Diabetes-related factors and atherosclerosis regression

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Diabetes-related factors and atherosclerosis regression

Tatjana Josefs

@ Tatjana Josefs, Maastricht 2020

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Diabetes-related factors and atherosclerosis regression

DISSERTATION

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the Board of Deans, to be defended in public on

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by

Tatjana Josefs

Promoters:

Prof. Dr. C.G. Schalkwijk (Maastricht University)

Prof. Dr. E.A. Fisher (NYU School of Medicine)

Co-Promoter:

Dr. K. Wouters (Maastricht University)

Assessment Committee:

Prof. Dr. C. Reutelingsperger (Chair, Maastricht University)

Prof. Dr. E.A.L. Biessen (Maastricht University)

Prof. Dr. M.P.J. de Winther (Amsterdam UMC)

Dr. M. Westerterp (University of Groningen)

Dr. R. Koenen (Maastricht University)

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1

General Introduction

Diabetes and Cardiovascular Diseases (CVD)

Type1 & Type2 Diabetes Mellitus (T1DM, T2DM) are common diseases with increasing prevalence worldwide.¹ In the United States alone, diabetes is the most expensive chronic disease, reaching about \$327 billion in 2017.² The global costs of diabetes are estimated around \$727 billion.³ Subjects suffering from diabetes, with the majority being diagnosed as T2DM⁴, have a significantly increased risk of atherosclerosis and associated cardiovascular events (CVE).⁵ This is due to the fact that metabolic risk factors for atherosclerotic CVD are commonly found in subjects with diabetes. Hyperglycemia is an independent risk factor for CVD, next to genetic predisposition, hypertension and diabetic dyslipidemia. The adjusted relative risk for T2DM compared to non-T2DM patients is 1.7 for cardiovascular death, 1.8 for myocardial infarction, and 1.5 for stroke.⁶

Cardiovascular diseases (CVDs) are the deadliest disease worldwide and despite a steady decline in death from CVD due to advances in scientific discoveries, clinical cardiology as well as public health, it is still responsible for 30% of deaths.⁷ The global cost is estimated to be \$863 billion and is predicted to increase by \$1044 billion until 2030⁸ due to a rising and aging population and the high incidence of secondary complications after survival of CVD incident.

Atherosclerosis

Atherosclerosis is the underlying vascular pathology of myocardial infarction (MI) and stroke, the two main CVDs, and describes a chronic-inflammatory disease that involves the formation of lesions (=plaques) in medium-sized to large arteries.⁹ Atherosclerotic plaque progression begins in childhood¹⁰ and progresses silently before symptoms occur. A key initiating step of atherosclerosis is the deposition of apoB-containing lipoproteins in the intima, followed by accumulation of cholesterol, immune cells, foam cells, and smooth muscle cells leading to the progression of atherosclerotic plaques.¹¹ By the age of 40, 95% of people have some lesions and problems occur if the lesions lead to reduced blood flow or ruptures and occludes vessel, thereby interfering with tissue oxygenation.¹² Plaque rupture and thrombus

formation preceding acute coronary events such as MI and stroke are thought to be its main cause and the result of vulnerable (=rupture-prone) plaques.¹³ Prominent features of such vulnerable plaques (often referred to as thin-cap fibroatheroma) are large lipid- and macrophage-rich cores and necrotic areas covered by a thin fibrous cap.¹⁴ Additional characteristics associated with plaque vulnerability are the presence of cholesterol crystals, microcalcifications and intraplaque angiogenesis.¹⁴

Atherosclerosis regression describes the repair of blood vessels initiated by markedly reduced circulating cholesterol levels in animals and in humans¹⁵; cholesterol and macrophages leave the arterial wall and are replaced by collagen, reducing plaque vulnerability. Given that many patients start treatment once lesions have already formed, attaining atherosclerosis regression is an unmet, highly desirable, clinical goal.

Autopsy¹⁶⁻¹⁸ and clinical studies have confirmed the ability of human atherosclerotic plaques to regress. In clinical studies, plaque size and later arterial wall thickness (assumed to be equivalent to plaque volume) was measured using angiography or intravascular ultrasonography. However, reported changes in plaque size/volume¹⁹⁻²³ are rather small compared to ~80% reduction in clinical events in men with coronary artery disease (CAD) and ~50% in individuals suffering from the metabolic syndrome with CAD.²⁰ The modest quantitative changes suggest that changes in plaque composition might pre-dominate over change in plaque size, as already reported by Brown et al. in 1993.²⁴ In contrast, results from the GLAGOV trial, which reported a ~1% regression in plaque volume with addition of a proprotein convertase subtilisin/kexin type 9 (PCSK9 inhibitor) to statin therapy, did not find changes in plaque composition compared to statin monotherapy.²⁵ Further studies and especially advances in imaging modalities^{26,27} to establish the predictive ability of plaque composition and CVD risk are needed.

As current techniques to evaluate regression in humans are not optimal, the study of regression in experimental animal models is of high significance. Animal models of atherosclerosis regression, many pioneered by the Fisher laboratory, have significantly increased the understanding of the mechanisms underlying the resolution of lesions and show that regression does not simply involve the same mechanisms as atherosclerosis progression just in reverse order, but has its distinct processes. A couple of recent reviews describe the underlying mechanisms of atherosclerosis regression in detail.^{15,28-30} Further, processes leading to a stable plaque such as

deposition of collagen matrix can hinder a detection in reduced lesion size. Therefore, in animal models we have characterized atherosclerosis regression as not necessarily a reduction in lesion size, but as a reduction in lipid and/or macrophage content, a switch from a proinflammatory to a pro-resolving macrophage, smaller necrotic core size, and an increase in collagen.

Mouse Models to study atherosclerosis regression

Mice are resistant to atherosclerosis, which is likely due to their relatively low low-density lipoprotein cholesterol (LDL-C) levels and the fact that most of their plasma cholesterol is carried by high-density lipoproteins (HDL).¹⁵ To study atherosclerosis in mice, two genetically modified mouse models were developed in the early 1990s to hinder efficient lipoprotein clearance and thereby increase plasma cholesterol levels leading to atherosclerosis progression – 1. The apolipoprotein E deficient (*ApoE*^{-/-}) mouse model was created by the Breslow³¹ and Maeda³² laboratories; 2. The low-density lipoprotein deficient (*Ldlr*^{-/-}) mouse model was developed by the Brown and Goldstein laboratory³³. Feeding a western diet (WD; high fat, high cholesterol) can accelerate the development of atherosclerotic plaques in these models.¹⁵

There are several mouse models to study atherosclerosis regression, which are based on lowering LDL, raising HDL or treatment with anti-miR33³⁵. LDL-lowering strategies include re-expression of ApoE in *ApoE*^{-/-} mice^{36,37} and Ldlr in *Ldlr*^{-/-} mice³³ using an adenovirus; transplanting a plaque burdened thoracic arch or aortic arch segment to normolipidemic animals³⁸; Reversa mouse with conditional lowering of LDL cholesterol (*Ldlr*^{-/-} *ApoB*^{100/100} *Mttp*^{fl/fl} *Mx1Cre*^{+/+})³⁹; and switching *Ldlr*^{-/-} from WD to chow diet⁴⁰. Novel strategies have been developed to study regression in any mouse model, not being limited to mice with a genetic background of *Ldlr*^{-/-} or *ApoE*^{-/-}. These methods first induce LDLr deficiency using PCSK9-adenovectors⁴¹ or LDLrASO⁴², followed by induction of regression using antisense oligonucleotides against ApoB⁴³ or microsomal triglyceride transfer protein (MTTP)⁴¹. Raising HDL particle levels is achieved either genetically⁴⁴ or by infusion⁴⁵ of apoA1 or HDL respectively. Space does not permit full description of all atherosclerosis regression models but will be focused on the ones used in this Thesis and are all based on lowering LDL.

The aortic transplant mouse model was pioneered by the Fisher laboratory in

the early 2000s and involves the transplantation of an atherosclerotic arch from a hyperlipidemic donor mouse (e.g. *ApoE*^{-/-} or *Ldlr*^{-/-}) into a normocholesterolemic recipient mouse, interpositioned with the abdominal aorta and blood flow directed through the graft. This leads to a rapid environmental change and is not of transient nature as some other atherosclerosis regression models, hence leading to plaque regression over a surprisingly short time (<1 week).

In 2013, the Fisher laboratory showed that atherosclerosis regression could also be achieved by switching an *Ldlr*^{-/-} mouse from a WD to a chow diet over an extended period without any intervention.⁴⁰ This method, however, is slower and is variable in its effectiveness.⁴⁶

A novel regression mouse model is using a combination of LDLR antisense and sense oligonucleotides (ASO; SO) and was pioneered by the Goldberg laboratory in collaboration with the Fisher laboratory. In this model, LDL-C is increased by the injection of LDLR ASO, leading to a similar phenotype as *Ldlr*^{-/-} mice. GalNAc-conjugated SO are designed to bind and inactivate the LDLR ASO, and accelerates the return of LDLR expression and normal LDL-C levels.

Another novel mouse model is the PCSK9-adenovirus in combination with an ApoB ASO (MTTP inhibitor, which reduces very low-density lipoprotein (VLDL) secretion, is also an option). PCSK9 directs hepatic LDL receptors for degradation⁴⁷ and thereby reduces LDL receptor expression, hence increasing plasma LDL cholesterol (LDL-C). ApoB ASO reduces hepatic apoB mRNA expression, hence reduces VLDL secretion and decreased plasma cholesterol levels by about 90%.⁴³

Diabetes-associated factors are thought to contribute to the increased risk of developing CVD in individuals suffering from diabetes compared to non-T2DM individuals. Two of these factors are elevated TG levels, a feature of diabetic dyslipidemia, and hyperglycemia-induced neutrophilia. This Thesis is divided into two parts to study each of these factors in regard to atherosclerosis regression.

Triglycerides and their role in atherosclerosis

The question if triglycerides (TGs) are atherogenic has vexed clinicians for more than half a century and is still debated. Already in 1973 Goldstein et al. reported that

elevated TG levels are common in CVD patients⁴⁸ and in the early 1980s, LDL-C and TG were seen as equal contributors to atherosclerosis.⁴⁹ The role of LDL-C in CVD has been largely established, including by the Nobel prize winning discoveries by Brown and Goldstein in 1985 leading towards the development of the LDL-C lowering drug class called statin (3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) inhibitor). Statin-induced LDL-C reduction lowered clinical events and all-cause mortality (e.g. primary prevention: JUPITER Trial⁵⁰, secondary prevention: PROVE IT-TIMI 22⁵¹). While mean LDL-C levels in the population declined from 1999 to 2016⁵², TG levels have been increasing with the epidemic of obesity and diabetes. Further, although LDL-lowering reduced CVD risk and mortality, there is still residual risk of up to 70%. People with diabetes especially do not respond as robustly to lipid lowering therapies as non-diabetics, and even after lipid-lowering sustain increased risk.^{53,54}

Despite studies showing an association of elevated circulating TG-rich lipoproteins with increased CVD risk, it is still unclear if this is due to direct toxic effects on arteries or whether this is merely an association. It is challenging to disentangle the role of TG in CVD risk because elevated plasma TG levels are always accompanied by other factors that themselves are plausibly causally related to CVD, e.g. low HDL-C and high small dense LDL-C levels in diabetic dyslipidemia⁵⁵ as well as non-lipoprotein parameters such as hypertension, a procoagulant state, hepatosteatosis, or abdominal obesity⁵⁶. This uncertainty also led to a lack in treatment options for elevated TG and currently TG treatment is not included in the latest recommendations to lower CVD risk in the United States and optional in Europe. Given the increasing evidence that TG-rich lipoproteins can cause CVD and are not only an innocent bystander, further research is needed to finally disentangle the role of TGs in CVD.

Definition of hypertriglyceridemia(HyperTG)

The standard practice to measure TG levels is after 12h of fasting. Fasting TG levels of >1.7mmol/l (>150mg/dL) are generally accepted to be classified as HyperTG and can be further classified into borderline high (1.8-2.2mmol/L, 150-199mg/dL), high (2.3-5.6mmol/L, 200-499mg/dL), and very high

	TG	
	mmol/L	mg/dL
Normal	<1.7	< 150
borderline high	1.8 - 2.2	150 - 199
high	2.3 - 5.6	200 - 499
very high	>5.7	>500

Table 1. Classification of circulating triglyceride (TG) levels according to the national lipid association

(>5.7mmol/L, >500mg/dL) according the National Lipid Association (Table 1).⁵⁷ The prevalence of HyperTG in the USA was 47% in 2010, which was largely driven by lifestyle factors.⁵⁸ While in Europe, 27% had nonfasting TG levels of >2.0mmol/L in 2016.⁴⁹ Off note, nonfasting TG levels measured 1-6h after meals only change 0.2mmol/L (8mg/dL) compared to fasting TG levels, leading to the suggestion from the European Atherosclerosis Society to assess the plasma lipid profile in nonfasting blood.⁵⁹ Further, both non-fasting and fasting plasma TG levels have been associated with CVDs.

TG metabolism & causes of HyperTG

TGs are carried within chylomicrons and VLDL (=TG-rich lipoproteins, or TRL) and contain exogenous and endogenous TGs (Figure 1). Exogenous TGs enter the human body via the diet and together with apoB48 they are packed into chylomicrons within enterocytes. Chylomicrons are the largest lipoprotein particles and have a TG content of about 80-95%. Through interaction of chylomicrons with HDL particles in the intestinal lymph capillaries, chylomicrons acquire apoC-II, apoC-III, and apoE on their way through the lymphatic system until they enter the subclavian vein via the thoracic duct. Endogenous TG are synthesized in hepatocytes by glycerol-3-phosphate dehydrogenase using free fatty acids (FFA) and glycerol and form together with apoB100 VLDL particles and obtain apoC-I, apoC-II, apoC-III, and apoE during secretion from hepatocytes.

Both, chylomicrons and VLDL are hydrolyzed by lipoprotein lipase (LpL) on capillary endothelial cells in peripheral tissues, resulting in FFA and chylomicron remnants/smaller VLDL particles followed by intermediate-density lipoproteins (IDLs). FFA can enter cells and either be resynthesized into TGs and stored in adipose tissue (AT), or they can be oxidized, mainly in mitochondria, by various cell types for energy retrieval. Chylomicron remnants can be cleared by the LDLr or LDLr-related proteins on hepatocytes. IDLs can be similarly taken up by hepatocytes and catabolized or can undergo further hydrolysis by LpL or hepatic lipase forming LDL particles. Further changes in VLDL and LDL is done by cholesterol ester transfer protein (CETP), which exchanges TG and cholesteryl ester (CE) between these lipoproteins and HDL.

HyperTG is the result of an increase in TG-rich lipoproteins due to increased

synthesis of TG-rich lipoproteins, decreased catabolism, or the combination of both. The most common form of HyperTG is mild-to-moderate HyperTG (1.8 – 5.6mmol/L) which is characterized by an increase in primarily VLDL and its remnants⁶⁰. Mild-to-moderate HyperTG can be due to polygenic traits in the LpL gene and related factors (e.g. *GPIHBP1*, *APOC2*, *APOA5*, *GPD1*, *LMF1*). HyperTG also often occurs as a secondary event in patients with the metabolic syndrome and/or T2DM as part of the diabetic dyslipidemia (Figure 1). In T2DM, an insulin resistant state, the production of VLDL is increased while the hepatic uptake of VLDL, IDL and LDL is decreased. This together with a reduction in LpL activity results in an increase in circulating TRLs. The large amount and prolonged time of TRLs in the circulation drives the CETP reaction, leading to TG-enriched LDL and HDL that are more readily hydrolyzed by hepatic lipase⁶¹ and endothelial lipase⁶² and smaller HDL particles are cleared faster by the kidney. The result is atherogenic small dense LDL particles, reduced HDL-C concentrations and elevated TG levels - diabetic dyslipidemia. Further, HyperTG can be the result of unhealthy diet or lifestyle choices such as alcohol overuse, lack of exercise, hepatosteatosis, hyperthyroidism, pregnancy, nephrotic syndrome, certain medications such as glucocorticoids and weight regain after substantial loss.⁵⁶

Severe HyperTG is (>5.7 mmol/L) mainly associated with an increase in chylomicrons, hence also called chylomicronemia. The very rare monogenic chylomicronemia (also called familial chylomicronemia) is mainly caused by homozygous mutations in LpL gene (>90%; Figure1) or its cofactors⁶³ and leads to HyperTG by an increase of chylomicrons only.⁶⁴ Polygenic chylomicronemia is more common and, in contrast to monogenic chylomicronemia, is not only associated with an increase in chylomicrons, but also with an increase in VLDL, and remnants of both.^{63,65} Secondary factors, such as an unhealthy lifestyle, can exacerbate TG levels.

Figure 1. Simplified illustration of the TG metabolism and two causes leading to hypertriglyceridemia

TG, Triglyceride; FFA, free fatty acid; HL, hepatic lipase; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; HDL, high-density lipoprotein; VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; sdLDL, small dense LDL; LpL, lipoprotein lipase

Hypertriglyceridemia and CVD

Lipid-lowering agents, especially statins, have lowered CVD risk by 25-35%.⁶⁶ However, a prospective meta-analysis of data from 90,056 participants showed that there was a residual CVD risk in patients with preexisting CVD treated with statins that was not due to LDL-C, suggesting that it might be related to other lipoproteins such as TRL or HDL.⁶⁷ One of the first large studies describing the association of TG with CVD was the Framingham study in 1986.⁶⁸ Since then, the perception of TG's role in CVD has fluctuated over the past decades from being the cause of CVD to only being an innocent bystander. This fluctuation is mainly due to TG's close relationship with HDL-C (due to changes in LpL and CETP actions, see Figure1). Due to the fact that HDL-C and TG are so closely associated, it remained unclear which of these two lipid fractions were the real cause of increased CVD risk and a number of studies failed to show an independent association of TG with CVD risk. These studies report that after

adjustment for other risk factors, especially HDL-C, TG did not add any additional predictive value.⁶⁹⁻⁷² Factors making it difficult to detect an independent relationship could be the small numbers of participants of some of the studies, the TG measurement itself, which is known to be difficult because of the high variability of TG levels in a single individual, including the lack of its correction.⁷³⁻⁷⁸ At the same time, low HDL-C was independently associated with increased CVD risk⁷⁹⁻⁸¹. However, with HDL-C raising trials failing to show benefits on CV outcome and additional evidence from epidemiological and genetic studies, TG and remnant cholesterol might be more likely the cause for CVD risk rather than HDL-C.⁸²

Evidence for the independent role of TG in CVD is growing and indicates that individuals with HyperTG are at increased risk to develop CVD.⁸³ This has been shown in meta-analyses^{67,84-86}, case-control studies^{79,87-92} and genetic studies⁹³⁻⁹⁵. In 2007, a meta-analysis of the western population including 262,525 patients, an odds ratio of 1.72 (95%CI 1.56-1.90) for incident CVD has been reported for patients in the top tertile of TG levels after adjusting for common risk factors.⁸⁵ This odds ratio is similar to the one observed in the meta analyses of the Asian and Pacific population⁸⁴. The Copenhagen City Heart Study showed that for individuals with severe HyperTG (nonfasting, >6.6mmol/L; 580mg/dL) the risk for myocardial infarction was 5.1-fold increased compared to individuals with normal TG levels (0.8mmol/L;70mg/dL), 3.2-fold increased for ischemic heart disease, 3.2-fold increased for ischemic stroke, 2.2-fold increased for all-cause mortality.⁴⁹ Interestingly, the risk for ischemic heart disease in women with elevated plasma TG levels is higher than for men.^{86,96,97} Another meta-analysis by Murad et al. included 35 studies with > 920,000 subjects and reported an odds ratio of 1.80 (95%CI, 1.31-2.49) for cardiac events, 1.31 (95%CI, 1.15-1.49) for myocardial infarction and 3.96 (95%, CI 1.27-12.34) for pancreatitis.⁹⁸ TG is also an independent risk factor for CVD in individuals with diabetes⁹⁹⁻¹⁰², the metabolic syndrome¹⁰³⁻¹⁰⁶ or familial combined hyperlipidemia^{107,108}.

The most persuasive evidence for TG's causal role in CVD risk comes from recent genetic studies. In a large mendelian randomization trial of about 20,000 myocardial infarction cases and 50,000 control participants, a 1SD increase in TG levels was associated with a 54% increase in myocardial infarction risk, while low HDL-C showed no association.¹⁰⁹ Further, increased TG levels due to variants in

APOA5 increased the risk for coronary heart disease (OR 1.18, 95%CI 1.11-1.26).^{110,111} A study using individuals from the Copenhagen City Heart Study with variants in the LpL gene showed that a reduction in TG levels of 1mmol/L (88.5 mg/dL) was associated with a 50% reduction in all-cause mortality risk.⁹⁵ Also variants in the APOC3^{112,113} and ANGPTL^{114,115} genes point towards a casual effect of TG on CVD risk.

Mechanisms of TG atherogenicity

The exact mechanisms by which TG may promote atherosclerosis remain to be elucidated. Until recently, the general accepted view was that patients with HyperTG due to increased circulating chylomicrons were resistant to atherosclerosis¹¹⁶, because these lipoproteins were thought to be too large to transverse the endothelium. Animal models support the hypothesis that larger TG-rich lipoproteins are less atherogenic than smaller cholesterol-rich lipoproteins, however they are not totally harmless. While chylomicrons may be too big to enter the arterial wall, chylomicron remnants and VLDL and its remnants are able to enter arterial wall¹¹⁷ and are suitable for transcytosis in specialized clathrin-coated vesicles.^{118,119}

Although patients with severe HyperTG (chylomicronemia) have low risk for atherosclerosis^{120,121}, these subjects usually also have low LDL-C levels, likely contributing to the decreased CVD risk. However, for most of the population, HyperTG is in the range of mild to moderate HyperTG, tying increased atherosclerosis risk to smaller TG-rich particles and their remnants.

In 1973, Zilversmit hypothesized that postprandial increases in TG and elevated remnant lipoproteins cause atherosclerosis and proposed two possible mechanisms (Figure 2).¹²² Both are based on the hydrolysis of TG by LpL – one describes the atherogenic potential of remnant particles, the second one the lipolysis step itself.¹²² After crossing the endothelial barrier, remnants can be taken up by arterial macrophages directly¹²³, contributing to foam cell formation.^{124,125} Additionally, TRL remnants carry more cholesterol per particle than LDL due to their size.¹²⁶ For these reasons, remnants are seen as atherogenic and have been linked directly to plaque progression¹²⁷, rather than TG themselves.⁸² The importance of remnants in CVD risk was also confirmed in a meta-analysis using the Emerging Risk Factors Collaboration

data that detected TGs as a strong risk factor for CVD and stroke.⁷² This association attenuated after adjusting for HDL-C, but was lost after adjustment for non-HDL-C/remnants (VLDL, IDL, LDL).⁷² These remnant particles that can cause atherosclerosis are usually rapidly cleared by the liver. However, in individuals with T2DM/metabolic syndrome, hepatic clearance is delayed. Thus, prolonged exposure and increased concentrations raises the possibility for particles to enter atherosclerotic plaques.^{117,128} Additionally, the lipolysis step itself is thought to be atherogenic. LpL can hydrolyze TG into monoacylglycerols and FFA at the endothelial surface or within the arterial intima, which is associated with increased atherosclerosis and CVD.¹²⁹ Overall, uptake and lipolysis of TG-rich remnants have been shown to promote endothelial dysfunction, foam cell formation, increase inflammation, damage smooth muscle cell, and impaired vascular repair.^{124,127,130,131}

Figure 2. Two possible mechanisms for the atherogenicity of triglycerides.

Illustration from Goldberg et al. Triglyceride and Heart Disease. Still a hypothesis? *Arterioscler Thromb Vasc Biol.* 2011;31:1716-1725

Reducing TG levels & Clinical Trials

A key question is whether reducing TG levels is protective of CVD. So far, patients with TG levels >500mg/dl are considered at increased risk of pancreatitis^{57,132-134} and therefore receive TG lowering treatment. Regarding CVD, European Guidelines (ESC/EAS guidelines for the management of dyslipidemias¹³⁵ and European

guidelines on CVD prevention in clinical practice¹³⁶) recommend that individuals with triglyceride levels >1.7 mmol/l (>150 mg/dl) should be considered at increased risk of CVD, but did not include risk estimation of TG or recommended target values. Earlier guidelines from the adult treatment panel are in line with the European guidelines¹³⁷, but more recent guidelines from the ACC/AHA on the assessment of CVD risk don't mention TGs at all.¹³⁸ However, reducing TG could be beneficial for CVD, especially in individuals with preexisting coronary heart disease and diabetes.^{73,137,139,140}

Several clinical trials of TG lowering have reported benefits for CVD risk.^{60,82,141} Medications that have shown efficacy in reducing TG levels are fibrates, statins, niacin and omega-3 PUFA. **Fibrates** lower TG by 30-50% depending on the baseline TG levels and are the most efficient TG lowering drug⁷³. This TG reduction is due to an increase in FA oxidation, LpL synthesis and a reduction in apoC-III expression. A study using the fibrate gemfibrozil reduced CVD risk in patients with elevated TG levels.^{142,143} Further, a meta-analysis of 18 trials and 45 058 patients reported a 10% relative risk reduction for major CVD, predominantly by prevention of coronary events, in individuals with HyperTG alone or in combination with HDL-C with fibrate intake.¹⁴⁴ Also posthoc analyses of fibrate trials revealed that especially patients with low HDL-C (e.g. ACCORD trial <34mg/dL) and elevated TG (e.g. ACCORD >204mg/dL) levels as features of the metabolic syndrome and diabetes have CVD benefits from TG lowering using fibrates.^{145,146} **Statins** are used to lower LDL-C and have been shown to also reduce TG and non HDL-C by 10-15%.¹⁴⁷ A study of pravastatin in diabetics suggests that low HDL-C and elevated TG were significant predictors of increased CVD risk.¹⁴⁸ Unfortunately, combination therapies of statins and fibrates have failed to show additional CVD risk benefits.^{149,150} **Niacin** is a B vitamin that reduces TG production by the liver^{151,152} and lowers TG levels depending on its dose up to 50%¹⁵³. Clinical trials failed to show a decrease in CVD event when niacin is added to statin treatment (AIM-HIGH⁶⁶, HSP2-Thrive¹⁵⁴). However, posthoc analyses of the AIM-HIGH Trial suggested that there might be a benefit in individuals with TG >200mg/dL.¹⁵⁵ Daily intake of 3-4mg/day of **omega-3 PUFA** (eicosapentaenoic acid (EPA) + DHA) from marine source, but not plant-derived, has been shown to reduce TG levels by 45% in individuals with HyperTG.¹⁵⁶⁻¹⁵⁹ A recently published Trial, the REDUCE-IT Trial, showed that in statin treatment patients with elevated TGs and CVD or diabetes, treatment with icosapent-ethyl, a highly purified EPA, reduces CVD risk including first,

subsequent, and total ischemic events^{160,161}. However, it is still unclear if the reduction in ischemic events was due to lowering TG or due to a combination of effects involving plaque instability reduction and inflammation. Notably, no differences in HDL-C upon icosapent-ethyl were detected.

