatory and pro-migratory profile. Only potent Lp(a) reduction demonstrated a profound reduction in the reported pro-inflammatory and pro-migratory profile, as opposed to modest Lp(a) reduction, indicating the promising potential of potent Lp(a)-lowering strategies in reducing cardiovascular risk.

DISCUSSION

Using transcriptome analysis, we show that elevated Lp(a) promotes a distinct pro-inflammatory gene expression profile in circulating monocytes, which is more pronounced in patients with established CVD compared to healthy individuals. We also show that potent Lp(a)-lowering by the apo(a) antisense AKCEA-APO(a)-L_{Rx} markedly attenuates the inflammatory changes on transcriptional as well as functional level in monocytes of CVD patients with elevated Lp(a). In contrast, modest Lp(a)-lowering combined with robust LDL-C lowering following PCSK9ab had no effect on the gene expression profile nor on function of monocytes. Collectively, this data lends further support to Lp(a)-mediated pro-inflammatory effects on the innate immune system, which is reversible only following large reductions in Lp(a) (Take home figure).

As pointed out, we observed a marked, reversible pro-inflammatory Lp(a) signature in circulating monocytes, but we made several other key observations as well. First, we found a more pronounced inflammatory effect in CVD patients, which likely pertains to the fact that CVD patients are generally hallmarked by multiple CV risk factors, potentiating the pro-inflammatory gene expression signature[11]. Equally important is the observation that these CVD patients with elevated Lp(a) are still characterized by an inflammatory profile of monocytes, despite the use

of medication comprising statins and platelet inhibition, both with documented anti-inflammatory effects[12,13]. Yet, potent and highly selective Lp(a)-lowering by AKCEA-APO(a)-L_{Rx} reduced the augmented inflammatory gene expression in circulating monocytes of CVD patients with elevated Lp(a). This underscores a strong contributory inflammatory effect mediated by Lp(a), that seems to be independent from other risk factors[14]. Although the effect size on inflammatory gene expression was modest after Lp(a) lowering, it was associated with a marked reduction in transendothelial migration, a functional property of circulating monocytes regulated by inflammatory stimuli. Hence, prolonged and more potent absolute reductions of Lp(a) using optimally dosed apo(a)-antisense is expected to be associated with a more robust anti-inflammatory effect. A potent effect of Lp(a) on monocyte activation is substantiated by the absence of any change in monocyte gene expression or monocyte migratory capacity following PCSK9ab in patients with Lp(a) elevation. The latter concurs with our previous observation that PCSK9ab did not attenuate arterial wall inflammation in subjects with elevated Lp(a), in whom post-treatment Lp(a) levels were still elevated[5]. Taken together, these results support a causal role of Lp(a) in circulating monocyte activation *in vivo*.

The mechanisms by which Lp(a) elicits inflammation have remained incompletely understood. The discordance of a distinct anti-inflammatory effect of AKCEA-APO(a)-L_{Rx}, and the lack of this effect following PCSK9ab treatment, suggests that Lp(a) and LDL-C mediate monocyte activation via independent pathways. We previously reported that intracellular accumulation of LDL-C in circulating monocytes of patients with severe hypercholesterolemia is associated with monocyte activation[15]. Accordingly, LDL-C lowering following PCSK9ab reversed the observed pro-inflammatory activation of circulating monocytes in these patients, which coincided with less intracellular lipid accumulation. Lp(a) on the other hand, is on an equimolar basis more atherogenic than LDL-C, which is attributed to its additional apo(a)-tail and OxPL content[16]. Lp(a)-carried OxPL, has been identified as a critical signaling source for pro-inflammatory monocyte activation in subjects with elevated Lp(a)[4]. OxPL are a signaling source for danger associated molecular pattern (DAMP)-mediated monocyte activation via TLR recognition[17,18], which is in line with our observation that genes involved in the TLR pathway are upregulated at baseline in CVD patients with elevated Lp(a), and are downregulated after Lp(a) lowering by AKCEA-APO(a)-L_{Rx}. Also the observed trend in a positive correlation between reduction in transendothelial migration and reduction in OxPL-apoB levels suggests a role of OxPL in monocyte activation. Another intriguing finding in this study is the distinct upregulation of the IFNα/β and IFNγ pathways in both healthy subjects with elevated Lp(a) as well as CVD patients with elevated Lp(a). Since IFNγ

in vivo is predominantly produced by natural killer cells and T-lymphocytes rather than monocytes themselves[19], this finding could imply that other immune cells such as lymphocytes may act as intermediates in Lp(a)-induced monocyte activation in humans.

Clinical implications

The advent of potent Lp(a)-lowering strategies has ignited the debate whether Lp(a)-lowering strategies are capable of lowering CVD risk. In contrast to the linear relationship between LDL-C reduction and CV-benefit, the mandatory Lp(a) changes potentially mediating CVD-risk reduction remain to be established. Mendelian randomization studies[8] suggest that absolute reductions as high as 100 mg/dL may be required in order to achieve clinically relevant CV-risk reductions. In support, randomized-controlled trials with moderate Lp(a)-lowering compounds (percent reduction ranging from 20 to 25 % following nicotinic acid derivates and CETP-inhibitors, respectively[6,7,20]), failed to convey CV-benefit that can be attributed to Lp(a) reduction. In this *ex vivo* study, we showed a marked anti-inflammatory effect on circulating monocytes only after potent Lp(a)-lowering strategies in patients, in absence of any change in inflammatory profile following moderate Lp(a)-lowering. In accordance with the cumulating data supporting a strong and independent effect of inflammatory activation on CVD risk[21,22], our data support the benefit of potent Lp(a)-lowering in CVD prevention strategies. Moreover, the absence of an anti-inflammatory effect following potent LDL-C lowering implies that solely targeting other CV-risk factors than Lp(a) is unlikely to fully attenuate the increased CVD risk in patients with Lp(a) elevation. These findings require validation in the planned CV outcomes study for AKCEA-APO(a)-L_{Rx} (NCT04023552).

Study limitations

Several limitations merit closer attention. First, this study includes the results of two separate intervention studies, and therefore direct comparison of the transcriptomic data between all groups was not feasible. However, since the included studies were intervention trials, patients served as their own control for the transcriptome analysis. Secondly, we only focused on the inflammatory changes in transcriptome profile and monocyte phenotype and function. However, multiple pathways have been suggested to contribute to Lp(a)'s atherogenicity, also comprising pro-coagulant and pro-thrombotic effects, which we did not address in the current study. Thirdly, in the AKCEA-APO(a)-L_{Rx} trial patients were treated for 26 weeks, whereas in the ANITSCHKOW trial subjects were treated for 16 weeks. This may have contributed to an underestimation of the effect of PCSK9ab treatment. However,

no trend towards reduction of the inflammatory activity of circulating monocytes following PCSK9ab treatment was observed, making this an unlikely confounder.

CONCLUSION

This study supports the hypothesis that Lp(a) contributes to the pro-inflammatory activation of circulating monocytes in both healthy individuals and CVD patients, which is reversible only by large absolute reductions in Lp(a). Collectively, these *ex vivo* findings provide indirect translational evidence that treatment with AKCEA-APO(a)-L_{Rx} could lead to CV-benefit in CVD patients with elevated Lp(a).

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Conflict of interest
L.C.A.S., K.H.M.P., R.M.H., S.L.V., J.G.S., K.E.D., A.J.C., and M.B. have nothing to disclose. J.K. received a postdoctoral grant from Amsterdam Cardiovascular Sciences and a VENI grant from ZonMW (91619098). S.T. is a co-inventor and receives royalties from patents owned by UCSD on oxidation-specific antibodies and of biomarkers related to oxidized lipoproteins, has a dual appointment at UCSD and Ionis Pharmaceuticals, is a co-founder of Oxitope, Inc. and Kleanthi LLC and is a consultant to Boston Heart Diagnostics. E.S.G.S. reports that his institution has received lecturing fees and advisory board fees from Amgen Inc., Regeneron, Sanofi, Akcea, Mylan and Esperion, and a grant from Athera. M.P.J.W. is supported by the Netherlands Heart Foundation (CVON 2011/B019, CVON 2017-20); Spark-Holding BV (2015B002); the European Union (ITN-grant EPIMAC), and Foundation Leducq (LEAN-Transatlantic Network Grant).

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SUPPLEMENTARY MATERIAL

Supplementary methods and materials

Flow cytometry

For flow cytometry analysis, whole-blood (<2 hours after blood withdrawal) was used. After red blood cell lysis (eBioscience™ 1X RBC Lysis Buffer) and washing, cells were incubated with fluorescently labelled antibodies (see below). Samples were measured using FACS Canto II™ (BD Biosciences, San Jose, CA, USA), and were analyzed using FlowJo software version 10. Delta median fluorescence intensity (MFI) was obtained for each patient by subtracting median MFI of the isotype control from the median MFI of the corresponding fluorescent label. The following antibodies were used: PerCP-Cy™5.5 Mouse Anti-Human HLA-DR, Clone G46-6 (BD Biosciences), APC-H7 Mouse Anti-Human CD16, Clone 3G8 (BD Biosciences), PE-Cy™7 Mouse Anti-Human CD14, Clone M5E2 (BD Biosciences), FITC anti-human CD282 (TLR2) Clone T2.5 (Biolegend), Alexa Fluor® 647 Mouse anti-Human CD192 (CCR2) (BD Biosciences), FITC Rat Anti-Human CX3CR1 Clone 2A9 (BD Bioscience).