Overall, clinical trials that appear to confirm CVD benefits of TG-lowering therapy rely on subgroup analyses and trials designed to focus on subjects with HyperTG only are missing. Thus, current results are non-conclusive. The REDUCE-IT trial was the first published trial to determine the relationship between TG and atherosclerosis in a population with elevated plasma TG levels and there are currently further clinical trials ongoing (e.g. STRENGTH) with results being expected by 2022 (Reviewed in¹⁶²).

Neutrophils and their role in atherosclerosis

Neutrophils are polymorphonuclear cells and are, with about 75% of the leukocyte population, the most abundant immune cell in the adult human body.¹⁶³ Leukocytes can be divided in subtypes (neutrophils, lymphocytes, monocytes, basophils, eosinophils) and are essential mediators of the immune system that derive from hematopoietic stem cells (progenitor cells).¹⁶⁴ Hence, leukocytes are responsible for fighting against foreign elements and maintaining tissue homeostasis.¹⁶⁵ Since a couple of decades, sterile inflammation by activation and aggregation of leukocytes have been implicated in chronic inflammatory disease^{166,167} and recent results of clinical trials have affirmed the inflammation hypothesis in CVDs (CANTOS, COLCOT).^{168,169}

The relationship between CVD and leukocytes was first suggested over 80 years ago.¹⁷⁰ Since then, CVD and diabetes have both been associated with leukocytosis¹⁷¹, including an association between increased risk of CVD with elevated neutrophil levels.^{172,173} Diabetes is characterized by neutrophilia and monocytosis and clinical studies as well as experimental mouse data suggest that hyperglycemia leads to abnormalities in neutrophil chemotaxis, phagocytosis and bacteriocidal properties^{40,174}, while lowering blood glucose levels using anti-diabetic drugs has been shown to improve neutrophil activity.¹⁷⁵ Neutrophils are present in human¹⁷⁶ and mouse atherosclerotic lesions.¹⁷⁷⁻¹⁸⁰ Further, circulating neutrophil levels have been linked to atherosclerotic burden^{40,177,181} and neutrophil depletion reduces

atherogenesis.¹⁸⁰ In diabetic mice, reduction in circulating neutrophil levels by normalizing blood glucose levels was linked with improved atherosclerosis regression.⁴⁰ In contrast, neutrophils¹⁸² and neutrophil extracellular traps (NETs)¹⁸³ (discussed below) can also have beneficial effects in cardiac tissue healing after a myocardial infarction.

Despite all the evidence of the possible implication of neutrophils in the pathophysiology of CVD, neutrophils have been overshadowed by other leukocyte subtypes such as monocytes and macrophages, partly due to neutrophil's short life span with a half-life of approx. 6 to 8 hours in mice and humans.¹⁸⁴ However, activated neutrophils have increased longevity, which leads to the presence of primed neutrophils at sites of inflammation.¹⁸⁴ Further, recent discoveries showed that neutrophils contribute to chronic inflammatory disease via crosstalk with other cells.^{185,186} Especially the discovery of neutrophil's ability to form NETs, a process called NETosis, has sparked new interest in neutrophils under inflammatory conditions.¹⁸⁷

NETosis

The process of NET formation can unfold in two distinct forms – 'suicidal' (also called lytic) and 'vital' NETosis (Figure 3).^{188,189} Both forms differ in type of trigger, timing and mechanism of NETs release. 'Suicidal' NETosis requires a strong activation of nicotinamide adenine dinucleotide phosphatase (NADPH) oxidase over several hours, chromatin condensation via myeloperoxidase (MPO) and activation of neutrophil elastase (NE), resulting in neutrophil death.^{188,190} This form of neutrophil death is distinct from other forms of cell death such as apoptosis, necrosis or necroptosis.¹⁸⁸ 'Vital' NETosis upon stimulation by, e.g. LPS, entails vesicular trafficking of DNA to the extracellular space without membrane perforation. It is a rapid process compared to 'suicidal' NETosis and results in a viable anuclear neutrophil.¹⁹¹⁻¹⁹³

Irrespective of NETosis form, NETs consist of cytosolic and nuclear material including histones, NE, MPO, proteinase 3 (PR3), cathepsin and gelatinase.¹⁸⁷ Upon stimulation by pathogens, such as bacteria or fungi, neutrophils expel NETs to catch pathogens thereby protecting the host.^{187,194} Thus, NETosis belongs to one of several

strategies (e.g. degranulation, phagocytosis) of neutrophils to respond to damage/infection.¹⁹⁵

Our understanding of how NET formation is controlled is still limited. Several intracellular mechanisms and signaling pathways for NETs formation have been reported and recently reviewed in detail.¹⁹⁶ The original observation of NETosis describes a phorbol myristate acetate (PMA)-induced reactive oxygen species (ROS) production via the NADPH oxidase.¹⁸⁸ Downstream of ROS, peptidylarginine deiminase 4 (PAD4) and MPO lead to chromatin decondensation, a pivotal step of NETosis.^{190,197-200} Thereby PAD4 catalyzes the deamination of arginine residues of histone to citrulline.¹⁹⁷⁻¹⁹⁹ MPO promotes NE release from azurophilic granules²⁰¹ from where NE translocates to the nucleus and partially cleaves histones.¹⁹⁰ Recently, NE has been shown to also mediate the proteolytic activation of the pyroptosis-associated protein gasdermin D, which, along with caspase-11, mediate suicidal NETosis.^{202,203} Independently of the activation of the NADPH oxidase, also mitochondrial ROS formation can lead to NETosis via calcium influx.^{192,204,205} Notably, also other cell types like macrophages or mast cells can release decondensed chromatin – a process called ETosis.^{206,207}

Next to being an important part of the hosts' immune defense, NETs have also been reported to be implicated in many chronic inflammatory disease such as rheumatoid arthritis²⁰⁸, cancer²⁰⁹, diabetes²¹⁰ and atherosclerosis^{211,212}.

Figure 3. Formation of neutrophil extracellular traps (NETs)

NETosis and atherosclerosis

NETosis in context of atherosclerotic disease can be stimulated by infectious triggers such as *Tannerella forsythia*²¹³ and noninfectious triggers such as activated platelets²¹⁴, oxidized LDL²¹⁵ and cholesterol crystals²¹⁶.

In humans, circulating NETs markers (double stranded DNA, nucleosomes, citrullinated histone H4, MPO-DNA complexes) have been independently associated with atherosclerotic lesion size severity of coronary artery disease, a pro-thrombotic state and the occurrence of major adverse cardiac events.²¹⁷ NETs have been shown to be present in intact^{176,218,219} and complicated thrombotic plaques^{220,221} in humans as well as in atherosclerotic plaques of mice^{216,222,223}. Interestingly, autopsied plaques from MI patients revealed significantly more NETs in complicated plaques (e.g. superficial erosion or hemorrhage) compared to intact plaques.²²¹

Experimental mouse studies revealed that genetic and pharmacological inhibition of NETs formation leads to reduced plaque size, inflammation and plaque stability.^{216,223,224} Within the plaque, NETs components and especially the interrelationship between neutrophils and macrophages have been demonstrated to modulate atherosclerotic plaques by recruiting inflammatory monocytes, activation of the inflammasome and oxidative changes to lipoproteins.

NET components such as MPO trigger macrophages to release ROS and other cytokines.²²⁵ In turn, ROS can modify LDL to oxLDL, which drives foam cell formation.^{226,227} Released cytokines recruit immune cells and oxLDL can trigger neutrophils for further NETosis²¹⁵, augmenting plaque inflammation and formation. MPO or NADPH oxidase can also lead to oxidative modifications of HDL and thereby reduces its beneficial cholesterol efflux capacity.²²⁸ Other NETs components like cathepsin G, cathelicidins and cathelicidin-related antimicrobial peptide (CRAMP) exhibit monocyte-chemotactic activity.²²⁹⁻²³² CRAMP, together with self-DNA released by NETs or other dying cells, can trigger plasmacytoid dendritic cells (pDCs)-driven autoimmune activation²³³, which has been shown to aggravate atherosclerosis lesions.²³⁴ Recently, Warnatsch et al. has shown that NETs lead to inflammasome activation in macrophages.²¹⁶ In this setting, cholesterol crystal-activated NETs present the 1st signal for inflammasome activation in macrophages resulting in pre-mature interleukin (IL)-1 β , with cholesterol crystals being the 2nd signal, leading to

mature IL-1 β .²¹⁶ Subsequently, IL-1 β upregulates IL-17, which, in turn, enhances recruitment of immune cells to atherosclerotic site. Inflammasome activation itself can also lead to NET formation, which was shown by a study from Westerterp et al. using mice deficient for myeloid Abca1/g1 (ATP-binding cassette A1/G1), two key transporters in promoting cellular cholesterol efflux. Thereby intracellular cholesterol accumulation in myeloid cells induced NLRP3 inflammasome activation, which promoted neutrophil recruitment and NETs formation in atherosclerotic plaques and accelerated atherogenesis.²²²

NETosis and diabetes-induced atherosclerosis

Plasma NETs markers are not only correlated with severity of CAD, but also with severity of T2DM as judged by correlation of nucleosomes with HbA1c (hemoglobin A1c) in humans.²³⁵ Hyperglycemia seems to facilitate constitutively upregulated NETosis by priming neutrophils for NET formation²¹⁰, and by providing increased substrate for NETosis, which has been shown to require glucose²³⁶. The activation of neutrophils can be reversed by exposing neutrophils to physiological conditions.^{235,237} A study by Wong et al. showed that NETs impair wound healing in diabetic mice²¹⁰ and humans²³⁸ and a recent study by our lab showed that diabetic mice have increased amount of NETs in atherosclerotic plaques.⁴⁴ Given that a diabetic environment enhances processes that are related with NETosis, increased NET formation in atherosclerotic lesions from diabetic mice and the fact the wound healing resembles atherosclerosis regression in terms of the need of inflammation resolution, it is likely that NETs contribute to diabetes-impaired atherosclerosis regression and the increased CVD risk in patients suffering from diabetes.

NETs as a therapeutic target

Several possible therapeutic targets to reduce NETs have been reported.¹⁸⁵ Two targets have been studied in context of atherosclerosis – 1. PAD4 inhibition and 2. DNase1. PAD4 inhibition prevents NET formation via the PAD4-mediated conversion of arginine to citrulline, thus inhibiting histone citrullination and chromatin decondensation. A study in *ApoE*^{-/-} mice with *Pad4*^{-/-} myeloid cells showed diminished

plaque formation with reduced NETosis and atherogenic inflammation.²²³ Studies using Cl-amidine, a pan-PAD inhibitor, have shown to reduce atherosclerosis in *ApoE*^{-/-} mice by preventing NETosis and reducing the recruitment of neutrophils and monocytes.²²⁴ Further, in diabetic mice, *Pad4*^{-/-} improved wound healing.²¹⁰ In contrast, a study in *Ldlr*^{-/-} mice with *Pad4*-deficient bone marrow cells showed no altered atherogenesis.²³⁹ Further, Cl-amidine is not specific for PAD4 and targets all PAD isotypes, and additionally, PADs, including PAD4, have many functions such as the activation of dendritic²⁴⁰ and smooth muscle cells²⁴¹ and cannot undoubtedly demonstrate NET involvement. A recent study by Lewis et al. reported specific PAD4 inhibition, which was sufficient to disrupt NET formation in mice and humans²⁴² and further studies with this molecule are needed.

One of the major postulated side effects in inhibiting NETs formation via PAD4 is the reduced ability to fight infections.²⁴³ However, another study in PAD4-deficient mice did not show differences in morbidity compared to WT mice.²⁴⁴ Patients suffering from the Papillon-Lefèvre Syndrome (unable to produce NE) do not exhibit severe immunodeficiencies^{245,246}, but suffer from periodontitis due to lack or deficiency of NETs.²⁴⁷ Therefore, NETs ability to fight pathogens might depend on pathogen type and location and further, other anti-microbial defense mechanisms might be able to compensate for NETs deficiency.

Given that that DNase1 (Pulmozyme®) is clinically used for patients with cystic fibrosis²⁴⁸, where it has beneficial effects and shows no toxicity, DNase1 might be a better therapeutic approach than PAD4 inhibition. DNase1 is an endonuclease that clears NETs^{188,249} rather than blocking NET formation. Administering DNase1 has been shown to lower plaque area in *ApoE*^{-/-} mice^{216,250} and improve diabetes-impaired wound healing.²¹⁰ Mutations in DNase1 activity has been shown in autoimmunity, in which increased NET formation is associated with disease severity.^{251,252} Since administering DNase1, thus increasing amount of active DNase, can overcome deleterious effects of NETs, e.g. improve wound healing despite ongoing hyperglycemia, it suggests that DNase1 activity might also be decreased in diabetes. However, one study shows that DNase1 activity is actually increased in diabetes.²⁵³ Genetic deletion of DNase1 did not increase NET formation *in vitro*, while double KO of DNase1 and DNase1L3 did.²⁵⁴ Further, only double KO mice with chronic neutrophilia developed vascular occlusion in line with increased NET formation, while

expression of either endonuclease subtype was able to prevent it. This indicates that other endonucleases like DNase1L3 are also able to degrade NETs. Further, although DNase1 has been shown to efficiently remove dsDNA, it has little effect on other NET components, such as NE, that might continue to cause inflammation at the vessel wall.²⁵⁵ This is in line with a study in *ApoE*^{-/-} mice, which showed no effect of DNase1 administration on lesion size after 4 months of high-fat diet.²⁵⁶ Further studies in the implication of DNase1 in atherosclerosis and diabetes are needed, including studies to evaluate different endonuclease types.

Knowledge Gap

Individuals suffering from diabetes have an increased CVD risk despite lipid-lowering therapy. Two of the postulated reasons are diabetic dyslipidemia and hyperglycemia. The connection between low HDL-C and elevated TG with CVDs risk has been shown. Due to failure of beneficial CVD outcomes from HDL-C raising trials, assessment of other HDL functions such as CEC has become of interest. However, studies on how these HDL characteristics in diabetes associate with CVD are scant. The close relationship of HyperTG and HDL-C has been described, but how HyperTG affects other HDL characteristics than HDL-C is unclear. Although evidence is increasing that HyperTG is the causal reason for increased CVD risk, current clinical trials showing CVD benefits by lowering TG levels are still inconclusive and data from pre-clinical trials are missing.

Hyperglycemia is known as an independent risk factor for CVD. One hypothesis is that this is due to stimulation of myelopoiesis. Activation of the bone marrow is thought to increase circulating levels of monocytes and neutrophils. While the role of monocyte-derived macrophages is well established, less is known about neutrophils. Recent studies have described neutrophils ability to undergo a process called NETosis that leads to the formation of pro-inflammatory NETs. How NETs behave during atherosclerosis regression, especially in a diabetic state in which regression is impaired, has not been established yet.

Outline

In this Thesis, we have evaluated the effect of two hallmarks of diabetes in atherosclerosis regression (Figure 4). In Part1, I am discussing the role of diabetic dyslipidemia, namely low HDL-C and HyperTG, on atherosclerosis regression. In fact, my research focused on HDL characteristics other than HDL-C and their associations with CVD risk in a human population. In experimental mouse studies, I have studied the effect of HyperTG on some of these HDL characteristics as well as its impact on atherosclerosis regression. In Part2, I describe the effect of hyperglycemia-induced neutrophilia on the formation of NETs in atherosclerotic plaques.

Chapter 2 describes the associations of different HDL parameters with prevalent atherosclerosis and CVD/CVE in a total population as well as in subjects with prediabetes and T2DM in a human cohort. **Chapter 3** introduces the mouse model used to create mice with HyperTG and low HDL-C, i.e. the lipoprotein lipase (LpL) deficient mice, and describes the role of LpL *in vitro* and *in vivo*. Next, in **Chapter 4**, I investigated the effect of HyperTG due to LpL deficiency on HDL with and without hCETP expression, and in turn on atherosclerosis regression in mice and people. **Chapter 5** shows the role of NET formation in diabetes-impaired atherosclerosis regression in diabetic mice and in **Chapter 6**, I discuss different endonucleases, known to degrade NETs, and their involvement in atherosclerosis pro-and regression. In **chapter 7**, I provide a summary and conclusion on the findings of this thesis, including their impact for CVD.

Diabetes

Part 1 Part 2

Figure 4. Thesis Outline

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Part 1

Diabetic Dyslipidemia

**High-density Lipoprotein Cholesterol Efflux Capacity
is not associated with Atherosclerosis and
Prevalence of Cardiovascular Outcome: The CODAM
Study**

Tatjana Josefs, Kristiaan Wouters, Uwe J.F. Tietge, Wijtske Annema, Robin P.F. Dullaart, Tomas
Vaisar, Ilja C.W. Arts, Carla J.H. van der Kallen, Coen D.A. Stehouwer, Casper G. Schalkwijk,
Ira J. Goldberg, Edward A. Fisher, Marleen M.J. van Greevenbroek

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Abstract

Background: Cholesterol Efflux Capacity (CEC) is considered to be a key atheroprotective property of high-density lipoproteins (HDL). However, the role of HDL-CEC in atherosclerosis and cardiovascular (CV) risk is still controversial and data in individuals with diabetes are limited.

Objective: In this study, we have investigated the relationship of CEC and other HDL characteristics with clinical and subclinical atherosclerosis in subjects with elevated CVD risk and Type 2 Diabetes Mellitus (T2DM).

Methods: Using multiple linear regression analyses, we determined the relationship of HDL-CEC with carotid intima media thickness (cIMT, Z-Score), an endothelial dysfunction (EnD) Score (Z-Score), prevalent CV disease (CVD, n=150 cases) and history of CV events (CVE, n=85 cases) in an observational cohort (CODAM, n= 574, 59.6±0.3 yr, 61.3% men, 24.4% T2DM). Stratified analyses were performed to determine if the associations differed between individuals with normal glucose metabolism (NGM) and those with a disturbed glucose metabolism.

Results: HDL-CEC was not associated with either marker of atherosclerosis (cIMT, EnD Score) nor with CVD or CVE. In contrast, other HDL characteristics i.e. HDL-Cholesterol (HDL-C, Z-Score), apolipoprotein A-I (apoA-I, Z-Score), HDL size (Z-Score) and HDL particle number (HDL-P, Z-Score) were inversely and significantly associated with the EnD Score (β s -0.226 to -0.097, $p < 0.05$) and CVE (ORs 0.61 to 0.68, $p < 0.05$). In stratified analyses, HDL size and HDL-P were significantly associated with the EnD Score in individuals with NGM ($p_{\text{interaction}}$ 0.039 and 0.005, respectively), but not in those with (pre)diabetes. HDL-C and apoA-I were inversely associated with prevalent CVD in individuals with (pre)diabetes ($p_{\text{interaction}}$ =0.074 and 0.034, respectively), but not in those with NGM.

Conclusion: HDL-CEC is not associated with clinical or subclinical atherosclerosis, neither in the whole population nor in individuals with (pre)diabetes, while other HDL characteristics show atheroprotective associations. The atheroprotective associations of HDL-size and HDL-P are lost in (pre)diabetes, while higher concentrations of HDL-C and apoA-I are associated with lower prevalence of CVD in (pre)diabetes.

Introduction

High-density Lipoprotein (HDL) is well-known for its cardioprotective effect, resulting from, among others, its anti-inflammatory and endothelial protective properties. The key cardioprotective effect of HDL is thought to originate in its cholesterol efflux capacity (HDL-CEC), which represents the first step in reverse cholesterol transport^{1,2}. Since up till now, the results of HDL Cholesterol (HDL-C) raising trials have been disappointing^{3,4}, the importance of HDL function in cardiovascular diseases (CVD) has become a topic of major interest over the last few years.

HDL-mediated cholesterol efflux may lead to cholesterol reduction in the atherosclerotic plaque by promoting the removal of excess cholesterol from plaque macrophage foam cells and transporting it back to the liver for its excretion into the bile.^{5,6} So far, animal studies showed a link between macrophage-specific cholesterol efflux and prevention of atherosclerosis.^{7–10} Further, functional HDL was shown to promote atherosclerosis regression.^{11,12} However, it has also been shown that, besides anti-inflammatory effects¹³, cholesterol efflux can also exert pro-inflammatory effects on mouse and human macrophages, which are thought to be detrimental for atherosclerosis.^{14,15} In human studies an inverse association between HDL-CEC and the prevalence¹⁶ and incidence of CV events (CVE)^{17–19} was shown, which remained present after adjustment for CV risk factors, including HDL-C¹⁹, HDL-C and apoA-I^{16,18}, and HDL-C and HDL particle number (HDL-P)¹⁷. In contrast, another study showed that a higher HDL-CEC was associated with increased risk of incident CVE, also after adjustment for CV risk factors, including HDL-C and LDL-C.²⁰ The reasons for these apparent discrepancies are currently unclear.

Diabetes increases the risk of CVD by 2 to 4-fold.²¹ While the underlying mechanisms remain to be fully understood, altered levels or impaired functions of circulating lipoproteins are likely to be involved. Some studies have shown that HDL-CEC is impaired in patients with insulin resistance²² and type 2 diabetes mellitus (T2DM)^{18,23}, but other studies did not find any difference²⁴ or even increased HDL-CEC compared to healthy controls.^{25–29} While CV outcome studies demonstrated that HDL-CEC did not differ between non-T2DM and T2DM subjects, they showed that statin treatment in both groups leads to an increase in HDL-CEC, which correlates with beneficial changes on plaque morphology.^{30,31}

Taken together, the outcomes of human studies on the relationship between HDL-CEC and CVD have been controversial and data in individuals with diabetes are limited. The goal of this study was to evaluate the associations of HDL-CEC and other characteristics of HDL on (sub)clinical atherosclerotic disease, as represented by carotid intima media thickness (cIMT) and an endothelial dysfunction Score (EnD Score), prevalent CVD and history of CVE. In addition, we determined whether such associations differ between individuals with normal glucose metabolism (NGM) and those with prediabetes or diabetes (hereafter referred to as (pre)diabetes) and are the first to report on the relationship of CEC with CVD/CVE in individuals with (pre)diabetes. This evaluation was done in a well-established human cohort that is characterized by a moderately increased risk of cardiometabolic disease, i.e., the Cohort on Diabetes and Atherosclerosis Maastricht (CODAM). We hypothesized that a higher HDL-CEC is associated with less (sub)clinical atherosclerotic disease and that this association is attenuated in individuals with (pre)diabetes.

Patients & Methods

Study Population

CODAM includes 574 participants who were selected from a large cohort in the general population (>20,000) based on an elevated risk of T2DM and CVD as described elsewhere.³² Inclusion criteria were Caucasian descent, age >40 years, and one or more cardiometabolic risk factors (i.e. BMI > 25kg/m², use of anti-hypertensive medication, positive family history of T2DM, history of gestational diabetes and/or glycosuria). The CODAM study was approved by the Medical Ethics Committee of the Maastricht University Medical Centre. All participants gave written informed consent and the described methods were carried out in accordance with the approved guidelines. Of the 574 participants, 25 had missing data on HDL characteristics and 16 had missing covariates leaving n=533 for a complete case analyses with the EnD Score, CVD or CVE as outcomes (NGM n=279, (pre)diabetes n=254). N=37 also had missing data on cIMT leaving n=496 (NGM n=268, (pre)diabetes =228).

All participants were asked to stop their lipid-modifying medication 14 days and all other medications on the day before the measurements (>80% adherence). Blood samples were obtained by venipuncture after an overnight fast and plasma aliquots were stored at -80°C until use. All participants underwent an oral glucose tolerance

test (except those with established T2DM), and were categorized as having normal glucose metabolism (NGM), impaired glucose metabolism (IGM, i.e. prediabetes) or T2DM as previously described.^{33,34,32} Glycated hemoglobin (HbA_{1c}) was determined as previously described.²⁵

Cardiovascular Measures

Carotid Intima-Media Thickness (cIMT)

cIMT was measured at the left and right common carotid artery 10-20mm proximal to the carotid bulb with an ultrasound imaging device as reported previously.³⁵

Endothelial Dysfunction (EnD) Score

Soluble intercellular adhesion molecule (sICAM-1), soluble vascular cell adhesion molecule (sVCAM-1), and soluble endothelial selectin (sESel) were measured using ELISA assays in serum or EDTA plasma. The measurements were calibrated after cross-validation as described elsewhere.³⁶ Baseline von Willebrand factor (vWF) was measured with an in-house ELISA in citrated plasma as described.³⁷

Cardiovascular Events (CVE)

CVE were defined as the occurrence of myocardial infarction (MI), coronary bypass, percutaneous coronary intervention and /or stroke reported by questionnaires; as well as signs of MI on an electrocardiogram (Minnesota codes 1-1 or 1-2), as reported earlier.³²

Cardiovascular Disease (CVD)

Prior CVD was defined by self-reported history of CVE. CVD additionally included signs of coronary ischemia (Minnesota codes 1-3, 4-1, 4-2, 4-3, 5-1, 5-2, 5-3, or 7-1); non-traumatic limb amputation; and/or ankle-brachial index <0.9.³²

HDL Characteristics

HDL-C was determined in EDTA plasma using the HDL-C plus assay (Roche Diagnostics, Mannheim, Germany) and apoA-I was determined by immunoephelometric assays as reported earlier.³² HDL size and HDL-P were

measured using nuclear magnetic resonance spectroscopy as described previously (available at: www.nightingalehealth.com).³⁸

Cholesterol Efflux Capacity (HDL-CEC)

Measurement of Cholesterol Efflux Capacity was done exactly as described elsewhere.²⁴ Briefly, HDL-CEC towards apoB-depleted plasma was measured using THP-1 human monocytes that were differentiated into macrophages by the addition of 100 nM phorbol myristate acetate (PMA).^{39,40} Differentiated THP-1 macrophages were then loaded with acetylated LDL (50 µg protein/ mL) and 1 µCi/ml ³H-cholesterol (Perkin Elmer, Boston, MA) for 24 hours followed by equilibration for 24 hours in RPMI 1640 medium containing 2% bovine serum albumin.⁴⁰ After equilibration, efflux towards 2% apoB-depleted plasma was performed for 5 hours. ApoB-depleted plasma was generated by precipitating apoB-containing lipoproteins using polyethylene glycol (PEG 600, Sigma, St Louis, MO) in 10mM HEPES (pH 0 8.0= according to a commonly used protocol.^{16–18,24,41} CEC measurements were performed in duplicates and at the same time using the same reagents to limit potential variations due to different assay conditions. Values for HDL-CEC were normalized using a standard curve of different concentrations of pooled apoB-depleted control plasma to correct for potential inter-assay variation across plates, and expressed in arbitrary units.⁴¹ The intra-assay CV of the method was previously determined to be 5.4%, the inter-assay CV 7.9%.

24,41

Other covariates

BMI was calculated as weight (kg) /height (m) squared.⁴² Waist circumference was measured as before.³⁵ Smoking behavior, physical activity⁴³, dietary calorie intake³³, use of anti-hypertensive, glucose-lowering, and lipid-medication⁴² was assessed with on-site administered questionnaires. Blood pressure was measured as described before.²⁴ LDL-C was calculated with the Friedewald formula.³² Triglycerides (TG) and apoB were determined as reported earlier.³²

Statistical analyses

Categorical variables are presented as frequencies and percentages. Normally distributed continuous variables are presented as mean ± standard deviation, skewed

variables are presented as median with interquartile range (IQR). Skewed variables (*i.e.* fasting plasma glucose (FPG), HDL-P, TG and EnD markers) were ln-transformed to achieve normal distribution prior to analysis.

To define an overall measure of endothelial dysfunction, a composite score was calculated using sICAM-1, sVCAM-1, sE-selectin and vWF. For this, each individual ln-transformed biomarker measurements were first standardized and the individual Z-scores were averaged into the endothelial dysfunction score (EnD Score). To allow direct comparison of the effect sizes for the EnD Score and cIMT, cIMT was also standardized. For the same reason, HDL characteristics were also converted into their respective Z-scores.

Cross-sectional associations of HDL characteristics (HDL-CEC, HDL-C, apoA-I, HDL size, HDL-P) with the EnD Score (N=533) and with cIMT (N=496) were investigated using linear regression analysis. Logistic regression analyses were done for CVD (N=533, 150 CVD cases) and CVE (N=533, 85 CVE cases). All regression coefficients are presented as crude effect sizes (Model 1) and adjusted for several potential confounders: Model 2: adjusted for age (yrs) and sex, Model 3 (full model): additionally adjusted for smoking (%), physical activity (mets/day), caloric intake (KJ/day), use of glucose-lowering, lipid-modifying, and antihypertensive medication (all yes/no), BMI (kg/m²), systolic blood pressure (mmHg), diastolic blood pressure (mmHg), fasting plasma glucose (mmol/L), IGM (yes/no), T2DM (yes/no).

To determine if the associations differed between individuals with NGM and those with a disturbed glucose metabolism (pre-diabetes or diabetes), we repeated the analyses mentioned above stratified according to normal or disturbed glucose metabolism. We also performed an interaction analysis by adding interaction terms (HDL characteristic (e.g. HDL-CEC) x glucose metabolism (normal or disturbed)) to the main analyses.

HDL-CEC was significantly higher in women compared to men, but the strength of association of HDL-CEC with cIMT, EnD Score, CVD and CVE did not differ between women and men.

Statistical analyses were performed using IBM SPSS Statistics Version 23. For the main effects $P < 0.05$, and for interaction terms $P_{\text{interaction}} < 0.10$ was considered statistically significant.

Results

The CODAM study population

The overall characteristics of the CODAM study as a whole and across tertiles of HDL-CEC are shown in **Table 1**. For the total population, the mean age was 59.6 ± 7.0 yrs, 61.3% were men, and 24.2% had T2DM. Mean values for systolic blood pressure were 140.1 ± 19.0 mmHg, diastolic blood pressure 81.6 ± 9.1 mmHg and 20% were current smokers. 28.1% and 16.0% were previously diagnosed with CVD and CVE, respectively. Prevalence of normal glucose metabolism, prediabetes or diabetes and Hb1Ac levels were not significantly different among HDL-CEC tertiles while HDL-C, apoA-I, HDL size, and HDL-P were higher in those individuals who were in the highest CEC tertiles. cIMT, EnD, CVD and CVE did not differ among CEC tertiles.

The associations of HDL-CEC with the atherosclerosis surrogates (*i.e.* cIMT and EnD Score) are shown in **Table 2**. In crude analyses, cIMT was not associated with HDL -CEC ($\beta = -0.04$, [95%CI -0.13; 0.05], $p=0.413$), but was significantly and inversely associated with HDL-C, apoA-I, and HDL-size, but not with HDL-P. These associations were attenuated and no longer significant in fully adjusted analyses (HDL-C: $\beta = -0.07$ [-0.16; 0.02], $p=0.130$; apoA-I: $\beta = -0.04$ [-0.13; 0.05] $p=0.408$; HDL size: $\beta = -0.03$ [-0.12; 0.06] $p=0.527$). Likewise, HDL-CEC was not associated with the EnD Score, neither crude ($\beta = -0.01$ [-0.09; 0.08] $p=0.897$) nor after adjustment for potential confounders. In contrast, HDL-C, apoA-I, HDL size, and HDL-P, were all significantly and inversely associated with the EnD Score and remained so in the fully adjusted analyses (HDL-C: $\beta = -0.23$ [-0.31; -0.14] $p<0.001$, apoA-I: $\beta = -0.16$ [-0.24; -0.07] $p<0.001$, HDL size: $\beta = -0.12$ [-0.21; -0.03], $p=0.008$, HDL-P: $\beta = -0.10$ [-1.8; -0.01], $p=0.028$).

Table 1: Values are mean $\pm$ SD for continuous variables with normal distribution and median [Interquartile range] for continuous variables with skewed distribution.

* variables with skewed distribution were ln-transformed prior to t-test, [†] n=496, cIMT indicates carotid intima media thickness; EnD score, Endothelial Dysfunction Score; CVD, Cardiovascular Disease; CVE, CV Events; NGM, normal glucose metabolism; IGM, impaired GM; T2DM, Type 2 Diabetes Mellitus; Hb1Ac, hemoglobin A1c; FPG, fasting plasma glucose; BMI, body mass index; BP, blood pressure; CEC, cholesterol efflux capacity; HDL-C, high density lipoprotein Cholesterol; ApoA-I, apolipoprotein A-I; HDL-P, HDL particle number; LDL-C, low density lipoprotein Cholesterol; ApoB, apolipoprotein B; VLDL, very low density lipoprotein; TG, triglyceride.

Table 1. Characteristics of the study participants divided in CEC tertiles.

	total population N=533	CEC tertiles			p-value*
		Lowest (N=181)	Middle (N=180)	Highest (N=172)	
age (years)	59.6 ± 7.0	59.1 ± 7.4	60.0 ± 6.6	59.8 ± 6.8	0.385
sex (%men)	61.3	67.4	61.1	54.9	0.054
cIMT (mm) [†]	0.78 ± 0.16	0.78 ± 0.16	0.79 ± 0.15	0.77 ± 0.17	0.562
EnD Score (SD)	-0.00 ± 0.65	0.02 ± 0.66	-0.01 ± 0.62	-0.02 ± 0.7	0.79
CVD (%)	28.1	31	28.3	24.9	0.438
CVE (%)	16.0	16.8	18.3	12.7	0.332
Glucose metabolism (%)	52.5/23.3/24.3	52.2/21.2/26.6	47.2/28.9/23.9	58.4/19.7/22.0	0.11
Hb1Ac (%)	6.0 ± 0.8	6.0 ± 0.9	5.9 ± 0.8	5.9 ± 0.8	0.572
FPG (mmol/L)	5.6 [4.4 - 6.7]	5.6 [4.4 - 6.8]	5.7 [4.4 - 6.9]	5.5 [4.3 - 6.6]	0.321
Use of Medication (%)	18.8	20.7	20.6	15	0.304
	lipid-lowering	14.7	11.7	11	0.530
	glucose-lowering	42.9	38.3	34.1	0.231
Antihypertensive	38.5	28.7 ± 4.2	28.2 ± 4.4	28.7 ± 4.6	0.464
BMI (kg/m ²)	28.5 ± 4.4	22.3	23.3	15	0.137
smoking (%)	20.3	102.9 ± 11.2	100.7 ± 10.3	102.0 ± 11.4	0.32
Waist circumference (cm)	101.9 ± 10.9	95.2 ± 11.8	93.8 ± 11.9	95.5 ± 13.4	0.703
	men	6633 ± 4164	7158 ± 4427	6140 ± 3431	0.07
physical Activity (mets/day)	9264 ± 2815	9643 ± 2853	9219 ± 2838	8909 ± 2714	0.046
caloric intake (kJ/day)	81.6 ± 9.1	81.6 ± 9.3	81.8 ± 9.2	81.5 ± 8.7	0.961
diastolic BP (mmHg)	140.1 ± 19.0	139.1 ± 17.8	141.6 ± 19.4	139.6 ± 19.9	0.409
systolic BP (mmHg)	1.3 ± 0.3	1.0 ± 0.1	1.3 ± 0.1	1.7 ± 0.2	<0.001
HDL-CEC	1.2 ± 0.4	1.0 ± 0.3	1.2 ± 0.3	1.4 ± 0.4	<0.001
HDL-C (mmol/L)	1.5 ± 0.2	1.3 ± 0.2	1.5 ± 0.2	1.6 ± 0.2	<0.001
ApoA-I (g/L)	9.9 ± 0.2	9.8 ± 0.2	10.0 ± 0.2	10.0 ± 0.2	<0.001
HDL size (nm)	7.0 [5.8 - 8.2]	6.6 [5.6 - 7.5]	7.1 [6.0 - 8.1]	7.4 [6.1 - 8.8]	<0.001
HDL-P (μmol/L)	3.3 ± 0.9	3.3 ± 0.8	3.3 ± 0.9	3.3 ± 0.9	0.57
LDL-C (mmol/L)	1.1 ± 0.24	1.1 ± 0.2	1.1 ± 0.2	1.1 ± 0.3	0.309
ApoB (g/L)	36.7 ± 1.1	36.8 ± 1.0	36.7 ± 1.1	36.6 ± 1.1	0.101
VLDL size (nm)	1.4 [0.5 - 2.3]	1.4 [0.5 - 2.3]	1.4 [0.4 - 2.4]	1.2 [0.2 - 2.2]	0.174
TG (mmol/L)					

Because all the HDL characteristics differed between CEC tertiles (**Table 1**), we adjusted the analyses of CEC with cIMT and the EnD Score for HDL-C, apoA-I, HDL size and HDL-P (**Suppl. Table S1**). This did not change the lack of association of HDL-CEC with cIMT or the EnD Score. We also evaluated if the associations of the individual HDL-characteristics with the atherosclerosis surrogates were independent of plasma triglyceride (TG) and LDL-C levels (**Suppl. Table S2**). When the analyses presented in **Table 2** were additionally adjusted for TG or LDL-C, the associations between the HDL characteristics and cIMT remained essentially unaltered (**Suppl. Table S2**). Further, none of the previously observed inverse associations of HDL-C, apoA-I, HDL size and HDL-P with the EnD Score were affected by additional adjustment for LDL-C. Adjustment for TG did, however, slightly attenuate the associations of HDL-size and HDL-P with the EnD Score (for HDL size to $\beta = -0.08$ [-0.18; 0.02] $p=0.103$, and for HDL-P to $\beta = -0.08$ [-0.17; 0.01] $p=0.068$).

Table 2. Relationship of HDL-CEC and other HDL characteristics with cIMT and the EnD Score.

Model		cIMT (Z-Score)			EnD (Z-Score)		
		N= 496			N=533		
		std β	95% CI	p-value	std β	95% CI	p-value
HDL-CEC	1	-0.037	-0.126;0.052	0.413	-0.006	-0.092;0.080	0.897
	2	-0.027	-0.113;0.058	0.532	-0.047	-0.131;0.037	0.276
	3	-0.022	-0.106;0.062	0.603	-0.007	-0.085;0.071	0.863
HDL-C	1	-0.127	-0.215;-0.039	0.005	-0.292	-0.375;-0.209	<0.001
	2	-0.090	-0.179;-0.001	0.048	-0.335	-0.419;-0.252	<0.001
	3	-0.071	-0.164;0.021	0.130	-0.226	-0.312;-0.140	<0.001
ApoA-I	1	-0.089	-0.178;0.001	0.051	-0.195	-0.280;-0.110	<0.001
	2	-0.044	-0.135;0.048	0.350	-0.240	-0.327;-0.152	<0.001
	3	-0.039	-0.132;0.054	0.408	-0.158	-0.244;-0.073	<0.001
HDL size	1	-0.093	-0.180;-0.005	0.038	-0.176	-0.260;-0.091	<0.001
	2	-0.054	-0.144;0.035	0.235	-0.222	-0.309;-0.134	<0.001
	3	-0.030	-0.123;0.063	0.527	-0.118	-0.205;-0.031	0.008
HDL-P	1	-0.076	-0.166;0.013	0.095	-0.061	-0.147;0.026	0.168
	2	-0.021	-0.114;0.072	0.652	-0.085	-0.175;0.006	0.066
	3	-0.060	-0.154;0.034	0.208	-0.097	-0.184;-0.011	0.028

Table 2: Linear regression analyses, Model 1: crude, Model 2: adjusted for age, sex, Model 3 additionally adjusted for smoking, bmi, medication usage (antihypertensive, glucose- and lipid lowering), physical activity, caloric intake, systolic and diastolic blood pressure, fasting plasma glucose, and glucose metabolism state. Std β ; standardized regression coefficient.

Association of HDL characteristics with prevalence of CVD and CVE

Next, we performed logistic regression analyses with prevalent CVD and CVE as outcomes. HDL-CEC was not associated with CVD or CVE (**Figure 1**). Also, none of the other HDL characteristics were associated with CVD (**Figure 1, panel a**). On the other hand, HDL-C (OR 0.61 [95% CI 0.41; 0.90] $p=0.014$), apoA-I (OR 0.67 [95% CI 0.46; 0.96] $p=0.030$) and HDL-P (OR 0.87 [95% CI 0.48; 0.97] $p=0.031$) were inversely associated with prevalent CVE, whereas HDL size was not (**Figure 1, panel b**). This implies that one standard deviation higher HDL-C, apoA-I, and HDL-P were associated with approximately 1.6-, 1.5- and 1.2-fold fewer CVE cases, respectively. None of these associations were dependent on plasma TG levels or LDL-C (**Suppl. Table S3**).

Figure 1: Forrest plot indicating the relationship of HDL characteristics and prevalent cardiovascular disease (CVD) and CV events (CVE) risk in whole population as well as stratified analyses.

Logistic regression analyses were adjusted for age, sex, smoking, bmi, medication usage (antihypertensive, glucose- and lipid lowering), physical activity, caloric intake, systolic and diastolic blood pressure, fasting plasma glucose, and glucose metabolism state. CVD N=383, 150 CVD cases (NGM: N=279, 67 CVD cases; (pre)diabetes: N=253, 83 CVD cases) and CVE N=448, 85 CVE cases (NGM: N=279, 39 CVE cases, (pre)diabetes: N=254, 46 CVE cases).

1a **1b**

Effect of glucose tolerance status on the associations between HDL characteristics and prevalent cardiovascular outcomes

Interaction analyses suggested that the strength of the association of CEC with cIMT ($p_{\text{interaction}} = 0.025$) and the EnD Score ($p_{\text{interaction}} = 0.103$) might differ between those with NGM and those with (pre)diabetes. Stratified analyses showed that CEC was not significantly associated with cIMT (**Table 3**) or the EnD Score (**Table 4**) in either of the subgroups (cIMT: NGM $\beta = 0.06$ [-0.04; 0.17] $p=0.25$, (pre)diabetes $\beta = -0.13$ [-0.26; 0.01] $p=0.060$; EnD Score: NGM $\beta = -0.08$ [-0.18; 0.03] $p=0.142$, (pre)diabetes $\beta = 0.06$ [-0.06; 0.19], $p=0.312$; full model). Potential interaction was also observed for the strength of the association of apoA-I ($p_{\text{interaction}} = 0.067$) and HDL-P ($p_{\text{interaction}} = 0.038$) with cIMT but no significant associations were observed within the subgroups (NGM or (pre)diabetes, **Table 3**). The strength of the associations of HDL-size and HDL-P with the EnD Score also appeared to differ between NGM and (pre)diabetes ($p_{\text{interaction}} = 0.039$ and 0.006 , respectively). HDL-size and HDL-P were inversely associated with the EnD Score in those with NGM, while such association was not observed in individuals with (pre)diabetes (**Table 4**). Notably, a significant inverse association between HDL-C and the EnD Score was observed in NGM as in (pre)diabetes ($p_{\text{interaction}} = 0.508$). Additional adjustments for plasma TG and LDL-C levels did not essentially change the strength of these associations (**Suppl. Tables S4 and S5**).

Table 3: Linear regression analyses, Model 1: crude, Model 2: adjusted for age, sex, Model 3 additionally adjusted for smoking, bmi, medication usage (antihypertensive, glucose- and lipid lowering), physical activity, caloric intake, systolic and diastolic blood pressure, fasting plasma glucose, and glucose metabolism state. Std β ; standardized regression coefficient.

Table 4: Linear regression analyses, Model 1: crude, Model 2: adjusted for age, sex, Model 3 additionally adjusted for smoking, bmi, medication usage (antihypertensive, glucose- and lipid lowering), physical activity, caloric intake, systolic and diastolic blood pressure, fasting plasma glucose, and glucose metabolism state. Std β ; standardized regression coefficient.

Table 3. Relationship of HDL-CEC and other HDL characteristics with cIMT, stratified for normoglycemia and (pre)diabetes.

Model		cIMT (Z-Score)						p _{int}
		NGM N=268			IGM&T2DM N=228			
		std β	95% CI	p-value	std β	95% CI	p-value	
HDL-CEC	1	0.062	-0.056;0.179	0.303	-0.152	-0.286;-0.017	0.028	0.019
	2	0.059	-0.052;0.170	0.296	-0.132	-0.265;0.001	0.051	0.023
	3	0.061	-0.044;0.167	0.255	-0.129	-0.263;0.005	0.060	0.025
HDL-C	1	-0.057	-0.172;0.058	0.328	-0.181	-0.325;-0.038	0.014	0.184
	2	-0.035	-0.150;0.079	0.545	-0.138	-0.287;0.012	0.071	0.216
	3	-0.044	-0.158;0.070	0.446	-0.138	-0.293;0.017	0.081	0.169
ApoA-I	1	0.007	-0.111;0.125	0.905	-0.188	-0.325;-0.025	0.007	0.033
	2	0.031	-0.088;0.149	0.611	-0.132	-0.277;0.014	0.076	0.075
	3	0.011	-0.105;0.128	0.848	-0.118	-0.268;0.031	0.120	0.067
HDL size	1	-0.088	-0.196;0.021	0.114	-0.056	-0.207;0.095	0.465	0.737
	2	-0.061	-0.172;0.050	0.279	-0.009	-0.165;0.147	0.908	0.823
	3	-0.055	-0.167;0.056	0.329	-0.006	-0.170;0.159	0.947	0.910
HDL-P	1	-0.007	-0.130;0.115	0.909	-0.169	-0.298;-0.039	0.011	0.061
	2	0.043	-0.081;0.167	0.496	-0.112	-0.253;0.029	0.118	0.080
	3	0.006	-0.115;0.128	0.919	-0.132	-0.276;0.012	0.073	0.038

Table 4. Relationship of HDL-CEC and other HDL characteristics with the EnD Score, stratified for normoglycemia and (pre)diabetes.

Model		EnD (Z-Score)						
		NGM (N=279)			IGM & T2DM (N=254)			p _{int}
		std β	95% CI	p-value	std β	95% CI	p-value	
HDL-CEC	1	-0.079	-0.186;0.028	0.146	0.039	-0.094;0.172	0.564	0.170
	2	-0.105	-0.210;-0.001	0.047	0.039	-0.093;0.171	0.562	0.099
	3	-0.076	-0.177;0.025	0.142	0.064	-0.060;0.188	0.312	0.211
HDL-C	1	-0.269	-0.370;-0.168	<0.001	-0.242	-0.383;-0.101	0.001	0.755
	2	-0.330	-0.432;-0.228	<0.001	-0.278	-0.424;-0.132	<0.001	0.695
	3	-0.256	-0.362;-0.150	<0.001	-0.185	-0.330;-0.040	0.013	0.508
ApoA-I	1	-0.216	-0.321;-0.111	<0.001	-0.125	-0.160;0.010	0.069	0.290
	2	-0.288	-0.394;-0.181	<0.001	-0.144	-0.287;-0.001	0.049	0.162
	3	-0.225	-0.333;-0.117	<0.001	-0.071	-0.210;0.067	0.312	0.102
HDL size	1	-0.212	-0.310;-0.114	<0.001	-0.047	-0.195;0.100	0.528	0.062
	2	-0.277	-0.378;-0.176	<0.001	-0.077	-0.231;0.078	0.331	0.068
	3	-0.204	-0.309;-0.099	<0.001	0.03	-0.119;0.185	0.668	0.039
HDL-P	1	-0.193	-0.304;-0.083	0.001	0.038	-0.091;0.166	0.564	0.005
	2	-0.252	-0.366;-0.138	<0.001	0.045	-0.093;0.182	0.523	0.002
	3	-0.205	-0.319;-0.092	<0.001	0.037	-0.095;0.168	0.582	0.006

HDL-CEC was not associated with CVD in either NGM or (pre)diabetes (**Figure 1, panel a**). We did observe a difference in the strength of the association of HDL-C and apoA-I with CVD ($p_{\text{interaction}} = 0.074$ and 0.034 , respectively). In individuals with NGM (N=67 CVD cases) no association of HDL-C or apoA-I with CVD was observed (HDL-C: OR =0.84 [0.56; 1.25] $p=0.38$, apoA-I: OR 0.98 [0.67; 1.44] $p=0.91$), while in those with (pre)diabetes (N=83 CVD cases), an increase of one unit SD of HDL-C or apoA-I was associated with 1.5-fold fewer cases (HDL-C: OR=0.62 [0.40; 0.94] $p=0.026$; apoA-I: OR=0.61 [0.41; 0.90] $p=0.012$). Adjusting for LDL-C did not change these associations, but adjustment for TG attenuated the strength of association between HDL-C and CVD (**Suppl. Table 6**). No significant interaction with glucose metabolism status was observed for the associations with CVE ($p_{\text{interaction}}$ between 0.13 and 0.98) but for completeness the stratified analyses are included in **Figure1, panel b**. Notably, a significant inverse association between HDL-C and CVE was observed in those with NGM as well as those with (pre)diabetes ($p_{\text{interaction}} = 0.171$).

Discussion

In this observational human cohort, we show that HDL-CEC is not associated with parameters of atherosclerosis as reflected by cIMT and the EnD Score, nor with prevalent CVD or CVE, independent of glucose metabolism state. In contrast, other HDL characteristics, i.e. HDL-C, apoA-I, HDL size and HDL-P, were inversely associated with the EnD Score and with CVE. The inverse association between HDL-C and the EnD Score was observed in subjects with NGM, as well as those with (pre)diabetes, while the inverse associations with apoA-I, HDL size and HDL-P were found only in individuals with NGM. On the other hand, higher concentrations of HDL-C and apoA-I were associated with less CVD in individuals with (pre)diabetes, but not in those with NGM.