CD14+ monocyte isolation

The mononuclear fraction was isolated from whole-blood using Lymphoprep™ Density (d=1.077g/mL) gradient medium (STEMCELL technologies, Germany). After collecting the interphase layer and washing of the cells, monocytes were isolated using anti human CD14 magnetic beads and MACS® cell separation columns according to the manufacturer's instructions (Miltenyi Biotec, Leiden, The Netherlands). Isolated CD14+ monocytes were used for transendothelial migration assays and RNA analysis.

Transendothelial migration assay

Human Arterial Endothelial Cells (HAECs; Lonza, Walkersville, MD, USA) were cultured in EGM-2 medium (CC-3162; Lonza) and grown to confluence in fibronectin-coated (Sanquin Research, Amsterdam, the Netherlands) tissue-culture treated 6-wells plates (Corning, NY, USA) and stimulated overnight (18 hours) with tumor necrosis factor-α (TNFα; 10 ng/mL, Peprotech, Rocky Hill, NJ, USA). Next, the TNFα-containing medium was removed and exchanged for EGM-2. CD14+ monocytes (1*106 cells/mL) were added to the monolayer of HAECs, and subsequently incubated in a humidified atmosphere of 95% air and 5% CO2 at 37°C for 30 minutes. Cells were fixed in 3.7% formaldehyde (Merck), washed with phosphate-buffered saline and at least three images per condition were obtained using a Leica DM8i inverted fluorescent microscope using phase contrast imaging and an N Plan Achromat 10x objective. Images were analyzed using Image-J software (National Institutes of Health, USA).

RNA isolation and sequencing

For RNA analysis, CD14+ monocytes (1*106 cells) were lysed in TriPure Isolation Reagent (Roche) and stored in -80°C until further processing. RNA purification was performed according to the manufacturer's instructions. RNA quality was measured using Bioanalyzer software. RNA-seq libraries were generated from total RNA using the "NEBNext Ultra Directional RNA Library Prep Kit for Illumina" (NEB #E7420). Briefly, rRNA was depleted from total RNA using the rRNA depletion kit (NEB# E6310). After fragmentation of the depleted RNA, cDNA synthesis was performed. Sequencing adapters were ligated to the cDNA, and the libraries were amplified by PCR. Clustering and DNA sequencing on the Illumina cBot and HiSeq 4000 was performed according to manufacturer's protocols on 151-cycle paired-end flow cell lanes.

For the monocyte transcriptome data, base calling and demultiplexing of samples was performed with the Illumina data analysis pipeline RTA (v2.7.7) and Bcl2fastq (v2.20). Reads were aligned to the human genome version hg38 with STAR (v2.5.2b)[23] (RNA-seq) or HISAT2 (v2.1.0)[24]. Mapped reads were filtered on MAPQ >= 30. Reads were counted in exons and aggregated per gene using HOMER's (v4.9.1)[25] analyzeRepeats.pl script with the following parameters: -count exons -raw. RPKM values were obtained using the same script with the -rpkm flag instead of -raw. Differential expression of genes was assessed with DESeq2 (v1.22.2)[26] in an R (v3.5.3) environment. Briefly, genes were condensed to the highest expressed isoform and filtered to include only genes with median RPKM > 1 in at least one experimental group, after which differential expression was called. Results for the relevant contrasts were extracted and visualized using ggplot2 (H. Wickham. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York, 2016.) and pheatmap (Raivo Kolde, 2019): Pretty Heatmaps. R package version 1.0.12. https://CRAN.R-project.org/package=pheatmap). Pathway and gene ontology analysis was performed using EGSEA (v1.10.1)[27]. R code is available upon request.

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SUPPLEMENTARY TABLES

Table S1. Statistical comparison of baseline characteristics of healthy individuals with normal Lp(a) versus healthy individuals with elevated Lp(a)

	P-value Healthy individuals normal Lp(a) vs elevated Lp(a)
Age, years	0.993
Sex, n male (%)	0.841
BMI, kg/m^2	0.149
Smoking, n active (%)	1.000
SBP, mmHg	0.049
DBP, mmHg	0.304
Total cholesterol, mmol/L	0.321
LDL-cholesterol, mmol/L	0.169
HDL-cholesterol, mmol/L	0.094
Triglycerides, mmol/L	0.102
ApoB, g/L	0.306
Lipoprotein(a), mg/dL	<0.001
Leukocytes, 10^9/L	0.341
Neutrophils, 10^9/L	0.261
Lymphocytes, 10^9/L	0.944
Monocytes, 10^9/L	0.525
hs-CRP, mg/L	0.320

ApoB, apolipoprotein B; BMI, body mass index; DBP, diastolic blood pressure; HDL, high-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; Lp(a), lipoprotein(a); SBP, systolic blood pressure

SUPPLEMENTARY FIGURES

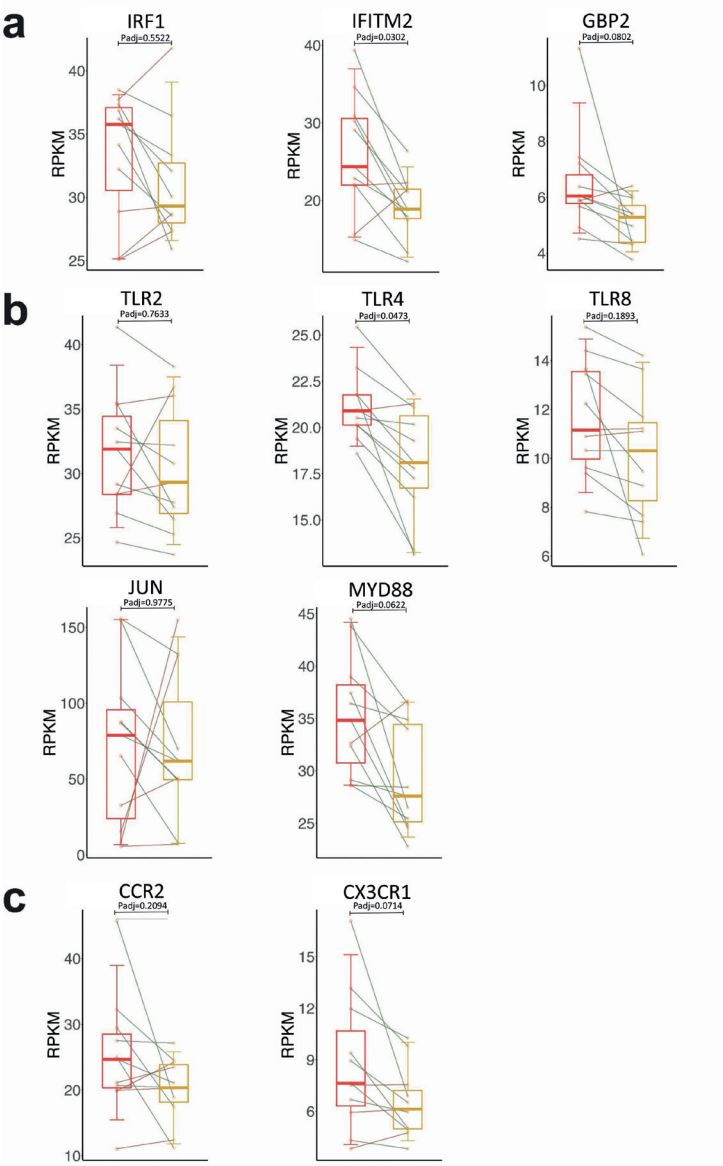

Figure S1. Effect of AKCEA-APO(a)-LRx on the expression of individual genes
Boxplots of gene expression (normalized read count) of IRF1, IFITM2, and GBP2 (A); TLR2, TLR4, TLR8, JUN, and MYD88 (B); CCR2 and CX3CR1 (C). Before (red box) and after (yellow box) AKCEA-APO(a)-LRx treatment. Each dot represents an individual patient; the line between the dots indicates paired measurements for one patient. (Padj values < 0.1 were considered statistically significant)

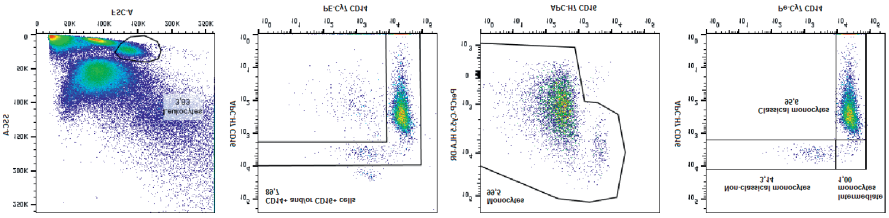

Figure S2. Gating strategy to define circulating monocyte populations
Circulating monocytes were gated as follows; the monocyte-containing leukocyte population was gated on the forward side scatter, and subsequently plotted in a CD14/CD16 plot. After selecting CD14+ and/or CD16+ cells, and excluding HLA-DR-CD16+ cells in a CD16/HLA-DR plot, classical (CD14++CD16-), intermediate (CD14++CD16+), and non-classical (CD14+CD16+) monocytes could be identified.