The role of HDL in atherosclerosis has undergone a major re-evaluation in the past 5 years, leading to intensified research on HDL-CEC⁴⁴. Thus far, the results of these efforts are not uniform and the final conclusion on whether and how HDL-CEC impacts atherosclerosis and CVD/CVE is yet to be made. Some studies showed that an increase in HDL-CEC could be beneficial for atherosclerosis¹⁶ as well as CVD/CVE^{17,18}, while others reported the opposite^{14,15,20}. In our current evaluation, we did not find any significant associations for HDL-CEC, which is in line with prevalent^{20,45}

and incident studies.⁴⁶ *Li et al*²⁰ reported that there was no association of HDL-CEC with prevalent CV risk in a cohort with stable angiographically confirmed CVD after adjustment for traditional risk factors, such as age, sex, smoking, diabetes, hypertension, LDL-C, and HDL-C. However, in the same cohort, they unexpectedly observed an association between higher HDL-CEC and increased incident CV events, suggesting a complex relationship between HDL-CEC and vascular disease. The relation of HDL-CEC might differ between atherosclerotic plaque development (=prevalent CVD) and plaque vulnerability-associated phenotypes e.g. myocardial infarction (=incident CVD). Further, a recent study showed that the association of cholesterol mass efflux capacity differs between coronary heart disease and stroke.¹⁹

In contrast to our null-findings for HDL-CEC, other HDL characteristics, i.e. HDL-C, apoA-I, HDL size and HDL-P, were inversely associated with the EnD Score, CVD and CVE. These HDL-characteristics were not associated with cIMT in our current evaluations, although the direction of the non-significant associations with cIMT were also inverse.

In the CODAM cohort, as in several other studies^{47–49}, we observed an inverse association of HDL-C with the EnD Score and CVE, which was independent of LDL-C. Despite these robust inverse associations of HDL-C with risk of atherosclerosis/CVE, pharmacological elevation of HDL-C has failed to reduce atherosclerosis⁵⁰ or cardiac events.^{3,4} For this reason, the attention has shifted not only from HDL-C to HDL-CEC, but also to further alternative indexes of HDL quantity and/or quality such as apoA-I, HDL size and HDL-P.⁵¹ As expected, we found inverse associations of apoA-I with atherosclerosis and CVD/CVE. ApoA-I is the main protein component (70%) of HDL. Several ways of atheroprotective mechanisms for apoA-I have been proposed and pharmacological interventions targeting apoA-I are in process (recently reviewed in^{51,52}). Also in line with our results is the current literature on HDL-P, which consistently reports an inverse association of HDL-P with atherosclerosis⁵³, CVE⁵⁴, future CAD risk^{53,55}, as well as a lower rate of coronary heart disease (CHD) death.⁵⁶ In our current evaluation, the association of HDL-P with the EnD Score and CVE was slightly attenuated after adjustment for TG, thereby remaining significant for CVE, but not for EnD Score. The inverse association of HDL size with the EnD Score was more strongly attenuated by adjusting for TG. These findings are consistent with the report of *Harchaoui et al*.⁵⁵, who showed that the relationship between HDL-size and CAD risk

was dependent on metabolic parameters, while the relationship of HDL-P with CAD was not.

Overall, HDL is a heterogeneous lipoprotein fraction encompassing particles that markedly differ in size, density, surface charge and composition.⁵⁷ It exerts several atheroprotective properties, such as, anti-apoptotic, anti-oxidant and nitric oxide promoting effects. However, which atheroprotective property is the most clinically relevant one, and what subfraction of HDL exerts which property, remains unknown. Notably, the metabolic milieu may affect various properties of HDL. Therefore, we investigated whether the observed associations differed between those with normoglycemia (NGM) and those with (pre)diabetes. The inverse associations of HDL-P and HDL-size with the EnD Score in subjects with NGM were fully absent in those with (pre)diabetes. In contrast, the inverse association for HDL-C was clearly present in both NGM and (pre)diabetes. Our study population displays the typical dyslipidemic phenotype, i.e. high TG and low HDL-C, but not the decreased HDL-size and lower HDL-P, that has been shown before^{58,59}. This suggests that in those with NGM, the protective effect of a certain number of HDL particles against development of EnD is better than for that same number of HDL particles in (pre)diabetes. Thus, HDL particle composition, perhaps partly reflected in HDL size, is of interest regarding its protective vascular properties. In this context, a possible compromised anti-oxidative capability of HDL, may be of importance.⁶⁰ It has been shown before that HDL is able to reduce cytokine-induced expression of VCAM-1, ICAM-1, and E-Selectin in vitro⁶¹ and in vivo⁶². The observation that the inverse associations of HDL size and HDL-P are lost in (pre)diabetes subjects, suggests the loss of their anti-inflammatory and endothelium-protective properties in people with impaired glucose metabolism and insulin resistance. In contrast to our observations on the EnD Score, the association of higher concentrations of HDL-C and apoA-I with less CVD was only seen in individuals with (pre)diabetes. This was an unexpected observation that to some extent may be explained by more careful monitoring by health care professionals of individuals with (pre)diabetes for signs of development of CVD.

The main strength of this study is the relatively large population, the combination of several HDL characteristics, the availability of the EnD Score and cIMT in addition to CVD/CVE, and the approximately equal numbers of individuals with NGM and (pre)diabetes, which allowed us to perform sufficiently numbered stratified analyses.

Further, we are the first to report on the relationship of CEC with CVD/CVE in individuals with (pre)diabetes. The interpretation of our current study is limited by the fact that we measured only one functional aspect of HDL function, i.e. its CEC, which, is thought to reflect a key cardioprotective HDL functionality. We employed a well-validated HDL-CEC assay using human THP-1 cell-derived foam cells as cholesterol donor and apoB-depleted plasma as cholesterol acceptor. Significant positive associations of HDL-CEC with other HDL characteristics (HDL-C, apoA-I, HDL size, HDL-P) substantiate its measurement (Supplemental Figure 1). However, so far, a gold standard to measure HDL-CEC in humans does not exist and the optimal method for HDL isolation remains undecided, likely leading to discrepancies between studies.⁶³ The Rader Assay, another HDL-CEC method using apoB depleted plasma on murine J774 cells, has been shown to correlate with CVD.¹⁷ Therefore, we remeasured HDL-CEC in a subset of 57 CODAM samples using the Rader Assay and observed a significant correlation with an intraclass correlation coefficient of 0.6 (data not shown). In our opinion, employing human THP-1 monocyte-derived macrophage foam cells for HDL-CEC measurements provides a closer approximation of the condition in human atherosclerotic plaques, due to the use of a homologous system (human cells and human apoB-depleted plasma) and the expression of all relevant efflux transporters (ATP-binding cassette transporter A1 (ABCA1), ABCG1 and scavenger receptor B1 (SR-B1)).⁴¹

Further, the associations of HDL characteristics with the two surrogate markers of atherosclerosis, EnD Score and cIMT, were not always fully concordant. This may partly be due to the fact that they represent different aspects of atherosclerosis and partly to the measurements themselves. cIMT is a widely used noninvasive measure of atherosclerosis⁶⁴ and has been identified as a reliable indicator of generalized atherosclerosis.⁶⁵ However, cIMT measurements are not standardized^{66,67} and the sonographer, image analyst as well as random error can introduce variability.⁶⁸ Therefore, the difference in association of these parameters with cIMT and the EnD Score may be due to the sensitivity and variability of the cIMT measurements itself. Further, the washout period for lipid-lowering therapy was 14 days and may not have been sufficient to eliminate all their metabolic effects. However, the CODAM study includes participants with pre-existing CVD, thus 14 days were considered the maximum period for safety reasons. For the extrapolation of our findings, it should be

considered that our study includes individuals with at least one cardiometabolic risk factors. Nonetheless, the cohort represents a large part of typical Western populations, namely middle-aged to elderly individuals with a moderately increased risk of cardiometabolic disease. Lastly, the cross-sectional approach of our study hampers a direct causal interpretation of the observed associations.

Conclusion

In summary, in a cohort of 533 patients with an elevated risk of CVD and T2DM, we observed that HDL-CEC was not associated with atherosclerosis or CVD/CVE, neither in the whole population nor in individuals with (pre)diabetes, while other HDL characteristics showed atheroprotective associations. Notably, the atheroprotective associations of HDL-size and HDL-P are lost in individuals with (pre)diabetes. The fact that, particularly in T2DM, higher apoA-I and HDL-C concentrations were related to less CVD in subjects with (pre)diabetes, independent of CVD risk factors, was an unexpected observation and might be related to more intense cardiovascular risk monitoring and awareness of those individuals.

Conflict of Interest:

The authors declare that they have no conflict of interest.

Contributions:

T.J. and M.M.J.vG performed the analyses and T.J. created the Figures and Tables. T.J., K.W. and M.M.J.vG wrote and edited the manuscript. U.J.F.T., W.A., R.P.F.D., T.V. performed HDL-CEC assays, I.C.W.A. contributed to the lipoprotein measurements, M.M.J.vG., C.J.H.K., C.D.A.S., C.G.S. are part of the CODAM steering committee, E.A.F and I.J.G were involved in setting up the study. All co-authors read and edited the manuscript.

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Supplemental Table 1. Relationship of HDL-CEC with cIMT & the End Score adjusted for other HDL characteristics.

		cIMT N = 496			End Score N=533		
Model		std β	95% CI	p-value	std β	95% CI	p-value
HDL-CEC	full model	-0.022	-0.106;0.062	0.603	-0.007	-0.085;0.071	0.863
	full model + HDL-C	0.002	-0.088;0.093	0.962	0.082	-0.001;0.164	0.052
	full model + ApoA-I	-0.010	-0.101;0.081	0.827	0.057	-0.027;0.141	0.182
	full model + HDL size	-0.015	-0.104;0.073	0.736	0.029	-0.053;0.111	0.487
full model + HDL-P		-0.002	-0.093;0.089	0.970	0.033	-0.052;0.118	0.445

Linear regression analyses, full model adjusted for smoking, bmi, medication usage (antihypertensive, glucose- and lipid lowering), physical activity, caloric intake, systolic and diastolic blood pressure, fasting plasma glucose, and glucose metabolism state. Additional adjustment for HDL-C, apoA-I, HDL size and HDL-P was done, respectively. Std β ; standardized regression coefficient.

Supplemental Table 2. Relationship of CEC and other HDL parameters with cIMT & EnD adjusted for plasma TG Levels and LDL-C.

	Model	cIMT			EnD Score		
		N= 489			N=533		
		std β	95% CI	p-value	std β	95% CI	p-value
HDL-CEC	full model	-0.022	-0.106;0.062	0.603	-0.007	-0.085;0.071	0.863
	full model + TG	-0.022	-0.106;0.062	0.602	-0.007	-0.085;0.071	0.858
	full model + LDL-C	-0.022	-0.106;0.061	0.600	-0.007	-0.085;0.072	0.864
HDL-C	full model	-0.071	-0.164;0.021	0.130	-0.226	-0.312;-0.140	<0.0005
	full model + TG	-0.097	-0.200;0.005	0.064	-0.218	-0.313;-0.123	<0.0005
	full model + LDL-C	-0.058	-0.152;0.035	0.221	-0.231	-0.318;-0.144	<0.0005
ApoA-I	full model	-0.039	-0.132;0.054	0.408	-0.158	-0.244;-0.073	<0.0005
	full model + TG	-0.044	-0.138;0.051	0.365	-0.145	-0.231;-0.058	0.001
	full model + LDL-C	-0.037	-0.129;0.056	0.436	-0.158	-0.244;-0.073	<0.0005
HDL size	full model	-0.030	-0.123;0.063	0.527	-0.118	-0.205;-0.031	0.008
	full model + TG	-0.052	-0.159;0.055	0.339	-0.084	-0.184;0.017	0.103
	full model + LDL-C	-0.022	-0.115;0.071	0.638	-0.119	-0.206;-0.031	0.008
HDL-P	full model	-0.060	-0.154;0.034	0.208	-0.097	-0.184;-0.011	0.028
	full model + TG	-0.065	-0.160;0.030	0.181	-0.082	-0.169;0.006	0.068
	full model + LDL-C	-0.057	-0.151;0.037	0.231	-0.097	-0.184;-0.011	0.028

Linear regression analyses, full model adjusted for smoking, bmi, medication usage (antihypertensive, glucose- and lipid lowering), physical activity, caloric intake, systolic and diastolic blood pressure, fasting plasma glucose, and glucose metabolism state. Additional adjustment for triglycerides (TG), and LDL-C was done, respectively. Std β ; standardized regression coefficient.

Supplemental Table 3. Relationship of CEC and other HDL parameters with CVD and CVE adjusted for plasma TG levels and LDL-C.

	Model	CVD			CVE		
		N=533, 150 CVD cases			N=533, 85 CVE cases		
		OR	95th CI	p-value	OR	95th CI	p-value
HDL-CEC	full model	0.999	0.796;1.253	0.992	1.049	0.766;1.437	0.765
	full model + TG	0.988	0.787;1.240	0.917	1.037	0.757;1.419	0.821
	full model + LDL-C	1.000	0.797;1.255	0.999	2.054	0.768;1.445	0.746
HDL-C	full model	0.800	0.608;1.053	0.112	0.606	0.406;0.904	0.014
	full model + TG	0.909	0.671;1.230	0.536	0.614	0.398;0.947	0.027
	full model + LDL-C	0.789	0.599;1.040	0.092	0.59	0.396;0.878	0.009
ApoA-I	full model	0.814	0.627;1.056	0.814	0.667	0.462;0.963	0.030
	full model + TG	0.845	0.649;1.101	0.213	0.676	0.467;0.977	0.037
	full model + LDL-C	0.814	0.628;1.056	0.122	0.674	0.467;0.972	0.035
HDL size	full model	0.872	0.676;1.126	0.295	0.870	0.618;1.223	0.422
	full model + TG	1.031	0.768;1.383	0.840	0.948	0.641;1.402	0.790
	full model + LDL-C	0.871	0.675;1.124	0.288	0.876	0.623;1.231	0.446
HDL-P	full model	0.897	0.696;1.156	0.400	0.679	0.477;0.965	0.031
	full model + TG	0.941	0.727;1.217	0.641	0.694	0.487;0.991	0.044
	full model + LDL-C	0.898	0.697;1.158	0.408	0.687	0.482;0.979	0.038

Logistic regression analyses, full model adjusted for smoking, bmi, medication usage (antihypertensive, glucose- and lipid lowering), physical activity, caloric intake, systolic and diastolic blood pressure, fasting plasma glucose, and glucose metabolism state. Additional adjustment for triglycerides (TG), and LDL-C was done, respectively.

Supplemental Table 4. Relationship of CEC and other HDL parameter with cIMT, stratified for normoglycemia and (pre)diabetes and adjusted for plasma TG levels and LDL-C.

	Model	cIMT						
		NGM N=268			IGM&T2DM N=228			
		std β	95% CI	p-value	std β	95% CI	p-value	
							p _{int}	
HDL-CEC	full model	0.061	-0.044;0.167	0.255	-0.129	-0.263;0.005	0.060	0.025
	full model + TG	0.062	-0.044;0.167	0.249	-0.130	-0.264;0.004	0.058	0.025
	full model + LDL-C	0.061	-0.044;0.167	0.252	-0.130	-0.263;0.004	0.056	0.023
HDL-C	full model	-0.044	-0.158;0.070	0.446	-0.138	-0.293;0.017	0.081	0.169
	full model + TG	-0.028	-0.151;0.096	0.659	-0.239	-0.416;-0.062	0.008	0.116
	full model + LDL-C	-0.029	-0.146;0.088	0.625	-0.134	-0.288;0.021	0.090	0.120
ApoA-I	full model	0.011	-0.105;0.128	0.848	-0.118	-0.268;0.031	0.120	0.067
	full model + TG	0.019	-0.099;0.137	0.750	-0.144	-0.297;0.009	0.065	0.063
	full model + LDL-C	0.018	-0.099;0.135	0.762	-0.126	-0.275;0.023	0.097	0.047
HDL size	full model	-0.055	-0.167;0.056	0.329	-0.006	-0.170;0.159	0.947	0.910
	full model + TG	-0.039	-0.166;0.087	0.540	-0.079	-0.275;0.117	0.428	0.789
	full model + LDL-C	-0.045	-0.158;0.068	0.433	-0.017	-0.181;0.147	0.836	0.733
HDL-P	full model	0.006	-0.115;0.128	0.919	-0.132	-0.276;0.012	0.073	0.038
	full model + TG	0.014	-0.108;0.137	0.818	-0.151	-0.297;-0.005	0.043	0.037
	full model + LDL-C	0.012	-0.109;0.134	0.841	-0.135	-0.278;0.009	0.066	0.030

Linear regression analyses, full model adjusted for smoking, bmi, medication usage (antihypertensive, glucose- and lipid lowering), physical activity, caloric intake, systolic and diastolic blood pressure, fasting plasma glucose, and glucose metabolism state. Additional adjustment for triglycerides (TG), and LDL-C was done, respectively. Std β ; standardized regression coefficient.

Supplemental Table 5. Relationship of CEC and other HDL parameter with END, stratified for normoglycemia and (pre)diabetes and adjusted for plasma TG levels and LDL-C.

	Model	End Score						
		NGM N=279			IGM&T2DM N=254			
		std β	95% CI	p-value	std β	95% CI	p-value	p _{int}
HDL-CEC	full model	-0.076	-0.177;-0.025	0.142	0.064	-0.060;0.188	0.312	0.103
	full model + TG	-0.074	-0.175;0.028	0.153	0.063	-0.061;0.187	0.317	0.109
	full model + LDL-C	-0.076	-0.177;0.026	0.142	0.062	-0.063;0.186	0.331	0.103
HDL-C	full model	-0.256	-0.362;-0.150	<0.0005	-0.185	-0.330;-0.040	0.013	0.508
	full model + TG	-0.259	-0.373;-0.144	<0.0005	-0.151	-0.317;0.015	0.074	0.471
	full model + LDL-C	-0.282	-0.390;-0.174	<0.0005	-0.184	-0.330;-0.039	0.013	0.459
ApoA-I	full model	-0.225	-0.333;-0.117	<0.0005	-0.071	-0.210;0.067	0.312	0.102
	full model + TG	-0.216	-0.325;-0.108	<0.0005	-0.046	-0.187;0.095	0.520	0.085
	full model + LDL-C	-0.231	-0.340;-0.123	<0.0005	-0.076	-0.216;0.063	0.281	0.100
HDL size	full model	-0.204	-0.309;-0.099	<0.0005	0.033	-0.119;0.185	0.668	0.039
	full model + TG	-0.207	-0.326;-0.088	0.001	0.158	-0.020;0.336	0.081	0.018
	full model + LDL-C	-0.217	-0.324;-0.111	<0.0005	0.028	-0.125;0.180	0.718	0.036
HDL-P	full model	-0.205	-0.319;-0.092	<0.0005	0.037	-0.095;0.168	0.582	0.006
	full model + TG	-0.195	-0.310;-0.081	0.001	0.061	-0.072;0.194	0.366	0.005
	full model + LDL-C	-0.210	-0.323;-0.097	<0.0005	0.034	-0.098;0.166	0.611	0.006

Linear regression analyses, full model adjusted for smoking, bmi, medication usage (antihypertensive, glucose- and lipid lowering), physical activity, caloric intake, systolic and diastolic blood pressure, fasting plasma glucose, and glucose metabolism state. Additional adjustment for triglycerides (TG), and LDL-C was done, respectively. Std β : standardized regression coefficient.

Supplemental Table 6. Relationship of CEC and other HDL parameters with CVD, stratified for normoglycemia and (pre)diabetes and adjusted for plasma TG levels and LDL-C.

	Model	CVD					
		NGM			IGM+T2DM		
		N=279; 67 CVD cases			N=254; 83 CVD cases		
		OR	95th CI	p-value	OR	95th CI	p-value
HDL-CEC	full model	0.868	0.612;1.232	0.428	1.033	0.752;1.419	0.839
	full model + TG	0.927	0.557;1.543	0.771	1.022	0.673;1.552	0.920
	full model + LDL-C	0.873	0.615;1.241	0.450	1.034	0.753;1.419	0.839
HDL-C	full model	0.835	0.558;1.250	0.382	0.617	0.403;0.944	0.026
	full model + TG	0.870	0.566;1.339	0.528	0.785	0.489;1.259	0.315
	full model + LDL-C	0.800	0.530;1.209	0.290	0.617	0.403;0.944	0.026
ApoA-I	full model	0.978	0.665;1.440	0.911	0.609	0.413;0.898	0.012
	full model + TG	0.993	0.674;1.464	0.973	0.639	0.428;0.954	0.029
	full model + LDL-C	0.969	0.659;1.426	0.873	0.605	0.409;0.895	0.012
HDL size	full model	0.813	0.559;1.183	0.279	0.678	0.454;1.012	0.057
	full model + TG	0.783	0.427;1.437	0.430	0.796	0.447;1.415	0.436
	full model + LDL-C	0.796	0.544;1.163	0.238	0.674	0.451;1.009	0.055
HDL-P	full model	0.971	0.649;1.451	0.884	0.779	0.556;1.092	0.148
	full model + TG	0.644	0.353;1.175	0.152	0.659	0.413;1.054	0.082
	full model + LDL-C	0.778	0.555;1.091	0.145	0.968	0.648;1.446	0.874

Logistic regression analyses, full model adjusted for smoking, bmi, medication usage (antihypertensive, glucose- and lipid lowering), physical activity, caloric intake, systolic and diastolic blood pressure, fasting plasma glucose, and glucose metabolism state. Additional adjustment for triglycerides (TG), and LDL-C was done, respectively.

Supplemental Figure 1. Association of HDL-C with HDL-C, apoA-I, HDL size and HDL-P.
Scatterplots showing the associations of HDL-C with HDL-C (*top left*), apoA-I (*top right*), HDL size (*bottom left*), and HDL-P (*bottom right*). N= 533

3

Role of LpL(Lipoprotein Lipase) in Macrophage Polarization In Vitro and In Vivo

Hye Rim Chang*, Tatjana Josefs*, Diego Scerbo, Namrata Gumaste, Yuning Hu, Lesley-Ann
Huggins, Tessa J. Barrett, Stephanie Chiang, Jennifer Grossman, Svetlana Bagdasarov,
Edward A. Fisher, Ira J. Goldberg

*equal contribution

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Abstract

Objective: Fatty acid uptake and oxidation characterize the metabolism of alternatively activated macrophage polarization in vitro, but the in vivo biology is less clear. We assessed the roles of LpL (lipoprotein lipase)-mediated lipid uptake in macrophage polarization in vitro and in several important tissues in vivo.

Approach and Results: We created mice with both global and myeloid-cell specific LpL deficiency. LpL deficiency in the presence of VLDL (very low-density lipoproteins) altered gene expression of bone marrow–derived macrophages and led to reduced lipid uptake but an increase in some anti- and some pro-inflammatory markers. However, LpL deficiency did not alter lipid accumulation or gene expression in circulating monocytes nor did it change the ratio of Ly6Chigh/Ly6Clow. In adipose tissue, less macrophage lipid accumulation was found with global but not myeloid-specific LpL deficiency. Neither deletion affected the expression of inflammatory genes. Global LpL deficiency also reduced the numbers of elicited peritoneal macrophages. Finally, we assessed gene expression in macrophages from atherosclerotic lesions during regression; LpL deficiency did not affect the polarity of plaque macrophages.

Conclusions: The phenotypic changes observed in macrophages upon deletion of *Lpl* in vitro is not mimicked in tissue macrophages.

Introduction

Macrophages (Mφs) are indispensable for maintaining tissue homeostasis and defending against multiple threats as members of the innate immune system. They have essential developmental roles and regulate normal physiology as “first responders” by communicating with the host’s adaptive immune system ¹. Although the role and phenotype of different tissue Mφs are diverse and environment-specific, the Mφ phenotype is often oversimplified as a dichotomy of classically activated Mφs (CAMφs or M1) and alternatively activated Mφs (AAMφs or M2). Key determinants of these two phenotypes are thought to include differences in the metabolic pathways used by these cells, and a number of studies, predominantly *in vitro*, suggest that metabolic substrate use controls Mφ polarization ^{2, 3}.

These two forms of Mφs defend against different pathogens. CAMφs are implicated in acute bacterial infection; they are inflammatory, and their metabolic phenotype is glycolytic and hypoxia inducible factor 1α-dependent ³. AAMφs play a significant role in parasitic infection. They utilize fatty acid oxidation (FAO) and play roles in resolving inflammation, including experimental atherosclerosis ⁴, and in wound healing. Canonical induction of M2 polarization is mediated by signal transducer and activator of transcription 6, which transcriptionally upregulates both peroxisome proliferator-activated receptors (PPARs; PPARδ, PPARγ) and PPARγ coactivator-1 beta (PGC1β) ³. PPARs and PGC1β activation induce anti-inflammatory markers (*Arg1*, *Ym/Chi3l3*, and *Fizz1/Relma*), FAO-related genes (*Cpt1a*, *Aox*) ^{3, 5}, and fatty acid (FA) uptake genes such as cluster of differentiation 36 (*Cd36*) and lipoprotein lipase (*Lpl*) ⁵.