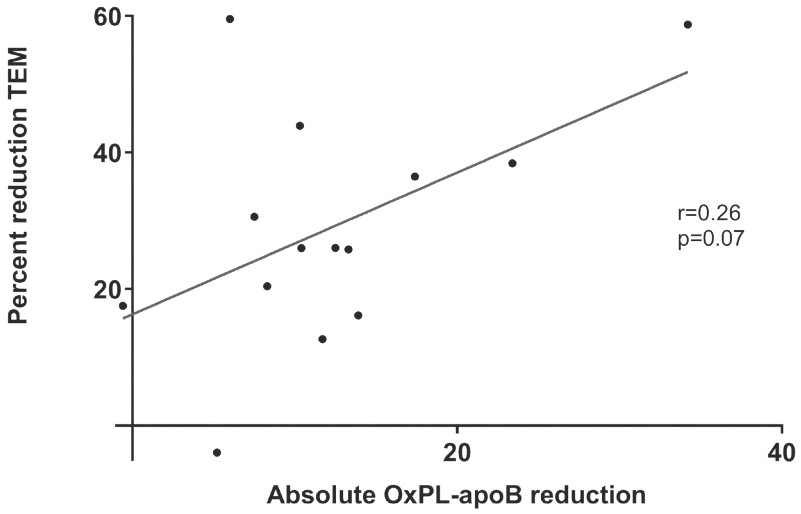

Figure S3. Correlation between absolute reduction in OxPL-apoB and percent reduction in trans-endothelial migration
Scatter plot representing the Pearson's correlation between absolute reduction in OxPL-apoB after 26 weeks of AKCEA-APO(a)-LRx treatment compared to baseline and percent reduction in TEM after 26 weeks of AKCEA-APO(a)-LRx treatment compared to baseline. ApoB, apolipoprotein B; TEM, transendothelial migration; OxPL, oxidized phospholipids

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Summary

The studies described in this thesis are just one grain of sand in the desert of research into atherosclerotic cardiovascular disease (CVD). Already two centuries ago, a German medical scientist called Virchow reported that atherosclerosis is not a simple lipid storage disease, but a complex chronic inflammatory process in the vessel wall[1]. However, in the following decades, the fast majority of research was focused on cholesterol metabolism even leading to multiple Nobel Prize winners[2]. It is therefore not surprising that cholesterol-lowering treatment currently forms the cornerstone of CVD treatment[3]. In fact, statins are one of the most prescribed medicines in the world. Given the enormous scope and depth of the current available research, it is alarming to note that CVD is still the number one cause of (premature) death worldwide.

To be able to tackle this therapeutic challenge, more research is needed. And although CVD is indeed one of the most researched medical topics globally, hopefully at the end of this chapter the necessity of doing more research will be evident; including the research described in thesis. Because after years of focusing on cholesterol metabolism and its impact on atherosclerosis, the last few years it became apparent that Virchow was not far from the truth. The inflammatory theory was proven by the ultimate *proof of the pudding*: selective inhibition of inflammation (in absence of associated lipid changes) in CVD patients led to a reduction in cardiovascular events and cardiovascular related mortality in a large randomized placebo-controlled trial[4]. However, the group with patients receiving the immunosuppressant showed a slightly, but significantly higher mortality rate due to severe infections (predominantly pneumonia) compared to the placebo group. This unfavorable side-effect profile emphasizes a big obstacle in targeting inflammation in CVD. It will be a challenge finding the right balance between suppressing inflammatory activity in the vessel wall, while allowing the immune system to activate and target deadly invaders such as bacteria and viruses.

Striving for this balance in targeting residual inflammatory risk, this thesis aimed to determine the impact of hyperlipidemia and lipid-lowering therapies on inflammatory activity in patients and define the maintaining factors of persistent inflammation post-treatment. By broadening the scientific horizon beyond the cardiovascular field, and using data from epidemiologic, murine, and ex vivo human (randomized controlled) studies, this thesis gives a unique translational insight into the origins of cardiovascular inflammation.

PART 1. THE VICIOUS INFLAMMATORY CIRCLE FORMED BY THE BONE MARROW-VASCULAR-AXIS

The first part of this thesis illustrates the vicious inflammatory circle in hypercholesterolemic patients which is formed by the bone marrow – vascular – axis. To understand this concept, it is important to remember that monocytes play a key role in atherosclerotic plaque formation as described in **chapter 1** of this thesis.

Previous research shows that in the process of atherosclerotic plaque formation, LDL-cholesterol instigates inflammation by activating monocytes. Notably, **chapter 2** of this thesis demonstrates that monocytes in patients with familial hypercholesterolemia remain in a hyper-responsive pro-inflammatory state despite normalization of LDL-cholesterol plasma levels post statin treatment. This type of immune memory is termed 'trained immunity'; and is characterized by increased cytokine production capacity. Additional analyses showed that transcriptional (RNA expression) and epigenetic (marks on DNA that regulate gene activation) reprogramming are underlying mechanisms of this acquired trained immunity phenotype.

The discrepancy of *long-term* immune memory in *short-lived* immune cells described in chapter 2 served as an important indicator of the existence of a bone marrow-vascular axis in human. Especially in inflammatory conditions, monocytes are destined to a short life-span of approximately 24 hours. The fact that circulating monocytes exhibit hypercholesterolemia-associated transcriptional and epigenetic alterations weeks after normalization of LDL-cholesterol levels, hints towards formation of already 'trained' monocytes in the bone marrow compartment. Although this phenomenon had been described previously in multiple murine studies[5,6], evidence from human studies were lacking as hematopoietic stem cells are a rare cell population and bone marrow material is not as easy to obtain as peripheral blood. However, in **chapter 3** these hurdles were overcome; this unique work provides both epidemiologic as well as mechanistic evidence that LDL-cholesterol drives pro-inflammatory monocyte production in the bone marrow compartment. First, analyses using data of over 12,000 individuals in the EPIC-Norfolk study demonstrate a positive association between LDL-cholesterol and monocyte percentage. To validate LDL-cholesterol-directed enhanced monocyte production at a cellular level, additional *ex vivo* bone marrow analyses before and after cholesterol-lowering treatment in patients with familial hypercholesterolemia (but without CVD) were performed. These paired experiments, using patients as their own controls, revealed myelomonocytic skewing and a promigratory profile of

hematopoietic stem cells using functional assays and transcriptomic analyses. These findings coincided with perturbed intracellular lipid homeostasis in these stem cells. Of note, compared to hematopoietic stem cells of a matched healthy control group, a lot of these findings reverted after 12 weeks of cholesterol lowering treatment. However, increased gene expression involved in monocyte and macrophage-mediated inflammation and migration persisted despite normalization of plasma LDL-cholesterol levels, hinting towards the existence of trained immunty. These *ex vivo* results were in line with the *in vitro* experiments described in **chapter 4**, as hematopoietic stem cells from healthy donors co-incubated with oxidized LDL-cholesterol revealed myeloid skewing using a dedicated colony forming assay (CFU-GM). Interestingly, retrospective analyses of CFU-GM capacity of hematopoietic stem cells harvested from cancer patients for autologous stem cell transplantation also demonstrated myeloid skewing in the patients who had already established cardiovascular disease. This finding suggests that hematopoietic stem cell reprogramming persists throughout all stages of atherosclerotic cardiovascular disease. Indeed, an additional *in vivo* functional imaging study using positron emission tomography with computed tomography (PET/CT) and the radioactive glucose analogue ^{18}F-FDG showed increased bone marrow activity in patients who suffered a prior myocardial infarction or ischemic stroke; potentially reflecting increased progenitor activity of hematopoietic stem cells residing in the bone marrow compartment. This was further substantiated by the correlation of plasma LDL-cholesterol levels and ^{18}F-FDG uptake in the bone marrow compartment. Interestingly, another strong correlation was found between ^{18}F-FDG uptake in the bone marrow compartment and the spleen. The spleen serves as an additional site of immune cell production in highly inflammatory conditions. In this context, previous murine studies have established that ischemic events such as a myocardial infarction or stroke trigger hematopoietic stem cell mobilization from the bone marrow to the spleen, transforming the spleen into an extramedullary production site of pro-inflammatory monocytes[7]. In other words, inflammation leads to ischemic events, and ischemic events trigger even more inflammatory activity. **Chapter 5** confirms this vicious inflammatory circle reporting increased levels of circulating monocytes and hematopoietic stem and progenitor cells in both the acute phase of myocardial infarction and three months post event. These findings coincided with increased uptake of DPA-714 (a PET tracer binding the TSPO receptor which is highly expressed in myeloid cells) in both the bone marrow compartment and the spleen. During follow-up, splenic DPA-714 uptake remained high three months post-event, and the number of circulating monocytes and hematopoietic stem cells were even increased up to 6 to 24 months post event. Circulating monocyte number as well as splenic activity are correlated with an increased risk of future cardiovascular

events[8], making these findings the last link in closing the vicious inflammatory circle in atherosclerosis.

In summary, the first part of this thesis showed that hypercholesterolemic patients are burdened by hypercholesterolemia-associated pro-inflammatory circulating monocytes that remain in a hyperresponsive state despite normalization of LDL-cholesterol by potent cholesterol-lowering treatment (chapter 2). Chapter 3 provides evidence that transcriptomic reprogramming of the long-lived hematopoietic stem cells in the bone marrow compartment causes long-term production of pro-inflammatory monocytes, explaining the persistent hyperresponsive state beyond the lifespan of circulating monocytes. Importantly, chapter 4 demonstrates that LDL-cholesterol has a direct impact on hematopoietic stem cell fate and differentiation, whilst also showing that hematopoietic activity is increased throughout all stages of atherogenesis, including in patients with established atherosclerotic cardiovascular disease. The ultimate complication of continuous influx of cholesterol and monocytes into the vessel wall is atherosclerotic plaque rupture causing an acute myocardial infarction or stroke. Chapter 5 visualizes the response of the bone marrow compartment and the spleen to an acute myocardial infarction and links the prolonged hematopoietic activity to increased numbers of pro-inflammatory circulating monocytes. Thereby, a tsunami of new monocytes enters the vessel wall leading to a vicious inflammatory circle in which inflammation leads to ischemic events that in turn leads to accelerated inflammation resulting in more ischemic events.

PART 2. BREAKING THE VICIOUS CIRCLE OF LIPOPROTEIN(A)-MEDIATED INFLAMMATION

Part 2 of this thesis focuses on the inflammatory response in patients with hyperlipoproteinemia(a). Whereas it is widely known that hypercholesterolemia causes cardiovascular disease, the detrimental relationship between hyperlipoproteinemia(a) and CVD is less established beyond the cardiovascular field. Lipoprotein(a) (Lp(a)) is an LDL-like particle, consisting of an apolipoprotein B-100 (apoB) molecule similar to LDL-cholesterol, but has an additional apolipoprotein(a) (apo(a)) tail[9]. Twenty percent of the general population has an Lp(a) level >50mg/dL which is associated with an increased CVD risk, making Lp(a) one of the most potent and prevalent genetic risk factors of CVD[10]. Prior studies have shown that Lp(a) elicits monocyte activation and thereby arterial wall inflammation similar to LDL-cholesterol[11]. However, **chapter 6** shows that in contrast to LDL-cholesterol, Lp(a) does not induce long-lasting reprogramming of hematopoietic stem cells.

Transplantation of bone marrow cells of mice with elevated Lp(a) into lethally irradiated mice without Lp(a) showed no differences in hematopoietic stem cell number and fate twelve weeks post-transplantation. Also 24 hours of *in vitro* stimulation of healthy hematopoietic stem cells with Lp(a) did not demonstrate long-lasting differences in stem cell behavior when analyzed 7 days later. In contrast, direct analysis after 24 hours of Lp(a) stimulation showed increased differentiation into pro-inflammatory monocytes, and 7 days of continuous Lp(a) stimulation induced myeloid stem cell fate and increased proliferation capacity. These data imply that Lp(a) directs hematopoiesis in favor of pro-inflammatory monocyte production without long-lasting reprogramming of hematopoietic stem cells. This would mean that Lp(a)-lowering therapy could lead to a rapid reversal of pro-inflammatory monocyte production in the bone marrow compartment and subsequently result in attenuated arterial wall inflammation. However, no specific Lp(a)-lowering therapies are yet available to treat patients with elevated Lp(a). Therefore, proprotein convertase subtilisin/kexin type 9 (PCSK9) antibody treatment was used in **chapter 7**, which gives a modest lowering of Lp(a) levels in addition to strong LDL-cholesterol reduction. In a randomized placebo-controlled trial, 16 weeks of PCSK9 antibody treatment in patients with elevated Lp(a) led to a 14% reduction in Lp(a) levels to an additional 61% reduction in LDL-cholesterol level without effect on arterial wall inflammation measured with ^{18}F-FDG PET/CT scan. Not only arterial wall inflammation remained unaffected, **chapter 8** also shows that in a subgroup of these patients, the pro-inflammatory phenotype of circulating monocytes is not attenuated post PCSK9 antibody treatment. Interestingly, a subgroup of patients with elevated Lp(a) who were also included in chapter 8, received a potent Lp(a)-lowering drug (AKCEA-APO(a)-L_{Rx}) in a randomized placebo-controlled phase 2 trial. This drug, based on antisense technology and still in developmental stage, led to a 47% reduction in Lp(a) and concomitantly reverted pro-inflammatory gene expression and function in circulating monocytes. The latter, including the results on the lack of reprogramming of hematopoietic stem cells in high Lp(a) conditions, suggests that the vicious inflammatory circle in patients with elevated Lp(a) can be broken by achieving large reductions in Lp(a).

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DISCUSSION, FUTURE PERSPECTIVES AND CONCLUSION

THE MORE YOU KNOW, THE LESS YOU KNOW *Aristotle*

Using a unique combination of translational methods, this thesis provides a framework for approaching the therapeutic challenge of residual inflammatory risk in CVD patients. This framework is timely in view of the rapidly evolving development of CVD treatment, and expectations are that its focus will shift towards anti-inflammatory regimens and specific lipoprotein(a)-lowering therapies. This is in light of the exceptionally advanced development of drugs targeting LDL-cholesterol, and with the outlook of promising new cholesterol-lowering drugs such as inclisiran[1], evinacumab[2] and bempedoic acid[3] added to an already strong lipid-lowering arsenal including statins and PCSK9-antibodies, it is anticipated that LDL-cholesterol lowering will no longer be a therapeutic challenge in the near future. On the other hand, targeting residual *inflammatory* risk has become a genuine ordeal, as previous CVD outcome trials using anti-inflammatory agents showed conflicting results. As described earlier, canakinumab (IL-1β antagonist) showed CV benefit at the cost of (fatal) side-effects due to infections in the CANTOS trial[4], while the CIRT trial using low-dose methotrexate was ended prematurely due to futility[5]. Colchicine showed CV benefit in patients with stable cardiovascular disease (LoDoCo2 trial)[6], as well as in patients who suffered a recent myocardial infarction (COLCOT trial)[7], although patients in the latter trial experienced significant more pneumonias after colchicine treatment versus placebo. Regarding Lp(a), a post-hoc analysis of the JUPITER trial showed that Lp(a) was a significant driver of residual risk in patients treated with potent statin treatment[8], whereas post-hoc analyses of the ODYSSEY[9] and FOURIER[10] trials suggest that additional LDL-cholesterol-lowering in combination with modest Lp(a)-lowering following PCSK9-antibody treatment only in part overcomes this risk. Summarizing, these trials exemplify that cracking a nut using a sledgehammer is not the way forward and emphasize the need of more targeted and ultimately more personalized therapy to tackle residual CV risk.

TACKLE THE PROBLEM AT ITS ROOTS

The first part of this thesis identifies a crucial role for hematopoietic stem cells in residual inflammatory risk, being the root of the hematopoietic tree and thus the source of all mature blood cells including inflammatory cells. The notion that cardiovascular inflammation being a multi-level process is not novel, and preclinical work already hinted towards the existence of a bone marrow – vascular axis[11]. Chapter 2 and 3 of this thesis confirm that hypercholesterolemia itself influences hematopoietic stem cell behavior *in patients*, and thereby exerts inflammatory effects even beyond normalization of cholesterol levels, and moreover, beyond the

lifespan of circulating monocytes. Murine work has attributed the changes in stem cell fate to hypercholesterolemia-induced epigenetic and metabolic alterations in these cells[12]. Chapter 2 and 3 hint towards similar mechanisms, although additional genome wide analysis of chromatin accessibility in hematopoietic stem cells and their progeny is warranted. Also, with the advances of single cell analysis the current dogma of stepwise lineage commitment during hematopoiesis is being challenged[13], making single cell epigenomics integrated with transcriptomic and metabolic profiling an interesting new step in elucidating the effects of hypercholesterolemia on hematopoietic stem cell behavior and fate. In respect of finding new druggable targets, epigenetic alterations are appealing targets, since these alterations are generally reversible. Also, 'resetting' hematopoiesis instead of 'suppressing' inflammatory cell production, would potentially mean less infectious side-effects. In this light, chapter 4 and 5 describe the pivotal role of extramedullary hematopoiesis following hematopoietic stem cell mobilization post ischemic event, highlighting another crucial mechanism by which *enhanced* monocyte production could be targeted in both the acute and chronic phase of CVD without complete suppression of monocytopoiesis. Finally, it would be of pathophysiological and clinical interest whether indeed intracellular lipid accumulation drives the reprogramming of hematopoietic stem cells, and if so, whether promoting cholesterol efflux from these cells would revert these changes.

A NEW KID ON THE BLOCK

Strengthened by Mendelian Randomization data[14, 15] and with the advent of specific Lp(a)-lowering drugs[16], residual risk determined by elevated Lp(a) has gradually regained interest by clinicians in the past few years[17]. Whereas the first part of this thesis showed that LDL-cholesterol acts as an important instigator of a vicious inflammatory circle in CVD patients, the second part of this thesis surprisingly showed that Lp(a) does not fulfill this detrimental role. Chapter 6 and 8 link the lack of long-term reprogramming of hematopoietic stem cells to the reversibility of Lp(a)-associated monocyte activation following specific Lp(a)-lowering treatment. Equally important, chapter 7 and 8 also show that persistent inflammation following inadequate Lp(a)-lowering treatment serves as an important pathophysiological mechanism contributing to Lp(a)-associated residual risk. In line with the aforementioned Mendelian Randomization studies, these chapters underpin the need of specific and large reductions in Lp(a) to fully mitigate Lp(a)-associated inflammation, and thus Lp(a)-associated residual risk. The ongoing phase-3 HORIZON trial[18], using a potent (GalNAc) antisense oligonucleotide drug directed against the *LPA* gene, is the first CV outcome trial to test whether *large* reductions

in Lp(a) (up to 90%) will lead to clinical benefit. On the other hand, although not primarily investigated in high Lp(a) patients, the ongoing phase-3 ZEUS trial[19] will test whether the Lp(a)-lowering *and* anti-inflammatory drug ziltivekimab will lead to cardiovascular risk reduction following both interleukin-6 inhibition, in addition to *modest* Lp(a)-reduction. Also, the discrepancy in ability to prime hematopoietic stem cells by LDL-cholesterol versus Lp(a) strengthens the hypothesis of NLRP3 inflammasome-mediated hematopoietic stem cell priming and trained immunity[12], since cellular LDL-cholesterol overload has been linked to NLRP3 inflammasome priming[20], in contrast to oxidized phospholipids that form an important pro-inflammatory component of Lp(a)[20]. Finally, extrapolating these findings beyond the realm of cardiovascular research, it would be of interest if cholesterol overload has similar effects in other types of stem cells and respective associated diseases, including leukemic stem cells[21] and intestinal stem cells[22].