As noted above, AAMφs perform oxidative phosphorylation of FAs and also of glucose ⁶ and glutamine ⁷, with FAO being the dominant source of energy. Uptake and use of FAs have shown to be critical to conversion of Mφs to AAMφs *in vitro* ⁵, but the *in vivo* biology is less clear. LpL is the rate-limiting enzyme for the conversion of circulating triglycerides (TGs), within chylomicrons and very low-density lipoproteins (VLDL), to free FAs (FFAs). It is also responsible, in part, for regulating plasma TG levels. Further, LpL increases the association of lipoproteins with proteoglycans, often referred to as bridging, and hydrolyses circulating lipoprotein, which is critical for lipid uptake in skeletal muscle, heart, and brown adipose tissue (AT) ⁸⁻¹⁰. LpL deletion reduces cellular uptake of both FFA and VLDL that occurs via non-receptor (bridging) pathways ¹¹; *in vitro* studies have documented the importance of this process in monocytes and

Mφs¹²⁻¹⁵. Notably, macrophage LpL deficiency does not affect receptor-mediated endocytosis¹⁶. Several *in vitro* studies have shown that LpL-mediated VLDL lipid accumulation in monocytes induces expression of inflammatory genes such as interleukin-1 beta (*Il1β*) and tumor necrosis factor alpha (*Tnfa*)^{17, 18}. This indicates that VLDL-derived lipid uptake is inflammatory in monocytes. In contrast, LpL-mediated VLDL lipid uptake has been proposed to suppress Mφ inflammation via PPARδ activation^{19, 20}, suggesting that LpL-mediated lipid uptake promotes an AA phenotype. In contrast to these purported roles in driving Mφs to a less inflammatory and more reparative phenotype, Mφ-derived LpL is atherogenic²¹. These data imply that pathways of lipid uptake and their effects on Mφ polarization found *in vitro* may differ from what occurs *in vivo*.

Thus, we asked whether the influences of lipid uptake pathways on Mφ polarity are microenvironment-specific. To answer this, we assessed the roles of LpL in Mφ polarization *in vitro* and in several important depots *in vivo*, including AT, the peritoneal cavity, and atherosclerotic plaques. Our data indicate that LpL affects both lipid uptake and Mφ polarity. The situation *in vivo* is more complicated as complete LpL deficiency also causes marked hypertriglyceridemia. However, this issue was controlled for, in part, by also studying mice with a myeloid-specific LpL deletion. Finally, our data show that LpL exerts distinct effects in each tissue depot *in vivo*, an observation that cannot be easily modeled *in vitro*.

Materials and Methods

The authors declare that all supporting data are available within the article and its online supplementary files.

Animals: Global inducible *Lpl* knockout (*iLpl*^{-/-}) mice were generated by crossing *Lpl*^{fl/fl} mice with Tg^{CreER} transgenic mice (The Jackson Laboratory)²², which harbor the tamoxifen-inducible Cre recombinase driven by the chicken beta actin promoter/enhancer coupled with the cytomegalovirus (CMV) immediate-early enhancer (β -actin-*MerCreMer*). Myeloid cell-specific *Lpl* knockout (*Mac-Lpl*^{-/-}) mice²¹ were generated by crossing *Lpl*^{fl/fl} mice with *LysMCre* mice (The Jackson Laboratory).

Cell culture: Bone marrow (BM) cells were isolated by flushing cells from the femurs or tibiae of mice. Cells were differentiated into BM-derived Mφs (BMDMs) in normal

(5 mM) glucose DMEM with 10% Fetal Bovine Serum (FBS), 1% penicillin/streptomycin, and murine M-CSF (10 ng/mL; PeproTech) for 7 days. The BMDMs were cultured in low (1 mM) glucose or normal (5 mM) glucose or high (25 mM) glucose DMEM for 24 hours with or without murine IL-4 (20 ng/mL; PeproTech) and/or human VLDL (100 µg/mL; Alfar Aesar) plus 5% FBS for 24 hours.

Plasma Lipid Measurement: Total triglyceride (TG) and total cholesterol (TC) were measured by using Infinity Total Triglyceride Reagent (Thermo Fisher Scientific; #TR22421) and Infinity Total Cholesterol Reagent (Thermo Fisher Scientific; #TR13421). Non-esterified FAs (NEFAs) were measured by using Wako Diagnostic NEFA reagents (Wako Life Sciences, Inc).

Lipoprotein Fractionation: Equal amounts of plasma (70 µl-100 µl) were used for sequential density ultracentrifugation to separate VLDL ($d < 1.006$ g/mL), low-density lipoprotein (LDL) ($d = 1.006-1.063$ g/mL), high-density lipoprotein (HDL) ($d = 1.063-1.21$ g/mL) in a TLA 100 rotor (Beckmann Instruments). Fractions were used to measure TC and TG as described above.

Glucose and Insulin Measurement: Blood glucose was measured by using OneTouch Ultra2 meter (One Touch). Plasma insulin was measured by using Mouse Ultrasensitive Insulin ELISA kit (Alpco; #80-ISMSU-E01).

White blood cell counts: Total white blood cell counts in freshly collected mouse blood were obtained using a hematology cell counter (Oxford Science Inc.).

Blood Leukocytes: Monocytes (total and subsets) and neutrophils were identified from whole blood as previously described ²³.

Hematopoietic stem cells: Hematopoietic stem and progenitor cells from the BM and spleen were analyzed by flow cytometry as previously described ²³.

Adipose tissue macrophage (ATM) isolation: ATMs were isolated as previously described ²⁴.

Peritoneal macrophage isolation: Peritoneal exudate cells were harvested by peritoneal lavage with FACS buffer (PBS+ 0.2%BSA+ 5mM EDTA) at a volume of 10 mL per mouse. Total peritoneal exudate cells were sorted by using FACS Arian (BD Bioscience). The gating strategy to sort large peritoneal Mφs (LPMs) and small peritoneal Mφs (SPMs) was as previously described ²⁵ and is shown in Supplemental Figure 6.

Seahorse extracellular flux analysis: BMDMs from *Lpl^{fl/fl}* and *iLpl^{-/-}* were seeded (300,000 cells per well) in 5mM glucose supplemented with 2%FBS into XF24 cell culture microplates (Seahorse Bioscience) and stimulated with IL-4 as described above. After 24 hours, oxygen consumption rates (OCR) were measured using an XFe24 instrument (Seahorse Bioscience). For cellular mitochondrial oxidation assessments, the XF Cell Mito Stress Test Kit was used according to the manufacturer's protocol.

Neutral lipid content measurement: Adipo-Red (Lonza) was used according to the manufacturer's protocol to measure intracellular lipid content in BMDMs. Freshly isolated monocytes, ATMs, and peritoneal Mφs were stained with Bodipy (1:500 dilution; Sigma) for 30 mins in dark on ice to fluorescently label intracellular lipid accumulation. Total neutral lipid content was quantified by using flow cytometry (LSRII) and analyzed by using FlowJo software.

Aortic Transplant. The aortic transplant model has been described before ^{4, 26-29} A plaque burden aortic arch from *Ldlr^{-/-}* mice was transplanted into the recipient mice (*iLpl^{-/-}* or *Lpl^{fl/fl}* mice), which was inter-positioned with the abdominal aorta. During the transplantation, blood flow was directed through the graft. At time of the transplant, all recipient mice were approximately 22 weeks old and maintained on a normal rodent diet. All mice were sacrificed 14 days after the aortic arch transplantation. AHA guidelines for experimental atherosclerosis studies have been followed ³⁰.

Laser capture microdissection and quantitative Real Time PCR. CD68⁺ cells were selected from atherosclerotic plaques by laser capture microdissection. All laser capture microdissection procedures were performed under RNase-free conditions. Aortic root sections were stained with hematoxylin-eosin as previously described ^{31, 32}.

Immunohistochemistry and plaque assessment. Grafted arches were removed after perfusion with 10% sucrose in saline, and embedded in optimum cutting temperature (OCT, Tissue-Tek; Sakura Finetek USA) block and freezed. Serial sections at (6 μm thick) were cut and stained for CD68 (Bio-Rad MCA1957) and CD206, also known as Mannose Receptor (MR) (Bio-Rad MCA2235). Negative controls were performed using an isotype control (Rat IgG2a) instead of the primary antibody (Abcam ab18450). ImagePro Plus 7.0 software was used to determine CD68⁺ and MR⁺ areas. Overlay was done using Adobe Photoshop CC2018.

Statistical Analyses: Data are presented as mean $\pm$ SD. Normality test (Shapiro-Wilk normality test ($\alpha=0.05$) and visual inspection of Q-Q plots) and homogeneity of variance (Brown-Forsythe test) were performed. Depending on the results, parametric or non-parametric tests were chosen as indicated in the figure legends. * $p<0.05$; ** $p<0.01$. All calculations were performed using Microsoft Excel and Graphpad Prism 7.

Results

LpL deficiency in M ϕ s affects their polarization and lipid accumulation *in vitro*:

LpL mediates the hydrolysis of VLDL-derived TG and activates PPAR δ ¹⁴, which suppresses inflammatory pathways in cultured M ϕ s ²⁰. To assess whether LpL deficiency affects VLDL-dependent lipid uptake and alternative activation (AA) under various culture conditions, BMDMs from wild type and M ϕ -specific LpL deficient mice (*Lpl*^{fl/fl}; *LysMCre*, *Mac-Lpl*^{-/-}) were cultured with 100 μ g/mL of human VLDL plus 5% FBS for 24 hours in low-glucose (1 mM) containing media. LpL actions enhance M ϕ s phagocytic function only in low glucose media ¹⁶ when FAs are likely to be a more critical source of energy substrate. Efficient *Lpl* deletion in BMDMs is shown in Figure 1A. As expected, LpL deficiency led to a marked reduction in FFA in the VLDL-containing culture system (Figure 1B). Loss of LpL in M ϕ s significantly reduced intracellular neutral lipids as measured by bodipy and Nile-Red staining (Figure 1C, 1D). To assess whether lack of LpL alters known PPAR δ -targeted genes, we measured a lipid droplet-related gene, perilipin 2 (*Plin2*) and a FAO-related gene, carnitine palmitoyltransferase 1A (*Cpt1a*). Consistent with the intracellular lipid droplet results, *Plin2* and *Cpt1a* gene expression were decreased in *Mac-Lpl*^{-/-} BMDMs (Figure 1E, 1F). In order to examine whether lack of LpL induces other metabolic-related gene expression as a compensatory mechanism, we measured glucose transporter 1 (*Glut1/Slc2a1*) and FA synthase (*Fasn*) mRNA levels. *Lpl* deficiency increased both *Glut1* and *Fasn* mRNA expression upon VLDL treatment (Figure 1G, 1H), suggesting that lack of LpL-mediated FA uptake increases glucose uptake and *de novo* FA synthesis. These results demonstrate that M ϕ -derived LpL is indispensable for VLDL-mediated FA uptake under a low glucose culture condition. Next, we investigated whether loss of LpL-mediated VLDL FA uptake impairs M2 polarization and/or augments M1 polarization. Although *Arg1* gene expression was decreased, as had been suggested by Chawla et al. ⁵, mRNA levels of other M2 markers, *Ym1* and *Mrc1*,

were increased in Mac-*Lpl*^{-/-} BMDMs in the presence of VLDL, as compared to *Lpl*^{fl/fl} BMDMs (Figure 1I, 1J, 1L). Among inflammatory genes, *Tnfa* was increased in Mac-*Lpl*^{-/-} BMDMs (Figure 1M), but *Nos2* gene expression showed a trend to decreased expression in Mac-*Lpl*^{-/-} BMDMs in the presence of VLDL (Figure 1O). These results show that lack of LpL in Mφs reduces lipid uptake and alters both inflammatory- and anti-inflammatory genes in VLDL-enriched culture conditions. However, the mixed phenotypic responses indicate a level of complexity in Mφ polarization due to different transcriptional regulation within both the inflammatory and AA pathways.

Figure 1: BMDMs from *Lpl*^{fl/fl} and Mac-*Lpl*^{-/-} mice were used to measure metabolism- related and M1/M2-related gene expression in the presence of human VLDL (100ug/mL). (A) The gene expression of *Lpl* in BMDMs. (B) Conditioned media FFAs concentration from cultured BMDMs from *Lpl*^{fl/fl} and Mac-*Lpl*^{-/-} mice. (C) Fluorescent staining of nuclei by DAPI and neutral lipid by bodipy dye in VLDL treated BMDMs (Blue: nuclei, Green: neutral lipids). (D) Quantification of neutral lipid content measured by Nile-red stain in VLDL treated BMDMs (RFU: Relative Fluorescent Unit). (E-F) The expression of PPARδ-targeted genes (*Plin2*, *Cpt1a*) in BMDMs. (G-H) The expression of *Glut1* and *Fasn* in Mac-*Lpl*^{-/-} BMDMs. (I-L) The gene expression of anti-inflammatory genes (*Arg1*, *Ym1*, *Fizz1*, *Mrc1*) in VLDL treated BMDMs. (M-P) The expression of inflammatory genes (*Tnfa*, *I1b*, *Nos2*, *Mcp1*) in VLDL treated BMDMs. N=6/group. *p<0.05, **p<0.01. Results are represented as median with 25th and 75th percentiles, capped bars indicate 10th and 90th percentile and compared using Two-way ANOVA, Sidak's multiple comparison.

Figure 1. LpL is required for VLDL-derived lipid uptake in macrophages and regulates both inflammatory and anti-inflammatory genes in the presence of VLDL containing low glucose media *in vitro*.

To investigate whether LpL regulates M ϕ polarity in response to the standard inducer of AAMs *in vitro*, we cultured *Lpl^{fl/fl}* and *Mac-Lpl^{-/-}* BMDMs with IL-4 (20 ng/mL) and IL-4 plus VLDL for 24 hours under low glucose conditions. Surprisingly, IL-4 stimuli alone increased *Plin2*, *Cpt1a*, and *Fasn* mRNA levels in *Mac-Lpl^{-/-}* BMDMs, as compared to *Lpl^{fl/fl}* BMDMs (Figure 2A, 2B, and 2D). Since IL-4 induces FAO ⁵, lack of FA uptake perhaps stimulated cells to increase *de novo* FA synthesis. Culturing M ϕ s with IL-4 plus VLDL led to a significant decrease in *Plin2* and *Cpt1a*, but a trend of increase in *Glut1* and a significant ~ 5-fold increase in *Fasn* gene expression in *Mac-Lpl^{-/-}* BMDMs (Figure 2A, 2B, 2C, and 2D), similar to *in vitro* findings in Figure 1 E-H. This suggests that in M2-like M ϕ s, LpL-mediated VLDL-derived lipid uptake regulates PPAR δ -targeted genes, but lack of LpL seem to induce metabolic compensations via *de novo* FA synthesis.

As expected, upon IL-4 treatment alone, lack of LpL prevented up-regulation of *Arg1* (Figure 2E). Deletion of LpL did not affect *Ym1*, *Fizz1* and *Mrc1* gene expression (Figure 2F-H). Upon IL-4 and VLDL treatment, *Lpl* deficiency did not affect *Arg1*, *Ym1*, and *Mrc1* but dramatically increased *Fizz1/Relma* expression. Huang et al. demonstrated that glucose-mediated FA synthesis is necessary for M2 activation as measured by *Fizz1/Relma* and programmed death-ligand 1 expression ⁶. Thus, up-regulation of *Fizz1* expression is probably due to glucose-mediated FA synthesis, as we found an increase in both *Glut1* and *Fasn* mRNA levels in *Mac-Lpl^{-/-}* BMDMs. To test the metabolic status of M ϕ s in LpL depleted conditions, we performed OCR experiments in cultured *Lpl^{fl/fl}* and *Mac-Lpl^{-/-}* BMDMs incubated with IL-4 (20 ng/mL) (Figure 2 I-J). The results showed that OCR is increased when LpL is depleted, being in accord with the changes we found in *Cpt1a* gene expression. Overall, our data show that in AA M ϕ s, LpL is necessary for FA uptake, but it is not anti-inflammatory under the low glucose condition *in vitro*. Further, increase in *Glut1* mRNA level suggests that LpL deficient M ϕ s switch to a greater use of glucose.

Figure 2. LpL is necessary for VLDL-derived FA uptake, but it is not anti-inflammatory under low-glucose condition *in vitro*. BMDMs from *Lpl*^{fl/fl} and *Mac-Lpl*^{-/-} mice were used to measure metabolism-related and M2-related genes in the presence of IL-4 (20ng/mL) or IL-4 plus human VLDL. (A) The expression of a lipid droplet-related protein, *Plin2*, gene in BMDMs. (B) The expression of a fatty acid oxidation-related gene, *Cpt1a* in BMDMs. (C) The expression of a glucose transporter, *Glut1* (*Slc2a1*), gene in BMDMs. (D) The expression of fatty acid synthase gene in BMDMs. (E-H) The expression of canonical M2 (anti-inflammatory) genes in BMDMs. (I) Oxygen consumption rate comparing IL-4 treatment in *Lpl*^{fl/fl} and *Mac-Lpl*^{-/-}. N=3/group. *p<0.05, **p<0.01. Results are represented as median with 25th and 75th percentiles, capped bars indicate 10th and 90th percentile and compared compared using Two-way ANOVA with Sidak's multiple comparison test.

To test whether FFAs not derived from LpL-hydrolysis alter the phenotype of *Mac-Lpl*^{-/-} BMDMs, we incubated BMDMs with or without oleic acid (OA) (300 μM) for 24 hours. OA significantly decreased *Lpl* expression, but increased *Cpt1a* gene expression in *Lpl*^{fl/fl} BMDMs (Supplemental Figure 1). OA treatment also significantly lowered *Mrc1*

gene expression in Mac-*Lpl*^{-/-} BMDMs with a trend towards decreased expression in *Lpl*^{fl/fl} BMDMs. Unexpectedly, we did not find altered expression of other anti-inflammatory related-genes such as *Ym1/Chil3* or *Fizz1/Relma*, whereas inflammatory genes increased upon OA treatment (Supplemental Figure 1). Although OA treatment is generally known to induce M2 genes (*Arg1*, *Mrc1*)³³⁻³⁵, our data suggest that OA-dependent M2 activation can be time- or dose-dependent. The pattern of gene expression in response to OA treatment was similar in *Lpl*^{fl/fl} compared to Mac-*Lpl*^{-/-} BMDMs, suggesting that FA uptake does regulate anti-inflammatory or inflammatory gene expression independent of LpL expression, whereas VLDL-derived FA requires LpL expression to invoke an inflammatory response.

In order to determine whether increasing glucose concentration from low glucose culture conditions (1 mM) to normal (5 mM) affects Mφ polarity *in vitro*, we performed the same experiment with both media. In the normal glucose condition, *Lpl* deletion increased *Plin2*, *Cpt1a*, and *Fasn* mRNA levels (Supplemental Figure 2A, 2B and 2E) but did not alter *Glut1* expression in the presence of VLDL (Supplemental Figure 2D). These data indicate that under the normal glucose condition, *in vitro*, Mφs mainly utilize glucose as the energy fuel as they do *in vivo*³⁶; thus, lack of LpL-mediated FA uptake does not impact *Glut1* expression. With regards to Mφ polarity, similar to low glucose culture conditions, *Arg1* was significantly decreased in Mac-*Lpl*^{-/-} BMDMs, whereas other typical M2 genes such as *Ym1* and *Fizz1* were up-regulated in the presence of VLDL (Supplemental Figure 2F, 2G, and 2H). Upon IL-4 stimulation in the normal glucose condition, we found a similar pattern to the low glucose conditions, suggesting that increasing glucose concentration from 1 mM to 5 mM does not significantly impact *Lpl* deficient Mφ polarity. The changes that we observe only matter to M2-induced conditions using VLDL and/or IL-4, as culture conditions without substrate added to the enzyme and no induction for anti-inflammation served as baseline conditions.

Since our results show that *Lpl* deficiency up-regulates *Glut1* and *Fasn* gene expression under low glucose and up-regulates *Fasn* gene expression under the normal glucose culture condition, we hypothesized that glucose-mediated FA synthesis is responsible for increasing some M2-related genes (*Ym1* and *Fizz1*). Thus, we performed the same *in vitro* experiment under high (25 mM) glucose conditions to examine whether an excess amount of glucose impacts both metabolic- and M2-related genes. Unlike low and normal glucose culture conditions, we found no

significant difference between *Lpl* expressing and non-expressing cells in all M2-related genes (*Arg1*, *Ym1*, *Fizz1*, *Mrc1*) in high glucose containing media (Supplemental Figure 3G, 3H, 3I and 3J). These data indicate that with excess glucose, Mφs mainly use glucose as their energy source; thus, ablating LpL-mediated FA uptake does not affect either metabolism or polarization.

The impact of myeloid-specific *Lpl* ablation on circulating monocytes in obese mice:

While we could reproduce many of the *in vitro* findings of previously reported studies^{14, 19}, we found that these effects varied by culture condition. For this reason, we next investigated the role of LpL in monocytes and Mφs in a lipid-rich microenvironment *in vivo*. In order to induce a hyperlipidemic condition, we used mice fed a HFD. Obesity increases circulating levels of myeloid-derived cells³⁷. This diet also enables us to examine ATMs, which are limited in animals fed a normal rodent diet. Thus, we sought to determine whether myeloid cell-derived LpL regulates monocyte subpopulations (Ly6C^{hi} and Ly6C^{low} monocytes), lipid accumulation, and phenotype of monocytes. The detailed experimental design is shown in Figure 3A. We confirmed that *Lpl* was efficiently deleted in monocytes in *Mac-Lpl*^{-/-} mice (Figure 3B). Ablation of *Lpl* in myeloid-derived cells with HFD did not affect plasma glucose levels (Figure 3C). As expected, levels of plasma TG and TC in VLDL, LDL, and HDL in *Mac-Lpl*^{-/-} mice were not significantly different from TG and TC levels in *Lpl*^{fl/fl} mice (Figure 3D, 3E). Lack of LpL in monocytes with HFD did not affect circulating levels of Ly6C^{hi}- and Ly6C^{low}-monocytes (Figure 3F). BM progenitors (common myeloid progenitors and granulocyte-Mφ progenitors) in *Mac-Lpl*^{-/-} mice were not significantly different from *Lpl*^{fl/fl} mice (Supplemental Figure 4A), as circulating levels of monocytes were unaltered. Thus, differences in BM progenitors and circulating monocytes noted with normal rodent diets³⁸ were abrogated by the HFD, which is known to stimulate BM progenitors³⁷. Ablation of *Lpl* did not alter intracellular lipid content as measured by percent of bodipy+ cells from each monocyte population (Figure 3G, 3H) and bodipy+ geometric mean fluorescent intensity (gMFI) (Figure 3I). Consistent with the intracellular lipid content, expression of *Plin2* and other lipid-related genes were unaltered in *Mac-Lpl*^{-/-} mice (Figure 3J). Also, *Lpl* deficiency in monocytes did not lead to an increase in inflammatory gene expression (Figure 3K) and did not affect expression of anti-inflammatory-related

genes (Figure 3L). These results indicate that myeloid cell-derived LpL *in vivo* is not required in monocytes for lipid accumulation and is not inflammatory in these cells.

Figure 3. Myeloid-cell derived LpL does not affect systemic metabolism and circulating levels of monocytes, and is not required for lipid accumulation in monocytes *in vivo*. Littermate controls (*Lpl^{fl/fl}*) and *Mac-Lpl^{-/-}* (*Lpl^{fl/fl}*; *LysMCre*) mice were studied for plasma metabolic parameters, circulating levels of monocytes, and lipid content and phenotype of monocytes. (A) Experimental design: *Lpl^{fl/fl}* and *Mac-Lpl^{-/-}* mice were fed with a HFD (60% kcal fat) from 8 weeks old of age until sacrifice (26 weeks old of age); Body weight and plasma parameters were measured between 24 weeks of age and 25 weeks of age; Circulating monocytes (Ly6C^{hi} and Ly6C^{low}), adipose tissue (PGAT, SCAT, and BAT), and adipose tissue macrophages (CD45⁺F4/80⁺CD11b⁺ and CD45⁺F4/80⁺CD11b⁺CD11c⁺) were obtained at 26 weeks old of age. (B) The expression of *Lpl* in monocytes (CD45⁺CD115⁺) that were isolated by fluorescence-activated cell sorting (FACS) method. (C) Plasma fasting glucose level. (D) Levels of plasma triglyceride in VLDL, LDL, and HDL fraction. (E) Levels of plasma total cholesterol in VLDL, LDL, and HDL fraction. (F) Representative flow cytometry plots of blood leukocytes and quantified number of circulating Ly6C^{hi} and Ly6C^{low} monocyte. (G) Circulating Ly6C^{hi} and Ly6C^{low} monocytes from HFD *Lpl^{fl/fl}* and HFD *Mac-Lpl^{-/-}* mice were analyzed using

flow cytometry for neutral lipid content (BODIPY); Representative flow cytometry histogram plots are shown for BODIPY fluorescence. (H) Quantified percentage of BODIPY fluorescence in Ly6C^{hi} and Ly6C^{low} monocytes. (I) Quantification of BODIPY geometric mean fluorescence intensity (gMFI) in Ly6C^{hi} and Ly6C^{low} monocytes. (J) The expression of metabolism-related genes (*Plin2*, *Cpt1a*, *Cd36*, *Glut1*) in total monocytes. (K) The expression of inflammatory genes (*Tnfa*, *Il1b*) in total monocytes. (L) The expression of anti-inflammatory genes (*Ym1* and *Tgfb1*) in total monocytes. N=6/group. *p<0.05, **p<0.01. Results are represented as mean $\pm$ SD (B, D, E) and median with 25th and 75th percentiles, capped bars indicate 10th and 90th percentile (F-L) and *Lpl*^{fl/fl} and Mac-*Lpl*^{-/-} compared using unpaired *t*-test (B-E, J-L). Two-way ANOVA with Sidak's multiple comparison test was used for (F-I).