CONCLUSION

The work presented in this thesis provides a stepping stone to tackle the therapeutic challenges of residual inflammatory risk in CVD patients. First, this thesis identifies LDL-cholesterol as instigator of a vicious inflammatory circle that is formed by the bone-marrow-vascular-axis. Second, inadequately lowered lipoprotein(a) plasma levels drive persistent inflammation, even on a background of normal to (very) low plasma LDL-cholesterol levels. With the upcoming of new anti-inflammatory regimens and specific Lp(a)-lowering drugs, recognizing the different drivers of residual inflammatory risk is pivotal, as they require their own specific therapeutic intervention beyond LDL-cholesterol-lowering.

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APPENDICES

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NEDERLANDSE SAMENVATTING

De studies beschreven in dit proefschrift vormen slechts een korreltje zand in de woestijn van wetenschappelijk onderzoek naar atherosclerose en het uiteindelijke gevolg daarvan: hart- en vaatziekten (HVZ). Bijna 200 jaar geleden beschreef de Duitse arts-onderzoeker Virchow al dat atherosclerose niet een simpele cholesterol stapelingsziekte is, maar het resultaat van een complex en chronisch ontstekingsproces in de vaatwand[1]. Echter, in de daaropvolgende decennia was het leeuwendeel van het onderzoek gericht op het cholesterolmetabolisme, wat uiteindelijk zelfs heeft geleid tot meerdere Nobel Prijs winnaars[2]. Het is daarom ook niet verwonderlijk dat cholesterolverlagers tegenwoordig de hoeksteen van de behandeling van atherosclerotische HVZ vormen[3]. Sterker nog: de cholesterolverlagende statines zijn één van de meest voorgeschreven medicijnen ter wereld. Toch is het alarmerend om vast te stellen dat ondanks al dit onderzoek HVZ wereldwijd nog steeds de nummer 1 doodsoorzaak is.

Er is dus meer onderzoek nodig om de aanpak van HVZ te verbeteren. En hoewel HVZ inderdaad een van de meest onderzochte medische onderwerpen wereldwijd is, zal hopelijk aan het einde van dit hoofdstuk de noodzaak om meer onderzoek te doen duidelijk worden; inclusief de noodzaak van het beschreven onderzoek in dit proefschrift. Want na jaren van focus op de impact van het cholesterolmetabolisme op atherosclerose, werd de laatste jaren duidelijk dat Virchow niet ver van de waarheid zat. Zijn ontstekingstheorie werd bewezen door de ultieme *proof of the pudding*: selectieve remming van ontsteking (zonder dat daarbij veranderingen in cholesterolwaardes optraden) in patiënten met HVZ verminderde het risico op hart- en herseninfarcten en het overlijden hieraan in een grote gerandomiseerde placebogecontroleerde studie[4]. Echter werd er een dure prijs betaald in deze studie: patiënten waarin ontsteking werd geremd overleden net iets, maar wel significant vaker aan infecties (voornamelijk longontsteking) vergeleken met de groep patiënten die met het placebo-middel werden behandeld. Dit ongunstige bijwerkingenprofiel legt meteen het belangrijkste pijnpunt bloot in het vinden van de balans tussen enerzijds het immuunsysteem onderdrukken waardoor aderverkalking in de vaatwand wordt tegengegaan en anderzijds het lichaam nog wel in staat te stellen om zich te verweren tegen dodelijke bacteriën en virussen.

Om deze balans te kunnen vinden, wordt er in dit proefschrift onderzocht wat de impact is van hoog cholesterol en cholesterolverlagende behandelingen op de algehele ontstekingsactiviteit in patiënten. Daarnaast wordt onderzocht welke factoren het ontstekingsproces onderhouden ondanks cholesterolverlaging. Door

de horizon te verbreden voorbij het cardiovasculaire onderzoeksdomein en door gebruik te maken van gegevens uit epidemiologische, muizen- en *ex vivo* humane (gerandomiseerde gecontroleerde) studies, geeft dit proefschrift een uniek, translationeel inzicht in de oorsprong van ontsteking in hart- en vaatziekten.

DEEL 1. DE VICIEUZE CIRKEL VAN ONTSTEKING GEVORMD DOOR DE BEENMERG – VAATWAND – AS

In deel 1 van dit proefschrift wordt duidelijk dat de vicieuze cirkel van ontsteking in patiënten met verhoogd cholesterol wordt gevormd door de beenmerg–vaatwand–as. Om dit concept te begrijpen, is het belangrijk om in het achterhoofd te houden dat monocyten een hoofdrol spelen in vaatwandontsteking, zoals beschreven in **hoofdstuk 1** van dit proefschrift.

Uit eerder onderzoek is reeds gebleken dat gedurende het proces van aderverkalking, het slechte cholesterol (LDL-cholesterol) ontsteking veroorzaakt door monocyten te activeren. **Hoofdstuk 2** laat zien dat de monocyten van patiënten met een familiare vorm van hoog LDL-cholesterol geactiveerd zijn, maar dat deze 'verhoogde staat van paraatheid' niet vermindert ondanks normalisatie van LDL-cholesterol waardes na statine therapie. Dit type immuun geheugen in monocyten wordt ook wel 'getrainde immuniteit' genoemd en wordt gekenmerkt door een beter vermogen van monocyten om ontstekingseiwitten te produceren. Aanvullende analyses lieten zien dat dit komt door reprogrammering van monocyten op het niveau van RNA en epigenetica (markeringen op het DNA die genen aan en uit zetten).

De discrepantie tussen het bestaan van een *lange*termijngeheugen in *kort*levende immuuncellen zoals beschreven in hoofdstuk 2 was een belangrijke hint naar het bestaan van een beenmerg–vaatwand–as in patiënten. Vooral bij actieve ontsteking hebben monocyten een zeer korte levensduur van ongeveer 24 uur. Het feit dat monocyten in de bloedbaan *weken* na normalisatie van LDL-cholesterolwaardes nog steeds RNA en epigenetische veranderingen vertonen die juist geassocieerd zijn met hoge cholesterolwaardes, duidt erop dat deze monocyten reeds met ingebouwde 'training' worden gemaakt door bloedvormende stamcellen in het beenmergcompartiment. Hoewel dit fenomeen eerder is beschreven in meerdere muizenstudies[5,6], is dit nog nooit aangetoond in patiënten, omdat bloedvormende stamcellen een zeldzame cel populatie vormen en beenmergmateriaal niet zo gemakkelijk te verkrijgen is zoals perifeer bloed. In **hoofdstuk 3** werden deze hindernissen echter overwonnen en levert deze unieke studie zowel epidemiologisch als mechanistisch bewijs dat LDL-cholesterol de productie van geactiveerde

monocyten in het beenmergcompartiment van patiënten stimuleert. Ten eerste tonen analyses waarbij gegevens van meer dan 12.000 personen in de EPIC-Norfolk-studie zijn gebruikt een positieve associatie tussen LDL-cholesterol en het percentage monocyten in het bloed. Om ook op cellulair niveau te bevestigen dat LDL-cholesterol de productie van monocyten in het beenmergcompartiment stimuleert, werden aanvullende *ex vivo* beenmerganalyses voor én na cholesterolverlagende behandeling uitgevoerd bij patiënten met familiair verhoogd cholesterol, maar zonder HVZ. Deze gepaarde experimenten, waarbij patiënten als hun eigen controles werden gebruikt, toonden met behulp van functionele experimenten en RNA-analyses aan dat de bloedvormende stamcellen van deze patiënten een voorkeur hadden om uit te groeien tot (voorlopers van) monocyten en dat zij kenmerken vertoonden die stamcelmobilisatie vanuit het beenmergcompartiment naar het bloed stimuleren. Daarbij gingen deze bevindingen ook gepaard met een verstoord cholesterolmetabolisme in de onderzochte stamcellen. Een andere belangrijke bevinding is dat veel van deze veranderingen terugkeerden naar het niveau van bloedvormende stamcellen van een gematchte gezonde controlegroep na cholesterolverlagende behandeling gedurende 12 weken. Echter bleef de expressie van genen die betrokken zijn bij monocyt- en macrofaag-gemedieerde ontsteking en migratie verhoogd ondanks normalisatie van het LDL-cholesterolgehalte, wat verder bekrachtigd dat 'getrainde immuniteit' wordt gedreven vanuit het beenmerg. Deze *ex vivo* resultaten sloten aan op de *in vitro* experimenten beschreven in **hoofdstuk 4**, aangezien ook bloedvormende stamcellen van gezonde donoren die gestimuleerd waren met geoxideerd LDL-cholesterol een voorkeur hadden tot het uitgroeien in de voorlopers van monocyten in een speciale kolonievormende experiment (CFU-GM). Interessant is dat retrospectieve analyses van CFU-GM-capaciteit van bloedvormende stamcellen afkomstig van kankerpatiënten die een autologe stamceltransplantatie ondergingen, maar ook bijkomend HVZ hadden, eveneens een voorkeur hadden tot het uitgroeien in de voorlopercellen van monocyten ten opzichte van de groep kankerpatiënten zonder HVZ. Deze bevinding suggereert dat herprogrammering van bloedvormende stamcellen voorkomt in alle stadia van atherosclerotische hart- en vaatziekten. Een aanvullend *in vivo* functioneel beeldvormingsonderzoek, waarbij gebruik werd gemaakt van positronemissietomografie met computertomografie (PET/CT) en de radioactieve glucose-analoog ^{18}F-FDG, toonde inderdaad verhoogde beenmergactiviteit aan bij patiënten die eerder een hart- of herseninfarct hadden doorgemaakt; mogelijk als gevolg van verhoogde delingsactiviteit van bloedvormende stamcellen in het beenmergcompartiment. Dit werd verder bevestigd door de correlatie van plasma LDL-cholesterolspiegels en ^{18}F-FDG-opname in het beenmergcompartiment. Interessant genoeg werd er tevens een sterke correlatie gevonden tussen ^{18}F-FDG-