The effect of *Lpl* deletion on ATMs: Next, we investigated whether deletion of LpL in myeloid-derived cells affects ATM lipid uptake and polarity. Visceral AT-specific acute silencing of *Lpl* in ATMs using glucan-encapsulated siRNA particles showed that *Lpl* deficiency in ATMs slightly decreased ATM neutral lipid content in leptin deficient (*ob/ob*) mice ³⁹. However, it is less clear whether LpL affects subpopulations and polarization. Because acute *Lpl* silencing affected lipid content in ATMs *in vivo*³⁷ and we found a significant reduction in lipid content of BMDMs *in vitro* (Figure 1), we postulated that LpL affects the phenotype of Mφs residing in a high-fat environment. For that reason, we first investigated the role of LpL in ATMs of obese mice.

ATMs are thought to consist of two major subpopulations: F4/80+CD11b (FBs) and F4/80+CD11b+CD11c+ (FBCs). Although several studies described that FBs are less inflammatory, while FBCs are more inflammatory ⁴⁰, Xu et al. ²⁴ demonstrated that FBCs have more neutral lipid content, increased transcriptional profile of lysosomal-dependent lipid metabolism, and are not more inflammatory than FBs. So, the data are inconsistent. In our experiments, ablation of LpL in myeloid-derived cells did not alter total ATM (F4/80+) content (Figure 4A). Unexpectedly, we found that *Lpl* deficiency in ATMs did not lead to fewer Cd11c+ ATMs (Figure 4B, 4C) or less lipid accumulation measured by percent of bodipy+ cells (Figure 4D, 4E) and bodipy+ intensity as measured by bodipy gMFI (Figure 4E). Upon *Lpl* deletion, *Lpl* deficient ATMs showed approximately 20% higher *Glut1* mRNA expression than control Mφs (Figure 4G), suggesting a slight metabolic shift from FA uptake to glucose uptake in ATMs. Inflammatory and anti-inflammatory gene expression did not change (Figure 4H, 4I). Our findings are similar to a previous report showing that myeloid cell-specific GLUT1 overexpression did not alter inflammatory phenotype *in vivo* ³⁶. Here, we illustrate that

LpL is not necessary for the development of more lipid-laden ATMs (FBCs), intracellular lipid accumulation, and ATM polarization.

These results would not be expected if LpL-mediated lipolysis was needed to supply lipids and to activate PPARs, as occurs in other cells and tissues, such as the heart and skeletal muscle⁴¹. Therefore, lipid supply to circulating monocytes and Mφs must either come from FFAs, uptake of remnant lipoproteins that were partially hydrolyzed by LpL in other tissues, or endocytosis of TG-rich lipoproteins followed by intracellular lipolysis or *de novo* lipogenesis.

Figure 4. Myeloid-cell derived LpL is not required for more lipid-laden ATM development (FBCs), intracellular lipid accumulation, and ATM polarization.

HFD fed littermate controls (*LpL^{fl/fl}*) and *Mac-LpL^{-/-}* (*LpL^{fl/fl};LysMCre*) mice were studied for total ATM (CD45+F4/80+) content, ATM subpopulation (CD45+F4/80+CD11b+ and CD45+F4/80+CD11b+CD11c+), and ATM polarity. (A) Flow cytometry analysis of total ATMs (CD45+F4/80+). (B) Representative flow cytometry plots for ATM subpopulations; FBs (CD45+F4/80+CD11b+) and FBCs (CD45+F4/80+CD11b+CD11c+) and quantified FBs and FBCs. (C) Quantified percentage of FBs and FBCs from CD45+F4/80+ cells. (D) Representative flow cytometry histogram plots are shown for BODIPY fluorescence in FBs and FBCs from HFD *LpL^{fl/fl}* and HFD

Mac-*Lpl*^{-/-} mice. (E) Quantified percentage of BODIPY fluorescence in FBs and FBCs. (F) Quantification of BODIPY geometric mean fluorescence intensity (gMFI) in FBs and FBCs. (G) The expression of metabolism-related genes (*Lpl*, *Glut1*, *Plin2*, *Cpt1a*, *Cd36*) in FBCs. (H) The expression of inflammatory genes (*Tnfa*, *Nos2*, *Mcp1*) in FBCs. (I) The expression of anti-inflammatory genes (*Arg1*, *Ym1*, *Fizz1*) in total FBCs. N=6/group. *p<0.05, **p<0.01. Results are represented as median with 25th and 75th percentiles, capped bars at 10th and 90th percentile and compared using Two-Way ANOVA, Sidak's multiple comparison test (A-F) or unpaired *t*-test, or Mann-Whitney Test (*Glut1* *Plin2*, *Cd36*, *Arg1*, *Ym1/Chil3*, *Fizz1/Relma*) (G-I).

The impact of inducible total body *Lpl* deletion in myeloid-derived cells in obesity: To determine whether deletion of LpL in myeloid-derived cells is compensated by LpL in other tissues, we analyzed monocytes and ATMs in *iLpl*^{-/-} and *Lpl*^{fl/fl} mice. To induce obesity, we fed control *Lpl*^{fl/fl} mice and *iLpl*^{-/-} mice a HFD for 16 weeks. Tamoxifen was injected intraperitoneally into both groups, globally ablating *Lpl* in the experimental (*iLpl*^{-/-}) group (Figure 5A)^{22, 42}. We confirmed efficient *Lpl* deletion by measuring *Lpl* expression in AT (Figure 5B).

The circulating glucose level was decreased in HFD *iLpl*^{-/-} mice (Figure 5C), suggesting that lack of LpL-mediated lipid uptake causes an increase in glucose uptake into tissues as was found with neonatal LpL deficiency⁴³. As expected, global *Lpl* deletion also increased VLDL-derived TG levels from 88 mg/dL to 4000 mg/mL as well as LDL- and HDL-TG (Figure 5D). VLDL-derived TC levels significantly increased, whereas LDL-TC and HDL-TC decreased in *iLpl*^{-/-} mice (Figure 5E).

Induced loss of LpL also altered circulating white bloods cells in these HFD-fed mice. Ly6C^{hi} circulating monocytes were significantly increased in HFD *iLpl*^{-/-} mice relative to HFD *Lpl*^{fl/fl} mice (Figure 5F). The increase in levels of circulating Ly6C^{hi} monocytes occurred with increased granulocyte-Mφ progenitors in bone marrow (Supplemental Figure 4D). Although percentage of bodipy+ cells in both Ly6C^{low}- and Ly6C^{high}- monocytes was unaltered in HFD *iLpl*^{-/-} mice (Figure 5G, 5H), we found that bodipy+ gMFI was significantly higher in Ly6C^{low} monocytes as compared to those in *Lpl*^{fl/fl} mice (Figure 5I). These intracellular lipid content results indicate that although monocytes from both HFD *Lpl*^{fl/fl} and *iLpl*^{-/-} mice are fully loaded with lipid droplets, lipid droplet size and number are greater in monocytes in HFD *iLpl*^{-/-} mice. Thus, these lipids are obtained via a non-LpL-mediated lipid uptake.

To study the transcriptional profile and determine the role of LpL in monocytes in HFD *iLpL^{-/-}* mice, we used RNA sequencing as an unbiased approach. As described previously, we analyzed inflammatory- and anti-inflammatory genes, as well as metabolism-related genes. An inflammatory gene, *Tnfa*, was significantly decreased, but *Il1 β* was unaltered in HFD *iLpL^{-/-}* mice (Figure 5K), while anti-inflammatory genes were up-regulated (*Tgfb1*, *Ym1/Chi313*) in HFD *iLpL^{-/-}* mice (Figure 5L). *CD36* mRNA levels were significantly downregulated in HFD *iLpL^{-/-}* mice, whereas other metabolism-related genes showed a trend towards increased expression (*Plin2*, *Cpt1a*) or were unaffected (Figure 5J). However, KEGG pathway analysis showed that lack of *LpL* increased oxidative phosphorylation and lysosome-related pathways (Supplemental Figure 7), suggesting that *LpL* deficiency leads to a metabolic reprogramming by increasing endocytic TG-rich uptake for more oxidative phosphorylation. These data show that hypertriglyceridemia caused by global *LpL* deletion with HFD induces BM progenitors to produce more Ly6C^{hi} monocytes. Also, hypertriglyceridemia in HFD *iLpL^{-/-}* mice leads to an increase of lipid content in Ly6C^{low} monocytes, which induces M2 gene expression but decreases a M1 gene, *Tnfa*, in monocytes.

Figure 5: HFD fed littermate controls (*LpL^{fl/fl}*) and *iLpL^{-/-}* (*LpL^{fl/fl};β-actin-MerCreMer*) mice were studied for plasma metabolic parameters, circulating levels of monocytes, and lipid content and phenotype of monocytes. (A) Experimental design: *LpL^{fl/fl}* and *iLpL^{-/-}* mice were fed with a HFD from 8 weeks old of age for until sacrifice (30 weeks old of age); Body weight and plasma parameters were measured between 24 weeks of age and 30 weeks of age; Circulating monocytes (Ly6C^{hi} and Ly6C^{low}), adipose tissue (PGAT, SCAT, and BAT), and adipose tissue macrophages (CD45⁺F4/80⁺CD11b⁺ and CD45⁺F4/80⁺CD11b⁺CD11c⁺) were obtained at 30 weeks old of age. (B) The expression of *LpL* in adipose tissue (N=4-5/group). (C) Plasma fasting glucose level (N=11-17/group). (D) Levels of plasma triglyceride in VLDL, LDL, and HDL fraction (N=4-5/group). (E) Levels of plasma total cholesterol in VLDL, LDL, and HDL fraction (N=4-5/group). (F) Representative flow cytometry plots of blood leukocytes and quantified number of circulating Ly6C^{hi} and Ly6C^{low} monocyte (N=11-17/group). (G) Circulating Ly6C^{hi} and Ly6C^{low} monocytes from HFD *LpL^{fl/fl}* and HFD *iLpL^{-/-}* mice were analyzed using flow cytometry for neutral lipid content (BODIPY); Representative flow cytometry histogram plots are shown for BODIPY fluorescence (N=3-5/group). (H) Quantified percentage of BODIPY fluorescence in Ly6C^{hi} and Ly6C^{low} monocytes (N=3-5/group). (I) Quantification of BODIPY geometric mean fluorescence intensity (gMFI) in Ly6C^{hi} and Ly6C^{low} monocytes (N=3-5/group). (J) The expression of metabolism-related genes (*Plin2*, *Cpt1a*, *Cd36*, *Glut1*) in total monocytes (N=4/group). (K) The expression of inflammatory genes (*Tnfa*, *Il1b*) in total monocytes (N=4/group). (L) The expression of anti-inflammatory genes (*Ym1* and *Tgfb1*) in total monocytes (N=4/group). *p<0.05, **p<0.01. Results are represented as median with 25th and 75th percentiles, capped bars at 10th and 90th percentile and compared between HFD *LpL^{fl/fl}* and HFD *iLpL^{-/-}* using unpaired t-test.

Figure 5. Inducible *LpI* deletion increased plasma triglyceride level, circulating Ly6C^{hi} monocytes level, and anti-inflammatory genes in monocytes despite more lipid accumulation in monocytes.

The effect of inducible *Lpl* deficiency in adipose tissue macrophages in obesity:

Because myeloid cell-derived-specific *Lpl* deficiency did not alter ATM phenotype, we postulated that this is due to developmental compensation and early action of *LysMCre*. We used *iLpl^{-/-}* mice to determine whether deleting *Lpl* after inducing obesity affects ATM subpopulation, lipid content, and phenotype. ATM *Lpl* expression was decreased 90% in *iLpl^{-/-}* mice, however, total ATM (F4/80+) content did not differ between control and HFD *iLpl^{-/-}* mice (Figure 6A). With regard to subpopulations, percent of FBs from total F4/80+ cells was significantly higher, whereas FBCs from total F4/80+ cells was significantly lower in HFD *iLpl^{-/-}* mice as compared to HFD *Lpl^{fl/fl}* mice (Figure 6B, 6C). This suggests that LpL is necessary for lipid accumulation in ATMs and important for FBC ATM development in obesity. To further examine whether *Lpl* deletion affects lipid content in ATMs, we measured neutral lipid content in both FBs and FBCs. Intracellular neutral lipid accumulation measured by percentage of bodipy+ cells and bodipy+ gMFI in FBs and FBCs in HFD *iLpl^{-/-}* mice tended to be lower in HFD *iLpl^{-/-}* mice despite the marked hypertriglyceridemia in these mice (Figure 6D, 6E, and 6F). These data show that lack of *Lpl* in adipocytes decreases quantity of FBCs and prevents lipid accumulation in ATMs.

To examine whether LpL affects ATM phenotype, we measured both inflammatory and anti-inflammatory gene expression. Although *Glut1* gene expression was dramatically increased in total ATMs of HFD *iLpl^{-/-}* mice (Figure 6G), mRNA levels of inflammatory genes (*Il1 β* , *Tnfa*) tended to be lower (Figure 6H). One anti-inflammatory gene, *Ym-1*, increased, but other M2 genes (*Arg-1* and *Fizz-1*) were unaltered (Figure 5E and 6I) in total ATMs. A FAO-related gene, *Cpt1a*, and other lipid-related genes (*Cd36*, *Plin2*) were not affected (Figure 6G). Overall, our data show that although lack of LpL reduced the number of lipid-laden ATMs and appeared to shift M ϕ s to a more glucose-oxidizing phenotype (increased *Glut1* mRNA level), this phenomenon did not lead to M1 phenotype in ATMs.

Figure 6. Inducible *Lpl* ablation decreases CD11c+ ATMs (FBCs) content, ATM lipid accumulation, but does not affect ATM polarity despite a dramatic increase in *Glut1* expression. HFD fed littermate controls (*Lpl^{fl/fl}*) and *iLpl^{-/-}* (*Lpl^{fl/fl}*;β-actin-*MerCreMer*) mice were studied for total ATM (CD45+F4/80+) content, ATM subpopulation (CD45+F4/80+CD11b+ and CD45+F4/80+CD11b+CD11c+), and ATM polarity. (A) Flow cytometry analysis of total ATMs (CD45+F4/80+). (B) Representative flow cytometry plots for ATM subpopulations; FBs (CD45+F4/80+CD11b+) and FBCs (CD45+F4/80+CD11b+CD11c+) and quantified FBs and FBCs (N=11-17/group). (C) Quantified percentage of FBs and FBCs from CD45+F4/80+ cells. (D) Representative flow cytometry histogram plots are shown for BODIPY fluorescence in FBs and FBCs from HFD *Lpl^{fl/fl}* and HFD *iLpl^{-/-}* mice (N=3-5/group). (E) Quantified percentage of BODIPY fluorescence in FBs and FBCs (N=3-5/group). (F) Quantification of BODIPY geometric mean fluorescence intensity (gMFI) in FBs and FBCs (N=3-5/group). (G) The expression of metabolism-related genes (*Lpl*, *Glut1*, *Cd36*, *Cpt1a*, *Plin2*) in ATMs (N=4-5/group). (H) The expression of inflammatory genes (*Tnfa*, *Il1b*) in ATMs (N=4-5/group). (I) The expression of anti-inflammatory genes (*Arg1*, *Ym1*, *Fizz1*) in ATMs (N=4-5/group). *p<0.05, **p<0.01. Results are represented as median with 25th and 75th percentiles, capped

bars at 10th and 90th percentile and compared between $Lp^{fl/fl}$ and $iLp^{fl/-}$ using unpaired t-test (A-F, *CD36*, *Tnfa*, *Il1b*) or Mann-Whitney Test (*LpL*, *Glut1*, *Cpt1a*, *Plin2*).

The impact of myeloid cell-specific *LpI* deletion in peritoneal Mφs: Tissue-specific Mφs have distinct phenotypes and functions, depending on the tissue microenvironment. A previous report demonstrated that VLDL-derived FA uptake induces inflammation in peritoneal Mφs *in vitro*, suggesting that LpL-dependent lipid uptake is inflammatory¹³. We next assessed whether LpL affects peritoneal Mφ number and polarity, both in resident ($F4/80^{hi}MHCII^{low}$) and recruited ($F4/80^{low}MHCII^{high}$) populations. We induced Mφ recruitment using zymosan A (100 µg/mouse; Sigma) in mice fed a normal rodent diet, as shown in Figure 7A. We found that lack of LpL did not affect Mφ accumulation in the peritoneal cavity (Figure 7B, 7C, and 7D).

Figure 7. Myeloid-cell derived *LpI* deficiency does not affect number of peritoneal macrophages. Lean littermate controls ($Lp^{fl/fl}$) and $Mac-Lp^{fl/-}$ ($Lp^{fl/fl};LysMCre$) mice were studied for both resident peritoneal macrophages ($F4/80^{high}MHCII^{low}$) and recruited peritoneal macrophages ($F4/80^{low}MHCII^{high}$) under zymosan-induced peritonitis. (A) Experimental design: Zymosan (100 µg/mouse) was i.p injected into 12-week-old lean $Lp^{fl/fl}$ and $Mac-Lp^{fl/-}$ mice on day 0 to induce monocyte/macrophage recruitment to peritoneal cavity. All mice were sacrificed on day 3 for peritoneal macrophage analysis using flow cytometry. (B) Representative flow cytometry plots for peritoneal macrophages subpopulations. (C) Flow cytometry analysis of resident peritoneal macrophages. (D) Flow cytometry analysis of recruited peritoneal macrophages. N=3-5/group. *p<0.05.

Results are represented as median with 25th and 75th percentiles, capped bars at 10th and 90th percentile and compared using Two-Way ANOVA with Sidak's multiple comparison test.

Glut1 mRNA expression was also significantly up-regulated in Mac-*Lpl*^{-/-} LPMs as compared to *Lpl*^{fl/fl} LPMs (Figure 8B), indicating that genetic ablation of LpL may lead to a metabolic reprogramming. However, despite an increase in *Glut1*, both inflammatory and anti-inflammatory genes were not significantly different in LPMs between Mac-*Lpl*^{-/-} and *Lpl*^{fl/fl} mice (Figure 8E-I). These results show that independent of hyperlipidemic conditions, LpL deficiency seems to shift the energy source from FA to glucose, as measured by *Glut1* expression, this metabolic reprogramming does not impact M1/M2 phenotype of tissue Mφs both in AT and the peritoneal cavity.

Figure 8. Myeloid-cell derived *Lpl* deficiency increases metabolic genes, but does not profoundly affect canonical inflammatory and anti-inflammatory genes in resident peritoneal macrophages under zymosan stimuli. (A) The expression of *Lpl* in resident peritoneal macrophages (Cd45⁺F4/80^{high}MHCII^{low}). (B) The expression of *Glut1* in resident

peritoneal macrophages. (C) The expression of *Plin2* in resident peritoneal macrophages. (D) The expression of *Cpt1a* in resident peritoneal macrophages. (E) The expression of *Tnfa* in resident peritoneal macrophages. (F) The expression of *Ii1b* in resident peritoneal macrophages. (G) The expression of *Arg1* in resident peritoneal macrophages. (H) The expression of *Ym1* in resident peritoneal macrophages. (I) The expression of *Fizz1* in resident peritoneal macrophages. N=3-5/group. * $p < 0.05$, ** $p < 0.01$. Results are represented as median with 25th and 75th percentiles, capped bars at 10th and 90th percentile and compared using Two-Way ANOVA with Sidak's multiple comparison test.

The effect of inducible *Lpl* deficiency in peritoneal Mφs: In contrast to *Mac-Lpl*^{-/-} mice, there was more than a 60% decrease in both resident and recruited peritoneal Mφs in *iLpl*^{-/-} mice as compared to those in *Lpl*^{fl/fl} mice (Figure 9B, 9C, 9D). The dramatic reduction in the resident population was evident even in the control (PBS treatment) group, showing that LpL is required for maintaining the tissue resident Mφs in the cavity.

Figure 9. Inducible *Lpl* deletion dramatically decreases both resident and recruited peritoneal macrophages content upon zymosan stimuli. Lean littermate

controls (*Lpl*^{fl/fl}) and *iLpl*^{-/-} (*Lpl*^{fl/fl};β-actin-*MerCreMer*) mice were studied for both resident peritoneal macrophages (F4/80^{high}MHCII^{low}) and recruited peritoneal macrophages (F4/80^{low}MHCII^{high}) under zymosan-induced peritonitis. (A) Experimental design: Zymosan (100 μg/mouse) was i.p injected into 12-week-old lean *Lpl*^{fl/fl} and *Mac-Lpl*^{-/-} mice on day 0 to induce monocyte/macrophage recruitment to peritoneal cavity. All mice were sacrificed on day 3 for peritoneal macrophage analysis using flow cytometry. (B) Representative flow cytometry plots for peritoneal macrophages subpopulations. (C) Flow cytometry analysis of resident peritoneal macrophages. (D) Flow cytometry analysis of recruited

peritoneal macrophages. N=3-5/group. *p<0.05. Results are represented as median with 25th and 75th percentiles, capped bars at 10th and 90th percentile and compared using Two-Way ANOVA with Sidak's multiple comparison test.

These results are parallel to those found with angiopoietin-like protein 4 deficiency where greater LpL activity is associated with severe peritoneal inflammation ⁴⁴. The decrease in recruited population illustrates that LpL is also important in monocyte recruitment into the peritoneal cavity upon interaction with inflammatory stimuli such as zymosan. While we were analyzing the M1/M2 gene expression profile from the resident and recruited peritoneal Mφs, we were unable to obtain an optimum amount of RNA to measure those genes due to the low number of resident peritoneal Mφs (LPMS). The gene expression profile from the recruited population (SPMs), however, was analyzed. We found a pattern of increase in *Glut1* expression (Figure 10B) along with significant *Tnfa* and *Il1b* expression (Figure 10E) in *iLpL*^{-/-} mice relative to *LpL*^{f/f} mice. When we analyzed M2 genes, *Arg1* was significantly upregulated in *iLpL*^{-/-} mice compared to *LpL*^{f/f} mice, but not *Ym1* and *Fizz1* (Figure 10G, 10H, 10I). Because we measured the gene expression in the recruited population, it is difficult to make a parallel comparison with the results from the *Mac-LpL*^{-/-} mice. However, the gene expression profile from total peritoneal Mφs was identical to the gene expression from the recruited population (Supplemental Figure 9). These data show that LpL derived from non-myeloid cells is required for Mφ development, recruitment, and polarization in peritoneal cavity.

Figure 10. Inducible *Lpl* ablation increases metabolic-, inflammatory-, and anti-inflammatory genes in recruited peritoneal macrophages upon zymosan stimuli.

(A) The expression of *Lpl* in recruited peritoneal macrophages (Cd45⁺F4/80^{low}MHCII^{high}). (B) The expression of *Glut1* in resident peritoneal macrophages. (C) The expression of *Plin2* in resident peritoneal macrophages. (D) The expression of *Cpt1a* in resident peritoneal macrophages. (E) The expression of *Tnfa* in resident peritoneal macrophages. (F) The expression of *Il1b* in resident peritoneal macrophages. (G) The expression of *Arg1* in resident peritoneal macrophages. (H) The expression of *Ym1* in resident peritoneal macrophages. (I) The expression of *Fizz1* in resident peritoneal macrophages. N=3-5/group. *p<0.05, **p<0.01. Results are represented as median with 25th and 75th percentiles, capped bars at 10th and 90th percentile and compared using Two-Way ANOVA with Sidak's multiple comparison test.

The impact of inducible *Lpl* deletion on atherosclerosis plaque Mφs in the context of regression: We hypothesized that lack of Mφ-derived LpL would prevent the polarization of newly recruited Mφs to M2 and negatively affect regression. To test our hypothesis, we utilized a well-established aortic transplant methodology^{28, 29} to

create a plaque regression microenvironment in *Lpl^{fl/fl}* and *iLpl^{-/-}* mice as described in Figure 11A. Two weeks after inducing regression, we isolated CD68+ Mφs from plaques using laser capture microdissection^{26, 45} and measured gene expression. Consistent with the other tissue Mφs, *Lpl* deletion increased *Glut1* mRNA levels (Figure 11B; $p=0.08$). Similar to *in vivo* ATMs, the expression of *Plin2*, *Cd36*, and *Cpt1a* genes were not altered in *iLpl^{-/-}* mice. Although we found a pattern of decrease in *Mcp1* mRNA level in *iLpl^{-/-}* mice as compared to *Lpl^{fl/fl}* mice, unaltered expression of other typical inflammatory genes (*Tnfa*, *Ilb* and *Nos2*) was observed (Figure 11C). In contrast to our hypothesis, the anti-inflammatory genes (*Mrc1*, *Fizz1*, *Il10*) were not significantly decreased upon deletion of *Lpl* (Figure 11D). We confirmed our gene expression results for mannose receptor Mrc1/CD206, a representative M2 marker in mouse and human plaque Mφs⁴, by CD206 immunostaining in the regressing plaques. The quantified area of mannose receptor in *iLpl^{-/-}* mice was comparable with *Lpl^{fl/fl}* mice (Figure 11E), indicating that LpL does not affect plaque Mφ M2 polarization in regression. As expected, *Lpl* deficiency had no impact on regressing plaque area (Figure 11F). Of note, despite plaque area not having changed in the regression groups compared to baseline, amount of CD68+ cells were reduced, representing atherosclerosis regression (data not shown). Overall, we found that LpL is dispensable for M2 polarization in plaque Mφs and resolution of atherosclerosis.

Figure 11: (A) Experimental design: A plaque burden aortic arch from *Ldlr^{-/-}* mice was transplanted into the 22-week-old recipient mice (*iLpl^{-/-}* or *Lpl^{fl/fl}* mice, maintained on a standard laboratory diet), interpositioned with the abdominal aorta, and blood flow was directed through the graft. All mice were sacrificed 14 days after the aortic arch transplantation. (B) The expression of metabolism-related genes (*Lpl*, *Glut1*, *Plin2*, *Cd36*, *Fsn*, *Cpt1a*) in plaque macrophages. (C) The expression of inflammatory genes (*Tnfa*, *Il1b*, *Nos2*, *Mcp1*[*Ccl2*]) in plaque macrophages. (D) The expression of anti-inflammatory genes (*Mrc1*, *Fizz1*, *Il10*) in plaque macrophages. (E) Quantification of mannose receptor (Cd206) staining in the atherosclerotic plaque (as shown with area unit, μm^2 , and percent of the plaque size per field). (F) Quantification of plaque area. (G) Representative images for the co-localization of CD68+ and CD206+ staining and the quantification of double-positive (CD206+CD68+) cells. N=4-7/group (baseline n=4, *Lpl^{fl/fl}* n=7, *iLpl^{-/-}* n=7). * $p<0.05$. Results are represented as median with 25th and 75th percentiles, capped bars at 10th and 90th percentile and compared using unpaired *t*-test (*Lpl*, *Plin2*, *Glut1*, *Cd36*, *Nos2*, *Mcp1*, *Fizz1*/*Relma*, *IL-10*), Mann-Whitney test (*Fasn*, *Cpta1*, *Mrc1*), Welch's *t*-test (*Il1b*, *Tnfa*), or One-way ANOVA with Tukey's multiple comparison (E, F).

Figure 11. Inducible *LpI* ablation does not profoundly affect plaque area and inflammatory/anti-inflammatory genes in plaque macrophages in the context of atherosclerosis regression.

Discussion

Genetic ablation of *Lpl* *in vitro* and/or *in vivo* demonstrated distinct effects on M ϕ phenotype. It is widely accepted that M ϕ phenotype is associated with, and likely depends on, cellular differences in lipid and glucose metabolism^{3, 46}. Moreover, several *in vitro* studies indicate that LpL is a primary regulator of M ϕ lipid uptake¹⁴ and therefore a modulator of M ϕ polarity^{5, 19}. By comparing the role of this enzyme both *in vitro* and *in vivo*, our data challenge these assumptions. We show that deleting myeloid cell-derived LpL does not alter M ϕ polarity in obese AT, peritoneal cavity, and regressing atherosclerotic plaques. In all depots, cellular lipid metabolism does not correlate with M ϕ phenotype, and the results support a model in which tissue environment plays the central role in determining how subsets of M ϕ s respond to changes in ability to produce FFAs from TGs.

Although we were able to create a TG-rich environment both in the circulation and in AT by feeding the mice a HFD, we also created a robust inflammatory microenvironment with high concentrations of glucose, insulin, and FFAs—so called metabolic chronic inflammation. In a human monocyte cell line (THP-1), LpL-mediated VLDL uptake induces both intracellular lipid accumulation and inflammation, as measured by *Il1 β* and *Tnfa* mRNA expression^{17, 18}. However, *in vivo* monocytes, markers of metabolism and phenotype were unchanged in *Mac-Lpl^{-/-}* mice, indicating that the inflammatory microenvironment induced by a HFD *in vivo* adapted to compensate for the lack of LpL in monocytes. In ATMs, local FFA concentrations are likely higher than FFAs in systemic circulation due to the close proximity to adipocyte lipolysis. Thus, our findings in ATMs in *Mac-Lpl^{-/-}* mice—unaltered ATM subpopulation, lipid accumulation, and phenotype—are likely due to FFA uptake via FA transporters or the flip-flop pathway, endocytic TG-rich lipoprotein uptake, or accumulation of adipocyte-derived lipid vesicles⁴⁷. This may explain the lack of changes in inflammatory gene expression phenotype in *Mac-Lpl^{-/-}* mice despite increased *Glut1* mRNA expression in ATMs.

Another possible interpretation is that since *LysM-Cre* is effective starting from the development stage, ablation of LpL may have induced a compensatory mechanism in the early developmental stages of mice. We tested the latter hypothesis by using HFD *iLpl^{-/-}* mice. We found a significant increase in levels of circulating Ly6C^{hi} monocytes with a significant increase in lipid accumulation within Ly6C^{low} monocytes. Because we

did not find increased LDL receptor (*Ldlr*), VLDL receptor (*Vldlr*) (data not shown), or *Glut1* gene expression level, higher neutral lipid content in Ly6C^{low} monocytes in HFD *iLpl*^{-/-} mice is perhaps due to LpL-independent lipid uptake pathway such as FFA uptake via the flip-flop pathway, as serum FFA concentration was higher in HFD *iLpl*^{-/-} mice than in HFD *Lpl*^{fl/fl} mice (Supplementary Figure 4G). Interestingly, although levels of circulating Ly6C^{hi} monocytes were higher in HFD *iLpl*^{-/-} mice, a typical inflammatory gene, *Tnfa* gene expression, was lower, but anti-inflammatory genes were higher as compared to HFD *Lpl*^{fl/fl} mice. These results show that, as previously shown by Fisher and Pearce groups ^{4, 48}, Ly6C^{hi} monocytes have potential to become M2 Mφs.

Even though inducible LpL deletion in obese mice did not affect total ATM content (F4/80+ cells), it shifted the FB:FBC ratio. Obesity is associated with FBC populations as they accumulate more neutral lipids ²⁴, however, deleting *Lpl* in obese mice decreased FBCs and increased FBs. There was a pattern of a reduction in intracellular lipid content in FBCs and FBs ($p=0.07$) in HFD *iLpl*^{-/-} mice, as compared to ATMs in HFD *Lpl*^{fl/fl} mice. In contrast, ATMs in HFD Mac-*Lpl*^{-/-} mice and HFD *Lpl*^{fl/fl} mice showed no difference in intracellular lipid content. One explanation for this is that lipid accumulation in Mφs is dependent on adipocyte-derived LpL. Another explanation is that uptake requires systemic initial lipolysis of TG-rich lipoproteins by LpL to allow their internalization by the ATMs, e.g. via lipoprotein receptors. Lipoprotein receptor uptake of nascent TG-rich lipoproteins requires their partial lipolysis to remnant or intermediate density lipoproteins to allow optimal uptake via lipoprotein receptors. Total deficiency of LpL will eliminate these particles and might be the reason that systemic rather than Mφ specific LpL deficiency reduced Mφ lipid droplets. Such a conclusion would contrast with *in vitro* studies showing Mφ lipid accumulation from chylomicrons via LpL actions.

Although *Glut1* mRNA expression was dramatically increased in ATMs in HFD *iLpl*^{-/-} mice, this did not affect the M1/M2 phenotype in ATMs, contradicting the current dogma of Mφ polarization *in vitro* ². The dogma is that greater glucose utilization creates inflammatory Mφs. Our studies do not support this association as causative, as increased *Glut1* mRNA did not create a more inflammatory phenotype in LpL deficient Mφs. Our data are consistent with the *Glut1* overexpression mouse model phenotype ³⁶, which did not show more inflammation in Mφs. Moreover, our data support a recent finding where deletion of a key enzyme involved in FAO, carnitine

palmitoyltransferase II (Cpt2), showed no effect on M2 conversion in both BMDMs and ATMs ⁴⁹. Overall, our data demonstrate that myeloid cell-derived LpL is dispensable for lipid uptake and polarity, however, LpL derived from other cells/tissues are perhaps necessary for ATM lipid accumulation.

The peritoneal cavity is a unique tissue compartment in both mice and humans. In addition to its distinctive anatomical structure, the peritoneal cavity contains many types of immune cells, such as lymphocytes, Mφs, granulocytes, and mesothelial cells ⁵⁰, suggesting a complex cytokine profile in peritoneal fluid. Under normal physiological conditions, 91% of total myeloid cells (Cd11b+) are resident Mφs (LPMs), whereas 9% of total myeloid cells (Cd11b+) are recruited Mφs (SPMs) ⁵¹. Upon a 3-day period of zymosan stimulation, the LPM:SPM ratio shifts from 91:9 to approximately 60:40. We sought to determine the role of LpL-mediated lipid uptake in both LPMs and SPMs in basal and zymosan-stimulated states. We found an increase in *Glut1* and *Plin2* mRNA levels in *Mac-Lpl^{-/-}* mice under zymosan-stimuli in the resident population. These changes in metabolic-related gene expression are similar to the *Mac-Lpl^{-/-}* BMDMs cultured under low glucose media (Figure 1). When we measured glucose concentration in peritoneal cavity, it was low (below detection threshold of the glucometer), and TG concentrations were also lower than in the circulation (Supplemental Figure 8). Although the exact concentration of glucose in peritoneal cavity is unknown, our data suggest that the peritoneal cavity microenvironment is a low glucose milieu and *Lpl* deficiency in peritoneal Mφs induces metabolic compensation. However, these changes did not profoundly affect the inflammatory or anti-inflammatory state of the cells.

Of the tissue Mφs we studied, plaque Mφs are perhaps the most appropriate type to evaluate *in vivo* the role of LpL in both lipid metabolism and polarity, as the atherosclerotic lesion microenvironment offers immediate contact between Mφs and lipid particles. Mφ-expression of LpL is atherogenic and associated with greater vascular inflammation ²¹. The role of Mφ-derived LpL in regression, however, is yet to be defined. A recent study demonstrated that continued recruitment of inflammatory Ly6C^{hi} monocytes and their polarization to the M2 state are required for the resolution of atherosclerotic inflammation and plaque regression ⁴. This finding led us to question whether LpL has an impact on M2 polarization in plaque Mφs during plaque regression. We speculated that altering the most upstream pathway of lipid uptake by deleting LpL

would affect alternative activation and resolution of atherosclerosis. However, our data show that ablation of LpL does not affect M2 conversion in plaque Mφs and thus does not change the plaque area. In fact, *Lpl* deficient Mφs from the regressing plaques had no change in the typical M2-related genes, including *Mrc1* and *Fizz1*. This result is surprising because we observed a trend towards increased *Glut1* mRNA levels in the plaque Mφs, as we did in other *in vivo* Mφs and *in vitro* BMDMs. These findings again indicate a metabolic shift to glucose metabolism at least at the transcriptional level, but it neither led to an expression increase of inflammatory genes nor a decrease of anti-inflammatory genes. The lack of impact on M2 conversion of plaque Mφs is perhaps due to multiple types of Mφs within the lesion area. Since atherosclerosis regression is a dynamic condition with constant recruitment of Ly6C^{hi} monocytes and retention of foam cells (well known as inflammatory), the baseline of regression condition is most likely comprised of a mix of M1/M2 Mφs, as observed in human plaques ⁵². Thus, deleting LpL may have affected both types of cells and led to no effect on the total plaque Mφs phenotype. It should be noted that, although we see a significant decrease in LpL expressing macrophages in *iLpl*^{-/-} recipient mice, suggesting that the majority of LpL expressing macrophages from the donor has been replaced by recruited *Lpl*^{-/-} macrophages, we cannot exclude that there are LpL expressing macrophages left in the atherosclerosis lesions also affecting macrophage phenotype.

Overall, we discovered that, unlike previous *in vitro* studies, LpL-mediated FAs (PPARδ ligands) are not required for AA of Mφs *in vivo*. Our data clearly show that monocyte/tissue Mφ-derived LpL minimally regulates lipid accumulation in those cells *in vivo* and does not affect polarization. More importantly, LpL in other cells including myocytes, adipocytes, and endothelial cells ⁵³ distinctly affects lipid accumulation and the phenotypes of circulating monocytes and ATMs. Hypertriglyceridemia caused by global *Lpl* deficiency increases lipid accumulation in monocytes and shifts them to a more M2 phenotype. Global, but not Mφ-specific, *Lpl* deficiency in AT decreases lipid accumulation in ATMs and reduces FBC population without affecting M1/M2 polarity. Whether these findings were due to the global knockout causing changes in circulating TG-rich lipoproteins that made them reliant on Mφ LpL or due to a requirement for adipose LpL in the Mφ-lipid uptake process is unclear. Most likely, it suggests that multiple sources of lipids are responsible for creation of lipid-rich ATMs. Furthermore, our data support several recent and novel observations related to LpL and cellular lipid

metabolism. Most importantly, a LpL-rich cluster of cells has been found in the bone marrow using single cells RNA sequencing ⁵⁴. Thus, it is possible that LpL directs cellular phenotyping in ways that are exclusive of M ϕ lipid uptake. Within the AT, our data show that M ϕ LpL expression does not alter M ϕ lipid content and is not likely to be a major modulator of lipid uptake of the recently described adipose-derived exosomes ⁴⁷.

In conclusion, our current findings and the previous *in vivo* studies demonstrate that the dogma of M1/M2 dichotomy is only applicable in certain tissue culture conditions and clearly not *in vivo* physiology. The *in vivo* tissue microenvironment is far more complicated than culture conditions because it involves dynamic interactions between different cell types, energy sources, and many other factors.

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HRC conducted all *in vivo* and *in vitro* experiments and analyzed the data presented in Figures 1-10. She primarily directed the project and wrote the manuscript. TJ performed the experiments and the data analyses for Figure 11, and she wrote the sections describing the methods related to those results. Both HRC and TJ revised the manuscript. DS, YH, LG, and TB assisted with the *in vivo* mouse studies. NG, SC, JG, and SB contributed to parts of the *in vivo* and *in vitro* experiments and performed qRT-PCR and analyzed the data.

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Disclosures

None

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Supplemental Methods

Animals: All procedures were approved by the Institutional Animal Care and Use Committee at New York University Langone Health. Mice were maintained in a temperature-controlled (25°C) facility with a 12 h-light/dark cycle and given free access to water and food, except when fasting blood was obtained. Mice were fed a laboratory standard rodent chow diet or a high fat diet (HFD) (60% fat kcal; Research Diet #D12492), as indicated. Euthanasia was done by intraperitoneal injection of ketamine 100 mg/kg and xylazine 10 mg/kg followed by cervical dislocation.

Blood Leukocytes: Tail blood was collected into EDTA-lined tubes and immediately placed on ice. All subsequent steps were performed on ice. Red blood cells were lysed, and white blood cells were centrifuged, washed, and resuspended in FACS buffer. Cells were stained with a cocktail of antibodies against CD45-Alexa Fluor 450 (Invitrogen), Ly6-C/G-PerCP-Cy5.5 (BD Pharmingen), and CD115- APC (eBioscience). Monocytes were identified as CD45^{hi}CD115^{hi} and further identified into Ly6C^{hi} and Ly6C^{lo}; neutrophils were identified as CD45^{hi}CD115^{lo}Ly6-C/G^{hi} (Gr-1).

Hematopoietic Stem Cells: BM was harvested from femurs and tibiae by flushing with ice cold PBS. Spleens were minced into small pieces, gently rubbed against the mesh in a cell strainer and flushed with PBS. The cell suspension was centrifuged at 500 g for 5 mins. The supernatant was aspirated and the cells were resuspended in red blood cell lysis buffer for 5 mins. Lysis was stopped by adding an excess amount of flow buffer, and the cell suspension was once again centrifuged and aspirated. A single cell suspension was prepared by gently pipetting up and down with 200 µl of flow buffer. This was followed by incubation with a cocktail of antibodies against lineage-committed cells (B220, CD19, CD11b, CD3e, TER-119, CD2, CD8, CD4, Ly6-C/G: All FITC, eBioscience), Sca1-Pacific Blue, and ckit-APC Cy7). HSPCs were identified as lin⁻, Sca1⁺, and ckit⁺ (LSK), while the hematopoietic progenitor subsets were separated using antibodies to CD16/CD32 (FcγRII/III) and CD34. Common myeloid progenitors were identified as lin⁻, Sca1⁻, ckit⁺, CD34^{int}, FcγRII/III^{int}, and granulocyte-Mφ progenitors as lin⁻, Sca1⁻, ckit⁺, CD34^{int}, FcγRII/III^{hi}. BM Mφs were identified by negative gating against (B220, CD19, CD11b, CD3e, TER-119, CD2, CD8, CD4, Ly6 C/G: All FITC, eBioscience) and then CD115⁺, Gr1^{lo}, F4/80⁺, CD169⁺, and either CD11c positive or negative. Flow cytometry was performed using an LSRII (for analysis) or FACS Aria (for sorting), with both machines running FACS DiVa software.

All flow cytometry data were analyzed using FlowJo software (Tree Star Inc.).

Adipose Tissue Macrophage (ATM) Isolation: Freshly dissected adipose tissue was minced into fine pieces (<10 mg) and centrifuged to remove connective tissue debris. Minced tissues were digested by liberase TM (Roche) for 20 mins and centrifuged at 500 g for 5 mins. The pellets were collected as stromal vascular cells (SVCs), and buoyant cells were collected as adipocytes. The SVCs were resuspended in erythrocyte lysis buffer (BD Biosciences) and the erythrocyte-depleted SVCs were incubated with F4/80-APC, CD11b-PE, and CD11c-PETR for M ϕ purification. ATM subpopulations were separated by F4/80+CD11b+ (FBs) and F4/80+CD11b+CD11c+ (FBCs); the gating strategy is shown in Supplemental Figure V.

Quantitative Real Time-PCR Analyses: Total RNA was extracted from cells with silica-based membrane method by using PureLink RNA Mini kit (Ambion) for BMDMs or RNeasy Micro kit (Qiagen) for FACS-sorted cells. Complementary DNAs (cDNAs) were generated with a Verso cDNA synthesis kit (Thermo Fisher), and quantitative PCR was carried out with fluorescent double-stranded DNA dye (Fast SYBR Green Mastermix; Applied Biosystems). Data were normalized to *Rps3* with the $\Delta\Delta C(t)$ method and are presented as relative transcript levels. All primers are listed in Supplemental Table I.

Supplemental Figures

Supplemental Table I. List of primers used for quantitative PCR

Gene	Forward (5' - 3')	Reverse (5' - 3')
<i>Rps3</i>	ATCAGAGAGTTGACCGCAGTTG	AATGAACCGAAGCACACCATAG
<i>Lpl</i>	AGGTGGACATCGGAGAACTG	TCCCTAGCACAGAAGATGACC3
<i>Plin2</i>	TCTGCGGCCATGACAAGTG	GCAGGCATAGGTATTGGCAAC
<i>Cpt1a</i>	TGCACTACGGAGTCCTGCAA	GGACAACCTCCATGGCTCAG
<i>Glut1</i>	TCGTAACGAGGAGAACCG	GGCCGTGTTGACGATA
<i>Fasn</i>	TCTTTCTAACAACCACCCTCTGG	CTTCACGACTCCATCACGAATG
<i>Cd36</i>	CCTTAAAGGAATCCCCGTGT	TGCATTTGCCAATGTCTAGC
<i>Tnf</i>	TGGAAGTGGCAGAAGAGG	AGACAGAAGAGCGTGGTG
<i>Il1b</i>	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA
<i>Mcp1</i>	CCCAATGAGTAGGCTGGAGA	TCTGGACCCATTCTTCTTG
<i>Nos2</i>	CAGCTGGGCTGTACAAACCTT	CATTGGAAGTGAAGCGTTTCG
<i>Arg1</i>	TGGCTTGCGAGACGTAGAC	GCTCAGGTGAATCGGCCTTTT
<i>Ym1</i>	AGGCTTTGCGGTCCTGAT	CCAGCTGGTGAAGTAGCAGA
<i>Fizz1</i>	CCAATCCAGCTAACTATCCCTCC	AAGCCACAAGCACACCCAGT
<i>Mrc1</i>	TGATTACGAGCAGTGGAAGC	G TTCACCGTA-AGCCCAATTT
<i>Il10</i>	CTGGACAACATACTGCTAACCG	GGGCATCACTTCTACCAGGTAA

Supplemental Figure I. *Lpl* deficiency does not affect alternative activation of macrophages upon oleic acid treatment *in vitro*

BMDMs from *Lpl*^{fl/fl} and Mac-*Lpl*^{-/-} mice were used to measure metabolism- related and M1/M2-related gene expression in the presence of oleic acid (OA). (A) The gene expression of *Lpl* in BMDMs. (B) The expression of a lipid droplet-related protein, *Plin2*, gene in BMDMs. (C) The expression of a fatty acid oxidation-related gene, *Cpt1a* in BMDMs. (D-G) gene expression of anti-inflammatory genes (*Arg1*, *Ym1*, *Fizz1*, *Mrc1*) in OA treated BMDMs. (H-K) The expression of inflammatory genes (*Tnfa*, *Il1b*, *Nos2*, *Mcp1*) in OA treated BMDMs. N=3/group.*p<0.05, **p<0.01. Results are represented mean $\pm$ SD and compared using Two-way ANOVA, Sidak's multiple comparison.

Supplemental Figure II. BMDMs culture in normal glucose (5mM)

BMDMs from *Lpl^{fl/fl}* and *Mac-Lpl^{-/-}* mice were used to measure metabolism- related and M1/M2-related gene expression in the presence of human VLDL (100ug/mL), IL-4 (20ng/mL) and normal glucose (5mM). (A) The gene expression of *Lpl* in BMDMs. (B) The expression of a lipid droplet-related protein, *Plin2*, gene in BMDMs. (C) The expression of a fatty acid oxidation-related gene, *Cpt1a* in BMDMs. (D) The expression of a glucose transporter, *Glut1* (*Slc2a1*), gene in BMDMs. (E) The expression of fatty acid synthase gene in BMDMs. (F-I) The expression of canonical M2 (anti-inflammatory) genes in BMDMs. N=6/group. *p<0.05, **p<0.01. Results are represented mean $\pm$ SD and compared using Two-way ANOVA, Sidak's multiple comparison.

Supplemental Figure III. BMDMs cultured in high (25mM) glucose

BMDMs from *Lpl^{fl/fl}* and *Mac-Lpl^{-/-}* mice were used to measure metabolism-related and M1/M2-related gene expression in the presence of human VLDL (100ug/mL), IL-4 (20ng/mL) and high glucose (25mM). (A) The gene expression of *Lpl* in BMDMs. (B) The expression of a glucose transporter, *Glut1* (*Slc2a1*), gene in BMDMs. (C) The expression of a fatty acid oxidation-related gene, *Cpt1a* in BMDMs. (D) The expression of a lipid droplet-related protein, *Plin2*, gene in BMDMs. (E) The expression of fatty acid synthase gene in BMDMs. (F-I) The expression of canonical M2 (anti-inflammatory) genes in BMDMs. N=3-4/group. *p<0.05, **p<0.01. Results are represented mean $\pm$ SD and compared using Two-way ANOVA, Sidak's multiple comparison.

Supplemental Figure IV. Bone marrow progenitors and weight of tissue in *Mac-Lpl*^{-/-} and *iLpl*^{-/-} mice fed a HFD

(A) Bone marrow progenitors in *Mac-Lpl*^{-/-} (B) Adipose tissue weight (g) in *Mac-Lpl*^{-/-} (C) Liver weight (g) in *Mac-Lpl*^{-/-} (D) Bone marrow progenitors in *iLpl*^{-/-} (E) Adipose tissue weight (g) in *iLpl*^{-/-} (F) Liver weight (g) in *iLpl*^{-/-} (G) Free fatty acids (mmol/L) in *Mac-Lpl*^{-/-} and *iLpl*^{-/-} mice. N=4-7/group. *p<0.05, **p<0.01. Results are represented mean ± SD and compared using Two-way ANOVA, Sidak's multiple comparison.

Supplemental Figure V. Adipose tissue macrophages (ATM) gating strategy

Supplemental Figure VI. Peritoneal macrophages gating strategy

Supplemental Figure VII. KEGG Mapper Analysis- Oxidative Phosphorylation and Lysosome Pathway (Genes cut off q-value <0.05)

Supplemental Figure VIII. TG levels in plasma versus TG levels in peritoneal fluid

Supplemental Figure IX. Inflammatory and anti-inflammatory gene expression in total peritoneal macrophages upon zymosan stimuli

Peritoneal Macrophages from *Lpl^{fl/fl}* and *iLpl^{-/-}* mice were used to measure metabolism- related and M1/M2-related gene expression. (A) Expression of macrophage marker related genes (*F4/80*, *Cd11b*, *Ccr2*). (B) The expression of metabolism-related genes (*Glut1*, *Plin2*, *Cpt1a*, *Cd36*). (C) The expression of inflammatory genes (*Tnfa*, *Il1b*). (D) The expression of anti-inflammatory genes (*Ym-1*, *Arg1*). N=4/group, *p<0.05, **p<0.01. Results are represented mean $\pm$ SD and compared using Two-way ANOVA, Sidak's multiple comparison.

Supplemental Figure X. BMDM culture in low (1mM) glucose

Side-by-side comparison of anti-inflammatory genes from Figure 1 and 2. N=6/group. * $p < 0.05$, ** $p < 0.01$. Results are represented mean $\pm$ SD and compared using Two-way ANOVA, Sidak's multiple comparison.

Supplemental Figure XI. Control staining for CD68 and CD206

Negative controls were performed using an isotype control (Rat IgG2a) instead of the primary antibody.