opname in het beenmergcompartiment en de milt. Wanneer het lichaam in een verhoogde ontstekingsstaat verkeerd en dus extra immuuncellen nodig heeft, dient de milt (naast het beenmergcompartiment) als extra locatie waar productie van immuuncellen kan plaatsvinden. In deze context hebben eerdere muizenstudies aangetoond dat hart- en herseninfarcten mobilisatie van bloedvormende stamcellen van het beenmerg naar de milt veroorzaken, waardoor de milt wordt getransformeerd in een 'extramedullaire' (= bloedcelproductie buiten het beenmerg) productieplaats van geactiveerde monocyten[7]. Met andere woorden, ontsteking leidt tot infarcten en infarcten veroorzaken op hun beurt weer meer ontstekingsactiviteit. **Hoofdstuk 5** bevestigt deze vicieuze cirkel van ontsteking en rapporteert verhoogde niveaus van monocyten en bloedvormende stamcellen in de bloedbaan in zowel de acute fase van een hartinfarct als drie maanden na het infarct. Deze bevindingen gingen gepaard met een verhoogde opname van DPA-714 (een PET-tracer die de TSPO-receptor bindt die in hoge mate tot expressie wordt gebracht in (voorlopercellen van) monocyten) in zowel het beenmergcompartiment als de milt. Tijdens de follow-up bleef de opname van DPA-714 in de milt drie maanden na het hartinfarct verhoogd en was het aantal monocyten en bloedvormende stamcellen in de bloedbaan zelfs 6 tot 24 maanden na het infarct verhoogd. Het is eerder beschreven dat het aantal monocyten in de bloedbaan en de miltactiviteit zijn gecorreleerd met een verhoogd risico op toekomstige hart- en herseninfarcten[8], waarmee het bevestigt dat deze bevindingen de laatste schakel vormen van de vicieuze cirkel van ontsteking in atherosclerose.

Samenvattend toonde het eerste deel van dit proefschrift aan dat patiënten met verhoogd cholesterol geactiveerde monocyten in de bloedbaan hebben circuleren, die zelfs na normalisatie van LDL-cholesterol door krachtige cholesterolverlagende medicijnen in verhoogde staat van paraatheid blijven (hoofdstuk 2). Hoofdstuk 3 levert bewijs dat herprogrammering op het niveau van het RNA van de langlevende bloedvormende stamcellen in het beenmergcompartiment langdurige productie van geactiveerde monocyten veroorzaakt, wat verklaart dat de verhoogde staat van paraatheid van circulerende monocyten ook na hun gemiddelde levensduur van 24 uur wordt gezien. Belangrijk is dat hoofdstuk 4 aantoont dat LDL-cholesterol een directe invloed heeft op het uitgroeien van bloedvormende stamcellen in monocyten, zowel in patiënten met als zonder vastgestelde HVZ. Het uiteindelijke resultaat van een continue instroom van cholesterol en monocyten in de vaatwand is het scheuren van een atherosclerotische plaque, wat vervolgens leidt tot een acuut hart- of herseninfarct. Hoofdstuk 5 visualiseert de reactie van het beenmergcompartiment en de milt op een acuut hartinfarct en koppelt de aanhoudende beenmerg- en miltactiviteit aan een verhoogd aantal geactiveerde

monocyten in de bloedbaan. Als gevolg hiervan stroomt een tsunami van nieuwe monocyten de vaatwand binnen, wat leidt tot een vicieuze ontstekingscirkel waarin ontsteking leidt tot hart- en herseninfarcten, die op hun beurt weer leiden tot verergering van ontsteking, wat vervolgens weer resulteert in meer hart- en herseninfarcten.

DEEL 2. DE VICIEUZE CIRKEL VAN LIPOPROTEINE(A)-GEMEDIEERDE ONTSTEKING DOORBREKEN

Deel 2 van dit proefschrift richt zich op de ontstekingsreactie bij patiënten met een verhoogd lipoproteïne(a) (Lp(a)). Hoewel het algemeen bekend is dat verhoogde cholesterolwaardes HVZ veroorzaakt, is de nadelige relatie tussen verhoogd Lp(a) en HVZ minder bekend buiten het cardiovasculaire veld. Lp(a) is een LDL-achtig deeltje, bestaande uit een apolipoproteïne B-100 (apoB) molecuul vergelijkbaar met LDL-cholesterol, maar heeft hieraan vast nog een extra apolipoproteïne(a) (apo(a))-staart gekoppeld[9]. Twintig procent van de algemene bevolking heeft een Lp(a)-niveau >50 mg/dL, wat geassocieerd is met een verhoogd risico op hart- en vaatziekten. Dit maakt Lp(a) één van de krachtigste en meest voorkomende genetische risicofactoren van hart- en vaatziekten. Eerdere studies hebben aangetoond dat vergelijkbaar met LDL-cholesterol, Lp(a) zorgt voor monocytactivatie en daarmee vaatwandontsteking[11]. **Hoofdstuk 6** laat echter zien dat, in tegenstelling tot LDL-cholesterol, Lp(a) geen langdurige herprogrammering van bloedvormende stamcellen induceert. Transplantatie van beenmergcellen van muizen met verhoogd Lp(a) in muizen zonder Lp(a) liet twaalf weken na transplantatie geen verschillen zien in het aantal bloedvormende stamcellen en hun voorkeur in uitgroeien tot bloedcellen. Ook 24 uur *in vitro* stimulatie van gezonde bloedvormende stamcellen met Lp(a) vertoonde geen langdurige verschillen in stamcelgedrag bij analyse 7 dagen later. Daarentegen toonde directe analyse van bloedvormende stamcellen nadat zij 24 uur met Lp(a) waren gestimuleerd een voorkeur tot uitgroei in geactiveerde monocyten en na 7 dagen continue Lp(a)-stimulatie was de delingscapaciteit van de voorlopers van monocyten sterk verhoogd. Deze resultaten impliceren dat Lp(a) de productie van bloedcellen stuurt ten gunste van geactiveerde monocyten zonder dat het leidt tot langdurige herprogrammering van de bloedvormende stamcellen. Dit zou betekenen dat Lp(a)-verlagende therapie zou kunnen leiden tot een snelle omkering van de geactiveerde monocytproductie in het beenmergcompartiment en vervolgens zou kunnen leiden tot verminderde vaatwandontsteking. Er zijn echter nog geen specifieke Lp(a)-verlagende therapieën beschikbaar om patiënten met verhoogd Lp(a) te behandelen. Daarom werd in **hoofdstuk 7** proprotein convertase subtilisine/kexine type 9 (PCSK9) antilichaambehandeling gebruikt,

wat naast een sterke LDL-cholesterolverlaging een additionele milde verlaging in Lp(a)-waardes geeft. In een gerandomiseerde placebogecontroleerde studie leidde 16 weken behandeling met PCSK9-antilichamen bij patiënten met verhoogd Lp(a) tot een verlaging van 14% in Lp(a)-waardes en een verlaging van 61% in LDL-cholesterol. Echter ging deze lipidenverlaging niet gepaard met een verlaging van de vaatwandontsteking gemeten met ^{18}F-FDG PET/CT-scan. Niet alleen de vaatwandontsteking bleef onverminderd aanwezig, **hoofdstuk 8** laat tevens zien dat de circulerende monocyten in een subgroep van deze patiënten geactiveerd blijven ondanks behandeling met PCSK9-antilichamen. Interessant is dat in hoofdstuk 8 ook een subgroep van patiënten met verhoogd Lp(a) is onderzocht die behandeld werd met een medicijn (AKCEA-APO(a)-LRx) dat forse en specifieke reductie in Lp(a) gaf in een gerandomiseerde placebogecontroleerde fase 2 studie. Dit medicijn, gebaseerd op antisense-technologie en nog in de ontwikkelingsfase, leidde tot een Lp(a)-verlaging van gemiddeld 47% wat gepaard ging met vermindering van monocytactivatie op zowel genexpressie als functioneel niveau. Deze bevindingen, samen met het feit dat er geen aanwijzingen zijn voor langdurige herprogrammering van bloedvormende stamcellen door Lp(a), suggereert dat de vicieuze cirkel van ontsteking bij patiënten met verhoogd Lp(a) kan worden doorbroken door een grote absolute verlaging in Lp(a) te bereiken.

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PORTFOLIO

Name PhD student: L.C.A. Stiekema

PhD period: November 2015 – December 2019

Name PhD supervisor: Prof. dr. E.S.G. Stroes

	Year	ECTs
1. PhD training		
Courses		
BROK	2016	1.0
Advances in Lipid Management and CV-risk reduction, Boston, MA, USA	2016	1.0
Advanced Immunology course, Graduate School AMC	2017	2.9
Seminars, Workshops and Master classes		
Weekly journal club, department of Vascular Medicine, AMC	2015-2019	4.0
Weekly clinical education, department of Vascular Medicine, AMC	2015-2019	4.0
Oral and poster presentations		
Gordon conference, Atherosclerosis & inflammation in Sunday River, USA, poster presentation	2017	0.5
86th EAS Congress in Lisbon, Science at a glance presentation	2018	0.5
Amsterdam Cardiovascular Sciences retreat in Soesterberg, poster	2019	0.5
87th EAS Congress in Maastricht, Oral presentation 88th EAS Congress (virtual), Oral presentation	2019 2020	0.5 0.5
Conferences		
Rembrandt symposium in Noordwijkerhout	2015, 2016	0.5
Cardio Vasculaire Conferentie in Ede	2016	0.25
Lp(a) satellite meeting in Innsbruck, and Maastricht	2016, 2019	0.5
EAS Congress in Innsbruck, Prague, Lisbon, and Maastricht	2016-2019	3.0
Gordon conference, Atherosclerosis & inflammation in Sunday River, USA	2017	1.5
Symposium Cutting edge LIPID Science: From haystack needles to razor blade therapies in Amsterdam	2018	0.25
Amsterdam Cardiovascular Sciences retreat in Soesterberg	2019	0.25
2. Teaching and lecturing		
Update Lipoprotein(a), Cardiologists and Cardiologists in training , AMC and Bernhoven hospital	2017, 2018	0.5
Cardiovascular Research in Care (Atherosclerosis), Bachelor medical students, University of Amsterdam	2018, 2019	1.0
3. Other		
Member of employee council AMC Medical Research bv	2016-2018	
4. Parameters of esteem		
Young Investigator Award EAS (three years in a row)	2018-2020	

LIST OF PUBLICATIONS

Included in this thesis

1. Impact of cholesterol on proinflammatory monocyte production by the bone marrow.
Lotte C.A. Stiekema, Lisa Willemsen, Yannick Kaiser, Koen H.M. Prange, Nicholas J. Wareham, S. Matthijs Boekholdt, Carlijn Kuijk, Menno P.J. de Winther, Carlijn Voermans, Matthias Nahrendorf, Erik S.G. Stroes, Jeffrey Kroon
European Heart Journal, 2021

2. Short-term regulation of hematopoiesis by lipoprotein(a) results in the production of pro-inflammatory monocytes.
Johan G. Schnitzler*, Kikkie Poels*, Lotte C.A. Stiekema, Calvin Yeang, Sotirios Tsimikas, Jeffrey Kroon, Erik S.G. Stroes, Esther Lutgens, Tom T.P. Seijkens
International Journal of Cardiology, 2020

3. Potent lipoprotein(a) lowering following apolipoprotein(a) antisense treatment reduces the pro-inflammatory activation of circulating monocytes in patients with elevated lipoprotein(a).
Lotte C.A. Stiekema*, Koen H.M. Prange*, Renate M. Hoogeveen, Simone L. Verweij, Jeffrey Kroon, Johan G. Schnitzler, Kim E. Dzobo, Arjen J. Cupido, Sotirios Tsimikas, Menno P.J. de Winther*, Mahnoush Bahjat*
European Heart Journal, 2020

4. Treatment with statins does not revert trained immunity in patients with familial hypercholesterolemia.
Siroon Bekkering, Lotte C.A. Stiekema, Sophie J. Bernelot Moens, Simone L. Verweij, Boris Novakovic, Koen Prange, Miranda Versloot, Jeanine E. Roeters van Lennep, Henk Stunnenberg, Menno de Winther, Erik S.G. Stroes, Leo A.B. Joosten, Mihai G. Netea, Niels P. Riksen
Cell Metabolism, 2019

5. Persistent arterial wall inflammation in patients with elevated lipoprotein(a) despite strong low-density lipoprotein cholesterol reduction by PCSK9 antibody treatment.
Lotte C.A. Stiekema, Erik S.G. Stroes, Simone L. Verweij, Helina Kassahun, Lisa Chen, Scott M. Wasserman, Marc S. Sabatine, Venkatesh Mani, Zahi A. Fayad
European Heart Journal, 2019

6. Prolonged hematopoietic and myeloid cellular response in patients after an acute coronary syndrome measured with 18F-DPA-714 PET/CT.
Simone L. Verweij, Lotte C.A. Stiekema, Ronak Delewi, Kang H. Zheng, Sophie J. Bernelot Moens, Jeffrey Kroon, Charlotte I. Stroes, Miranda Versloot, Jan J. Piek, Hein J. Verberne, Erik S.G. Stroes
European Journal of Nuclear Medicine and Molecular Imaging, 2018

7. Increased haematopoietic activity in patients with atherosclerosis.
Fleur M. van der Valk, Carlijn Kuijk, Simone L. Verweij, Lotte C.A. Stiekema, Y. Kaiser, Sacha Zeerleder, Matthias Nahrendorf, Carlijn Voermans, Erik S.G. Stroes
European Heart Journal, 2016

Not included in this thesis

8. The therapeutic age-paradox coming to an end.
Lotte C.A. Stiekema, G. Kees Hovingh, Erik S.G. Stroes
European Heart Journal, 2020

9. Metabolic effects of PCSK9 inhibition with evolocumab in subjects with elevated Lp(a).
Xiang Zhang, Lotte C.A. Stiekema, Albert K. Groen, Erik S.G. Stroes
Lipids in Health and Disease, 2020

10. PCSK9 antibody alirocumab attenuates arterial wall inflammation without changes in circulating inflammatory markers.
Renate M. Hoogeveen, Tjerk S.J. Opstal, Yannick Kaiser, Lotte C.A. Stiekema, Jeffrey Kroon, Remco J.J. Knol, Willem A. Bax, Hein J. Verberne, Jan H. Cornel, Erik S.G. Stroes
JACC Cardiovascular Imaging, 2019

11. Plaque permeability assessed with DCE-MRI associates with USPIO uptake in patients with peripheral artery disease.
Kang H. Zheng, Jasper Schoormans, Lotte C.A. Stiekema, Claudia Calcagno, Iwona Cicha, Christoph Alexiou, Gustav J. Strijkers, Aart J. Nederveen, Erik S.G. Stroes, Bram F. Coolen
JACC Cardiovascular Imaging, 2019

12. Sympathetic neuronal activation triggers myeloid progenitor proliferation and differentiation.
Sathish Babu Vasamsetti, Jonathan Florentin, Emilie Coppin, Lotte C.A. Stiekema, Kang H. Zheng, Muhammad Umer Nisar, John Sembrat, David J. Levinthal, Mauricio Rojas, Erik S.G. Stroes, Kang Kim, Partha Dutta
Immunity, 2018

13. Cardiovascular disease risk associated with elevated lipoprotein(a) attenuates at low low-density lipoprotein cholesterol levels in a primary prevention setting.
Rutger Verbeek, Renate M. Hoogeveen, Anne Langsted, Lotte C.A. Stiekema, Simone L. Verweij, G. Kees Hovingh, Nicholas J. Wareham, Kay-Tee Khaw, S. Matthijs Boekholdt, Børge G. Nordestgaard, Erik S.G. Stroes
European Heart Journal, 2018

14. CCR2 expression on circulating monocytes is associated with arterial wall inflammation assessed by 18F-FDG PET/CT in patients at risk for cardiovascular disease.
Simone L. Verweij, Raphaël Duivenvoorden, Lotte C.A. Stiekema, Nick S. Nurmohamed, Fleur M. van der Valk, Miranda Versloot, Hein J. Verberne, Erik S.G. Stroes, Matthias Nahrendorf, Siroon Bekkering, Sophie J. Bernelot Moens
Cardiovascular Research, 2018

15. Remnant cholesterol elicits arterial wall inflammation and a multilevel cellular immune response in humans.
Sophie J. Bernelot Moens, Simone L. Verweij, Johan G. Schnitzler, Lotte C.A. Stiekema, Merijn Bos, Anne Langsted, Carlijn Kuijk, Siroon Bekkering, Carlijn Voermans, Hein J. Verberne, Børge G. Nordestgaard, Erik S.G. Stroes, Jeffrey Kroon
Arteriosclerosis, Thrombosis, and Vascular Biology, 2017

16. The maturation of a 'neural-hematopoietic' inflammatory axis in cardiovascular disease.
Lotte Stiekema, Johan Schnitzler, Matthias Nahrendorf, Erik Stroes
Current opinion in Lipidology, 2017

17. PCSK9 inhibitors: Pharmacology, adverse effects, and use.
Lotte C.A. Stiekema, John J.P. Kastelein, Erik S.G. Stroes, Robert S. Rosenson
UpToDate 2016 (www.uptodate.com/contents/pcsk9-inhibitors-pharmacology-adverse-effects-and-use)

18. Low rate of cardiac events in first-degree relatives of diagnosis-negative young sudden unexplained death syndrome victims during follow-up.
Christian van der Werf, Lotte Stiekema, Hanno L. Tan, Nynke Hofman, Marielle Alders, Allard C. van der Wal, Irene M. van Langen, Arthur A.M. Wilde
Heart Rhythm, 2014

ABOUT THE AUTHOR

Lotte Catharina Albertina Stiekema (15 maart 1989, Alkmaar) is opgegroeid in het Twentse Nijverdal en Almelo. Na het behalen van haar gymnasiumdiploma aan het O.S.G. Erasmus in Almelo, verhuisde zij in 2007 naar Amsterdam om geneeskunde te studeren aan het Academisch Medisch Centrum (AMC). Na wat omzwervingen over de wereld met onder andere stages in Paramaribo en Brussel en een taalcursus Frans in haar 'coschap wachttijd' in Lyon, behaalde zij in 2014 haar artsexamen.

Na haar afstuderen begon Lotte haar artsencarrière als arts-assistent (ANIOS) bij de afdeling Interne Geneeskunde in het OLVG Oost. Aansluitend startte zij in november 2015 met haar PhD-onderzoek bij de afdeling Vasculaire Geneeskunde in het AMC in de groep van prof. dr. Erik S.G. Stroes. Gedurende haar PhD onderzocht zij welke invloed lipiden hebben op de productie en functie van ontstekingscellen in de context van atherosclerose, wat uiteindelijk geresulteerd heeft in dit proefschrift.

Eind 2019 verhuisde Lotte samen met haar vriend Amit naar Londen (Verenigd Koninkrijk), waar zij voor 1 jaar als postdoctoraal onderzoeker verbonden was aan de afdeling Haemato-Oncology van het Barts Cancer Institute en St. Bartholomew's hospital (Barts Health NHS Trust). Hier deed zij translationeel onderzoek naar de samenstelling van metabolieten in de adem van leukemie- en lymfoompatiënten in de groep van dr. John C. Riches. Begin 2021 keerde zij weer terug naar Amsterdam, alwaar zij is gestart met de opleiding tot internist aan het Amsterdam UMC, locatie AMC.

DANKWOORD

En dan nu waarschijnlijk het meest gelezen – en daarmee meest impactvolle – hoofdstuk van mijn thesis: het dankwoord. Volledig terecht wat mij betreft, want onderstaande mensen hebben verreweg de meeste impact gehad op mijn PhD traject.

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Dear John, I'm very grateful that you gave me the opportunity to come to London and step into your world of cancer immunometabolism. Although 2020 had slightly other plans, I am proud that our study got initiated and I am confident it will lead to exciting new insights.

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diplomatieke buffers was geen crisis te gek voor jullie. Bedankt dat jullie altijd een rots in de branding waren.

Lieve collega's van het trialbureau, wanneer het op de vroege morgen nog donker en verlaten was op F4, kon ik er altijd op rekenen dat jullie al beneden zaten met een verse pot koffie. Hans en Sandra, dank jullie wel voor het regelen van de onmogelijke planning van de monocyten substudie tijdens de AKCEA. Liesbeth, bedankt voor al je hulp bij de ANITSCHKOW studie, zonder jou was het afronden van deze enorme studie een stuk minder soepel verlopen. Linda, dank voor de vele vers gezette koffies en bovenal de gezelligheid tijdens de kinderstudies. Wat hebben we gelachen! Marianne, Petra en Jet, we hadden geen gezamenlijke projecten, maar ook jullie bedankt voor alle hulp en gezelligheid! Nanet, mijn eerste Vasculicious wintersport roomie en gelukkig trendsetter voor vele wintersport weekenden mét trialbureau. Bedankt dat je antwoord hebt op elke vraag. Daniela, bedankt voor alle steun, adviezen en vooral ook je warmte door de jaren heen!

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Lieve Vasculicious, waar te beginnen? Wintersport, zomersport, karaoke, vasculaire gala's, congressen, vasculaire prof feestjes, F4-lunches, flaneersessies, bbq's, oktoberfest, borrels. Je zou bijna vergeten dat er ook nog gewerkt wordt, maar dat wordt er zeker. We doneren liters bloed en poep, liggen voor elkaar in de MRI en helpen elkaar met moeilijke infusen of statistiek. Het groepsgevoel zit zo diep in ons DNA, dat dit zich ook uitte buiten de F4-muren. Een greep uit mijn favoriete vasculaire momenten: met z'n allen meedoen aan de INTOX Kees-controlled

trial in de Oostenrijkse bergen, op de vrijmibo met z'n allen tegelijk opgeven als stamceldonor (meer donoren is meer beter), bij tropisch weer zwemmen in de plas of in de Amstel en elkaar elke zomer weer motiveren om samen te trainen voor de Dam-tot-Dam (ik stond erbij en keek ernaar). Wetende dat er altijd een leger aan collega's voor je klaar staat, maakte dat ik mijn PhD tijd als een plateaupiek heb ervaren! Bedankt voor alle mooie herinneringen!

En nog een paar collega's in het bijzonder: Renaat, hoewel we officieel geen gezamenlijke projecten hadden, hebben we officieus samen onze promotie uitgevoerd. Kamers op F4 en congressen gedeeld, uren samen in de celkweek en op de glitterbal doorgebracht. Gelukkig kent een plateaupiek geen einde. Kris, voor jou gaf ik met liefde mijn 1-persoonskamer op F4 op. Live, Laugh, Love; ondanks (of dankzij?) het zuurstoftekort hadden we een mooie tijd in dat hok(je). Laten we snel weer onze vriendjes mee uiteten nemen naar een goede Italiaan. Rèèèèèèns, wat een mooie en hysterische tijd hebben we beleefd, ik ben blij dat ik 120% daarvan op beeld heb staan. Merel, zonder jou geen team indohurk of team atrofiesta. Thijs, buuf, escalator, stop de bloeding, red een (jouw) leven. Guid, inderdaad, we go way back. Hopelijk gaan onze paden elkaar weer kruizen in de toekomst. Kang, door jouw nuchtere blik op mijn dagelijkse drama ben ik redelijk mindful mijn PhD doorgekomen. Onze nachtelijke studie waarbij we bijna het loodje hadden gelegd ga ik nooit meer vergeten. Jan, over nachtelijke studies ga ik maar niet beginnen.. Gelukkig hebben we in de celkweek ook vele uren gegierd van het lachen. Sooph, je hebt mij leren mono's isoleren en zoveel meer. Een betere start van mijn PhD had ik niet kunnen wensen! Fleur, zonder jou was er überhaupt geen vacature voor dit onderzoek, bedankt dat je niet alleen de weg hebt vrijgebaand, maar ook hebt geasfalteerd voor mij! Simoon, we houden nog steeds het record van meeste screeningen en inclusies en ik denk niet dat die snel verbroken zal worden. Wat een werk hebben we samen verzet, ongelofelijk. Je bent mijn voorbeeld hoe je wetenschappelijke presentaties (en verdediging!) moet geven. Amaaazing Eriks group, zonder jullie geen amazing. Ik vind het bijzonder hoe hard we met én voor elkaar hebben gewerkt. Ook op congressen konden ze niet om de 'Amsterdam group' heen. Ik ga de (zelf)humor en het lage niveau (I'm out) missen! YanNick, mooi dat jullie het stokje hebben overgenomen en inmiddels ook weer hebben doorgegeven!

Lieve Ry, Lex, Pat, Teun, Ro en Baar, gezelligheid kent geen tijd en tóch vliegt de tijd! Ik ben erg benieuwd of de tijdscapsule deze PhD had voorspeld...

Lieve PBs, wat een bijzonder fijne club! Door de jaren heen hebben we vele mijlpalen en successen samen gevierd (en dat waren er nogal wat!). Ik vind het bijzonder dat

jullie mij en de anderen altijd de wereld gunnen; ik kan me geen betere personal-cheerleaders-in-life voorstellen! Bedankt daarvoor!

Lieve paranimfen, lieve Els en Giel. Een logischer duo naast mijn zijde is er wat mij betreft niet. Els, als tweelingzus weet jij op elk moment van de dag hoe ik mij voel aan de hand van de stand van de sterren. Giel, als halve astronaut (of was het astroloog?), maar ook als medisch lotgenoot, voel ook jij als geen ander aan hoe het met mij gaat. Hoewel onze triangle nu geografisch een beetje is uitgelubberd, bestaat de trust nog zeker. Dank dank dank!

Lieve pap, mam en Peter, bedankt dat jullie er altijd voor me zijn geweest ondanks de afstand en ik jullie minder vaak zag dan dat ik eigenlijk wilde. Ik prijs me gelukkig dat ik altijd in een warm nest kon terugkeren en dat er door jullie wél *altijd wat in Almelo te doen was*! Toch ben ik erg blij dat jullie en Angela uiteindelijk voor het westen hebben gekozen. Pap, ik ben onder de indruk van je harde werken om de vicieuze cirkel te slim af te zijn en ik ben je drie (ex-)huisgenoten erg dankbaar voor hun onvermoeibare ondersteuning daarin!

Lieve Amit, het boekje over LDL grote C en Lp kleine a is eindelijk af! Ik kan niet wachten totdat je samen met Sal je tong gaat breken op dit proefschrift. Hoewel jij van mening bent dat jij vrij weinig met dit proefschrift te maken hebt, hoop ik dat je je na al die jaren realiseert dat er in beginsel (nee hoor, grapje natuurlijk) geen letter op papier had gestaan zonder jou. Bovendien was jij reviewer nummer 1 van het belangrijkste hoofdstuk in dit boek. Dank daarvoor!