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Reduced High-density Lipoprotein Cholesterol and Particle Number due to Hypertriglyceridemia does not affect Atherosclerosis Regression

Tatjana Josefs, Debapriya Basu, Tomas Vaisar, Britt Arets, Jenny E. Kanter, Lesley-Ann Huggins,
Yunying Hu, Jianhua Liu, Jay W. Heinecke, Karin E. Bornfeldt,
Ira J. Goldberg*, Edward A. Fisher*

*Equal contribution

In Revision

Abstract

Rationale: Hypertriglyceridemia (HyperTG) and low high-density lipoprotein cholesterol (HDL-C), both of which are regulated by lipoprotein lipase (LpL) activity, associate with increased cardiovascular disease (CVD). Genetic regulators of LpL actions track with CVD risk in humans. Whether this is due to changes in HDL-C or function, or circulating triglyceride (TG) levels is unresolved.

Objective: We created HyperTG and HDL-C reduction in atherosclerotic mice to allow the assessment of how HyperTG and reduced HDL affect regression of atherosclerosis and the phenotype of plaque macrophages.

Methods and Results: Atherosclerosis regression was studied in control LpL floxed (*LpL^{fl/fl}*) mice and tamoxifen inducible whole-body LpL KO (*iLpL^{-/-}*) mice with HyperTG ~500mg/dL and reduced HDL-C (~50% reduction). Atherosclerosis regression was studied using two models: 1) aortic transplant, and 2) inhibition of the LDL receptor using antisense oligonucleotides followed by re-expression after sense oligonucleotide treatment. In a subset of mice, we expressed cholesterol ester transfer protein (hCETP) to humanize the relationship between apoB-lipoproteins and HDL. HDL particle number (HDL-P), cholesterol efflux capacity (CEC) and HDL proteome were measured in HyperTG mice and humans. Surprisingly, HyperTG and reduced HDL-C levels due to loss of LpL did not affect atherosclerosis lesion size or macrophage content (CD68+ cells) in either model. Expression of hCETP and further reduction of HDL-C did not alter lesions. Sera from *iLpL^{-/-}* mice had a decrease in total CEC, but not ABCA1-mediated CEC. HyperTG humans, including those with LpL deficiency, had greater ABCA1-mediated CEC and total CEC per HDL-P.

Conclusion: Atherosclerosis regression is driven by LDL reduction and is not affected by HyperTG and HDL-C levels.

Introduction

Although high-density lipoprotein cholesterol (HDL-C) levels are inversely correlated with cardiovascular disease (CVD), recent genetic studies have questioned whether this correlation reflects causation.^{1, 2} Fasting levels of HDL-C and triglyceride (TG) are inversely related, thus it is difficult to dissect the importance of each of these lipids. Postprandial TG excursions also inversely correlate with HDL-C^{3, 4} and some investigators consider HDL-C as a marker for average circulating TG levels.² While several recent genome-wide association studies (GWAS) failed to directly link HDL modulating genes with CVD, genes affecting TG levels—lipoprotein lipase (LpL) and angiopoietin-like proteins 3 and 4—do associate with clinical CVD endpoints.¹

There are limited experimental models linking hypertriglyceridemia (HyperTG) and atherosclerosis, but a large series of experimental studies have documented benefits of raising HDL-C and apolipoprotein A1 (ApoA-I levels; reviewed in^{5, 6}). For example, preclinical studies have shown that increased expression of ApoA-I, the major protein in HDL, reduces atherosclerosis⁶⁻⁸ and normalizes the defect in atherosclerosis regression that occurs with diabetes⁹. Some clinical studies have infused HDL or HDL-like particles and showed reduced atherosclerosis development¹⁰ with acute beneficial changes in plaque composition (e.g. reduced lipid and macrophage content)¹¹. Methods to markedly induce ApoA-I expression in humans are not available. Other methods to increase HDL-C, such as blocking the exchange of cholesterol and TG between TG-rich lipoproteins and HDL by cholesteryl ester transfer protein (CETP) inhibition, were not beneficial when corrected for the expected benefits of LDL-C reduction by this treatment.¹²⁻¹⁴

One postulated reason for the lack of benefits with CETP inhibition in humans is that the increase in HDL-C does not reflect an increase in cholesterol efflux capacity (CEC) or reverse cholesterol transport (RCT). In humans, CEC has been touted as a better CVD risk marker than HDL-C.¹⁵⁻¹⁷ Moreover, the regression of lesions after marked LDL-C reduction is widely believed to rely on HDL-CEC to remove accumulated cholesterol. However, kinetic studies performed nearly 4 decades ago showed that the variation in HDL-C levels found in most humans did not affect overall cholesterol

balance, which implied that CEC does not differ among most people.¹⁸ Thus, the role of HDL particles and their functionality in atherosclerosis are unclear.

In the present report, we tested whether reduced HDL-C due to HyperTG resulting from LpL deficiency would affect atherosclerosis regression, as well as CEC, in mice and humans. We hypothesized that LpL-deficiency would decrease CEC by reducing HDL particle numbers (HDL-P) and that this in turn would impair atherosclerosis regression. We chose to study regression, in part, because during this process, cholesterol must exit from atherosclerotic plaques, the first step of CEC. Moreover, current approaches to CVD in humans have a greater focus on the resolution of atherosclerotic coronary lesions through marked cholesterol reduction.

Our surprising results show that though LpL deficiency induced a reduction in HDL-C, the now TG-rich HDL particles maintained CEC. This likely allowed normal atherosclerosis regression and illustrates that normal repair of lesions can occur in the setting of HyperTG and reduced HDL-C.

Materials and Method

Animals: Global inducible LpL knockout mice were generated by crossing floxed *Lpl* (*Lpl^{fl/fl}*) mice¹⁹ with β -actin driven tamoxifen-inducible Cre (*MerCreMer*) transgenic mice (Jackson Laboratory) to obtain the β -actin-*MerCreMer*/*LpL^{fl/fl}* offspring designated as *iLpL^{-/-}* mice, as previously described.²⁰ Mice received intraperitoneal (i.p.) injections of tamoxifen (Toronto Research Chemicals, #T006000) or 4-hydroxytamoxifen (Tocris) in corn oil (Sigma) at a dose of 40 mg/kg BW/day for 5 consecutive days. On the day of sacrifice, mice were anesthetized via intraperitoneal (i.p.) injection of ketamine (100mg/kg) and xylazine (10mg/kg). Blood was collected via cardiac puncture in EDTA-containing tubes and mice were perfused with 10% sucrose in saline solution (0.9% NaCl). All procedures were approved by the Institutional Animal Care and Use Committee at New York University Langone Health approved protocol #160907 ("Lipoprotein Lipase and ApoB"). Mice were maintained in a temperature-controlled (25°C) facility with a 12 h-light/dark cycle and given free access to water and food, except when fasting blood was obtained. Mice were fed a laboratory

rodent chow diet or western diet (WD; 0.3% cholesterol; Dyets Inc. D101977Gi) as indicated.

Plasma lipid measurements: Total cholesterol (TC) was measured using an enzymatic assay (Total Cholesterol E Kit, Wako Life Science, cat # NC9138103 or Infinity Total Cholesterol Reagent, Thermo Scientific, cat #TR13521). Plasma TG was measured using either enzymatic assay (L-Type Triglyceride M, Wako Life Science or Infinity Triglycerides, Thermo Scientific, cat #TR2242) or a fluorometric kit (BioVision, #K622-100).

Lipoprotein fractionation: Lipoprotein fractions were obtained by sequential density ultracentrifugation as described before²⁰, and TC and TG measured as described above. Briefly, equal amounts of mouse plasma (70–100 μ L) were used for sequential density ultracentrifugation to separate very-low-density lipoprotein (VLDL $d < 1.006$ g/mL), LDL ($d = 1.006$ – 1.063 g/mL), and HDL ($d = 1.063$ – 1.21 g/mL) in a TLA 100 rotor (Beckmann Instruments, Palo Alto, CA).

hCETP: hCETP expression was induced by injecting mice i.p. with either adeno-associated virus (AAV) hCETP (AAV8.TBG.PI.hCETP.SV40) or a LacZ control virus (AAV8.TBG.PI.LacZ.bGH) at a concentration of 5×10^{11} GC (genome copies)/mouse (Penn Vector Core) as done before.²¹ Plasma CETP activity was measured using a CETP Activity Assay Kit (Sigma Aldrich, MAK106). CETP expression in the liver was assessed by qPCR from homogenized liver samples (Primer sequence in Supplemental Table I).

Aortic transplant regression model: The aortic transplant model has been described before.²²⁻²⁵ Briefly, a plaque burdened aortic arch from a hypercholesterolemic *Ldlr*^{-/-} mouse (fed a WD for 16wks) was transplanted into a normocholesterolemic recipient mouse (*iLpl*^{-/-} or *Lpl*^{fl/fl}), interpositioned with the abdominal aorta and blood flow was directed through the graft. All recipient mice were ~22 weeks old and maintained on standard chow diet until sacrificed 14 days after aortic transplantation.

LDL receptor (LDLR) antisense oligonucleotide (ASO) regression model: This protocol was based on our published model.³⁸ Briefly, GalNAc-conjugated Gen 2.5 ASO targeting mouse LDLR was developed and provided by Ionis Pharmaceuticals. LDLR ASO was injected intraperitoneally at a dose of 5 mg/kg body weight once a week for 16 weeks. GalNAc-conjugated sense oligonucleotide (SO) designed to bind and inactivate the LDLR ASO was injected once i.p. at a dose of 20 mg/kg body weight to accelerate the return of LDLR expression.

Bone marrow transplant (BMT): Bone marrow cells were isolated from C57BL/6 mice by flushing tibiae and femur bones, followed by red blood cell lysis and BM cells suspended in DMEM (Dulbecco's Modified Eagle Media, Fisher Scientific). 5×10^6 cells/mouse were injected retro-orbitally into 8 weeks old irradiated (10.000 Gy) *iLpI^{-/-}* and *LpI^{fl/fl}*, followed by 4 weeks of antibiotic treatment administered in drinking water.

Atherosclerotic plaque assessment: Aortic arches were removed after perfusion with saline + 10% sucrose, embedded in OCT (TissueTEK) and frozen at -80°C. Serial sections (6µm thick) were cut using a cryostat and stained for CD68 (Bio-Rad, Cat. MCA1957)²⁵, inducible nitric oxide synthase (iNOS; Abcam, Cat. ab209027)²⁶ and Arginase1 (Thermo Fisher Scientific, Cat. 53-3697-82) to determine content and phenotype of macrophages. For further plaque characteristics, Movats²⁷ and Picrosirius Red (Abcam, Cat. ab246832)²⁸ stainings were performed as described previously. Picrosirius Red staining was visualized using a polarized light microscope (Zeiss Axio Observer) to determine collagen⁺ areas.

Aortic roots and brachiocephalic arteries (BCAs) were removed after perfusion with PBS. The root was embedded in OCT (TissueTEK), frozen, and stored at -80°C. Serial sections (6 µm) of roots were obtained by cryosectioning, followed by Mac-2²⁹ or CD68²⁵ immunostaining to determine macrophage content as described. BCAs were collected in 10% formalin, kept overnight at 4°C, and stored in 70% ethanol at 4°C for further processing. BCAs embedded in paraffin were sectioned (5 µm) and every fifth cross section stained using the Movat pentachrome method. The maximal lesion site was determined, and adjacent sections were immunostained using a Mac-2 antibody to detect lesion macrophages, as described previously.²⁹

ImagePro Plus 7.0 software was used to determine CD68⁺, Mac-2⁺ and collagen⁺ area. ImageJ 1.51r (NIH) was used to determine iNOS⁺, Arginase1⁺ cells and divided by total cells (DAPI nuclear staining) to get percentage of total cells. Acellular areas were measured to determine necrotic core area and fibrous cap was evaluated as none, very thin (<5layers), thin (5-10 layers) and thick (>10 layers) above the necrotic core using Movats stain.

Macrophage phenotyping: CD68⁺ cells were selected from atherosclerotic plaques from *iLpI^{-/-}* and *LpI^{fl/fl}* mice by laser capture microdissection (LCM) as previously described.^{30, 31} Briefly, 6µm frozen sections from aortic arches were stained with hematoxylin-eosin and foam cells identified under a microscope and verified by positive CD68 staining. All LCM procedures were performed under RNase-free conditions. After LCM, RNA was isolated using the PicoPure Kit (Molecular Devices, Inc., Sunnyvale, CA), treated with DNase and the quality and quantity determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA was converted to cDNA and amplified using the WT-Ovation Pico RNA Amplification Kit (NuGEN, San Carlos, CA). Data were normalized to *HRPT* with the $\Delta\Delta C(t)$ method and are presented as relative transcript levels. All primers are listed in Supplemental Table I.

HDL analyses: Cholesterol efflux capacity (CEC) was analyzed by methods based on methodology developed by Rothblat et al as described previously.³²⁻³⁴ Briefly, cAMP-stimulated J774 cells were radiolabeled with [³H]-cholesterol and incubated with ApoB depleted plasma for 4h. CEC was quantified as the percentage of label in the medium relative to total labelled cholesterol levels in the culture system after subtraction of values obtained without ApoB depleted serum. ABCA1-mediated CEC was determined by analogous method in BHK cells expressing ABCA1 under mifepristone control where the ABCA1-specific CEC represents the difference between CEC of mifepristone stimulated and non-stimulated BHK cells.

HDL particle number (HDL-P) and particle size distribution was quantified using calibrated differential ion mobility analysis.^{33, 35} Proteome of HDL isolated by sequential density ultracentrifugation was characterized and quantified using data independent

analysis (DIA) mass spectrometry.^{34, 36} Proteomics data were analyzed using Reactome (<https://reactome.org>).³⁷

Human subjects: Plasma was obtained from 9 donors with elevated plasma TG levels of which three were LpL deficient (Characteristics in Supplemental Table VI), and 6 control subjects with normal plasma TG levels. CEC, HDL-P and HDL proteomics analyses were performed as described above.

Statistical Analyses: Data are expressed as mean $\pm$ SEM. Data were tested on normality and equal variance and analyzed by appropriate parametric or non-parametric test as stated in each figure. $p \leq 0.05$ was considered significant (GraphPad Prism 7).

Results

LpL deficiency increased circulating VLDL-TG and reduced HDL-C, which was further reduced with hCETP expression

At ~5 weeks of age, *Ldlr*^{-/-} mice were weaned onto WD, which was continued for 16 weeks to promote advanced atherosclerotic lesion formation (baseline group; Figure 1A, Supplemental Figure 1A). Plaque-bearing aortic grafts were then transplanted into *LpI*^{fl/fl} (control) and *iLpI*^{-/-} mice (HyperTG, induced 3 weeks prior to transplantation) that were normocholesterolemic. Both recipient groups had been maintained on a standard laboratory diet prior to receiving aortic transplants; this diet was continued for 2 weeks to maintain normocholesterolemia and induce plaque regression. A subset of mice was injected with an AAV hCETP (5×10^{11} genome copies/mouse; Figure 1A, D) to investigate whether the absence of CETP expression in mice was a factor in the effects of HyperTG.

In contrast to the hypercholesterolemic baseline/donor mice (TC 867 ± 36 mg/dL), both aortic transplant recipient groups were normocholesterolemic (TC 79 ± 10 mg/dL; Figure 1B). Plasma TG levels were normal in *LpI*^{fl/fl} mice (~67 mg/dL) and, as expected, TG levels were elevated in *iLpI*^{-/-} mice (~461 mg/dL; Figure 1C). hCETP injection did not affect TC or total TG levels but led to an expected increase in circulating CETP activity (Figure 1D). Body weights of the mice did not change due to

the transplant procedure and were similar in all regression groups (Supplemental Figure 1B). Body weight was elevated in the baseline group due to WD feeding.

The changes in lipoprotein profile due to LpL deficiency and hCETP expression are shown in Figures 1E-F. The most remarkable lipid change that occurred with hCETP and HyperTG was a marked reduction in HDL-C with smaller changes in other lipoprotein parameters. VLDL-C increased in *iLpL^{-/-}* compared to *LpL^{fl/fl}* mice (*iLpL^{-/-}* 37 ± 7 versus *LpL^{fl/fl}* 13 ± 3 mg/dL, $P = 0.04$). As LpL-mediated lipolysis is required for LDL and HDL formation, LpL deficient mice had decreased LDL-C and HDL-C levels; HDL-C was decreased from 29 ± 2 to 17 ± 1 mg/dL ($P = 0.004$) and even further reduced with hCETP expression to 9 ± 2 mg/dL ($P < 0.0001$). As expected, VLDL-TG was increased in *iLpL^{-/-}* compared to *LpL^{fl/fl}* mice (*iLpL^{-/-}* 314 ± 40 vs. *LpL^{fl/fl}* 60 ± 15 mg/dL, $P < 0.0001$, Figure 1F). Neither LDL-TG nor HDL-TG differed between *LpL^{fl/fl}* and *iLpL^{-/-}* mice, but HDL-TG significantly increased with hCETP expression to the same extent in mice with and without *LpL* expression (average HDL-TG with hCETP 3.3 ± 1.4 mg/dL vs. no hCETP 1.2 ± 0.2 mg/dL). Thus, LpL deficiency induced HyperTG and reduced LDL-C and HDL-C. hCETP reduced HDL-C even further and created TG-enriched HDL as expected from its role in humans in exchanging VLDL-TG for HDL-C.

Figure 1. LpL deficiency increased VLDL-TG and reduced HDL-C, which was further reduced with hCETP expression. (A) Study Setup (B) Total plasma cholesterol levels at time of harvest (C) Total triglyceride (TG) levels in baseline and regression groups before and after the 2 weeks atherosclerosis regression period (D) CETP activity measured in circulation. Cholesterol (E) and triglyceride levels (F) of isolated lipoproteins. N= 5-24/group; data represented as mean $\pm$ SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, 1-way ANOVA with Tukey's multiple comparison test; HDL-C and HDL-TG Welch ANOVA with Games-Howell's multiple comparison test; VLDL-TG & LDL-TG Kruskal-Wallis Test with Dunn's multiple comparison test

HyperTG due to global LpL deficiency does not affect atherosclerosis regression in the aortic transplant mouse model

After two-weeks of plaque exposure to normocholesterolemia (atherosclerosis regression period), lesion areas were similar in all groups (Figure 2A-B). We considered as evidence of atherosclerosis regression decreases in lesion area or macrophage content because the two parameters can be independent of each other (e.g.³⁸) and either is considered to be a beneficial change.

The macrophage content (CD68+ area, Figure 2C) decreased in regression groups compared to baseline, but did not differ between mice with and without LpL/hCETP expression (baseline $21.4\% \pm 1.3$ versus average of 4 regression groups: $11.5\% \pm 1.4$, $P < 0.0001$). To further evaluate the plaques, we assessed necrotic core, fibrous cap and collagen. Necrotic core (% lesion area) did not significantly differ between groups (Figure 2A, C-D). Fibrous caps (Figure 2E) and collagen (Figure 2G) were increased compared to baseline ($P = 0.052$), but did not significantly differ between regression groups. Therefore, the favorable remodeling of the cellular composition of the plaques (in particular, reduced macrophage content, an index of the resolution of lesion inflammation) was not impaired by HyperTG and/or hCETP expression. Moreover, we could not correlate lesion area or macrophage content with the degree of HyperTG (Supplemental Figure II).

HyperTG due to non-macrophage LpL deficiency also does not affect atherosclerosis regression in the aortic transplant mouse model

LpL is also expressed by macrophages and has been shown to promote lipid uptake and foam cell formation.³⁹⁻⁴¹ To exclude possible counterbalancing effects of the putative atherogenic HyperTG and atheroprotective LpL deficiency in macrophages, we performed atherosclerosis regression studies using *LpL^{fl/fl}* and *iLpL^{-/-}* transplanted with WT bone marrow (Supplemental Figure III A-B). HyperTG mice with LpL expressing macrophages showed body weights and lipid levels similar to those in total LpL-deficient mice (Supplemental Figure III C-G). Unlike the effects of macrophage LpL to promote atherosclerosis^{39, 42}, the number of macrophages per lesion area (%CD68) during regression was similarly reduced in mice with and without macrophage LpL expression, showing a reduction of >50% compared to baseline (Supplemental Figure III H-I). Thus, knocking out macrophage LpL expression did not

alter regression. These data are similar to another study that showed LpL expression does not modify lesional macrophage phenotype in the setting of atherosclerosis regression.²⁰

Figure 2. LpL deficiency (+/- hCETP) does not impair atherosclerosis regression.

(A) Representative stainings; CD68, MOVATs, Pico Sirius Red Staining (brightfield & polarized light). Quantification of (B) Lesion Area (μm^2) and (C) CD68 Area (% of Lesion Area) (D) Necrotic Core (% of Lesion Area) (E) Fibrous Cap determined by number of layers above the Necrotic Core, Thick >10layers, Thin 5-10layers, Very Thin <5 layers (F) Collagen (% of Lesion Area) N=6-19/group, data represented as mean $\pm$ SEM, * $P < 0.0001$, 1-way ANOVA with Tukey's multiple comparison test; (F) Welch ANOVA with Games-Howell's multiple comparison test

LpL deficiency mediated HyperTG does not affect atherosclerosis regression in a non-invasive mouse model

To confirm the results on LpL and atherosclerosis regression in an independent model (Figure 3A), WD-fed *LpI^{fl/fl}* and *iLpI^{-/-}* (non-induced) mice were made hypercholesterolemic using LDLR ASO treatment for 16 weeks. At the end of 16 weeks, the ASO treatments stopped and one subset from each genotype was analyzed as the 'baseline'. The other subset of mice was treated with tamoxifen to induce LpL deletion at week 14. These mice were then treated with LDLR SO at week 16 to rapidly reverse hypercholesterolemia and to induce regression.⁴³ After another 3 weeks, these mice were studied to analyze the regressing plaques. As expected, plasma TC decreased in regression groups compared to baseline (Figure 3B), and plasma TG increased significantly in *iLpI^{-/-}* mice (Figure 3C).

As with the transplant model, with marked cholesterol reduction there were no changes in total plaque areas in the aortic root (Figure 3D). There was a marked reduction in CD68% area (Figure 3E), but HyperTG did not affect this. In parallel, we noted no changes in total plaque area in the BCAs (Figure 3F), but as in the root, there was a decrease in macrophage area (Figure 3G, $P < 0.05$). Again, HyperTG did not affect the extent of BCA regression.

Thus, in two independent models of atherosclerosis regression, HyperTG *iLpI^{-/-}* mice had similar changes as those in the control *LpI^{fl/fl}* mice after reversal of hypercholesterolemia, and in the LDL ASO model there was no evidence that the changes we observed were specific only to the aortic root.

LpL deficiency-mediated HyperTG with local lipolysis in plaques does not affect atherosclerosis regression in a non-invasive mouse model of regression

One possible cause for the postulated atherogenicity of HyperTG is the generation of local lipolysis products.⁴⁴⁻⁴⁶ *iLpI^{-/-}* mice would be protected from this type of injury due to generalized LpL deficiency. To determine the importance of local lipolysis we assessed atherosclerosis regression in mice with LpL expression in endothelial cells driven by a Tie2-Lpl transgene, described in⁴⁷. These mice were allowed to develop atherosclerosis using the LDLR ASO protocol⁴³ and regression was induced by reversing the hypercholesterolemia with SO. Total plasma cholesterol significantly decreased in regression groups after SO treatment (Supplemental Fig IV A). Mice with

total LpL deficiency and the Tie2-LPL transgene had mild HyperTG compared to LpL floxed mice with the Tie2-LPL transgene (Supplemental Figure IV B). We quantified aortic root total plaque area (Supplemental Figure IV C) and percentage of plaque that was occupied by macrophages (Figure IV D) along with BCA total plaque area (Supplemental Figure IV E) and percentage of the Mac-2 positive (i.e., macrophage positive) area in BCA plaques (Supplemental Figure IV F). There were no significant differences attributable to increasing the lipolytic capacity of the endothelial cells. Thus, greater local lipolysis did not alter regression.

Figure 3. LpL deletion does not alter atherosclerosis regression in a non-invasive model of regression.

(A) Study design: atherosclerosis was created in *LpI^{fl/fl}* and *iLpI^{-/-}* mice with LDLR antisense oligonucleotides (ASO) and western diet feeding for 16 weeks. One set of mice were analyzed at 16 weeks as the baseline group and the rest of the mice were treated with SO to induce regression and were analyzed after 3 weeks. The mice in regression group were also treated with tamoxifen at Week 13 to induce hypertriglyceridemia in *iLpI^{-/-}* mice. (B) Plasma total cholesterol (TC) and (C) triglyceride (TG) levels in baseline and regression groups. (D) Total plaque area, (E) % of macrophages (CD68+) within aortic root lesions, (F) total plaque area, (G) % of macrophage (Mac2+) in plaques within the BCA in the baseline and regression groups. n=7-15 /group, results expressed as mean $\pm$ SEM. * P < 0.05, ** P<0.01 and **** P < 0.0001 using 1-way ANOVA with Tukey's multiple comparison test.

Effects of HyperTG on plaque macrophage gene expression

Given the similarity in the results between the two regression models in terms of changes in plaque macrophage content, we wondered if there may have been altered phenotype related to HyperTG. Thus, RNA expression of isolated plaque macrophages and immunofluorescence staining were performed using samples from the transplant studies (Figure 4).

We have previously shown that transcriptomic profiling of atherosclerotic macrophages did not disclose significant differences in metabolism-related genes or pro- or anti-inflammatory genes in LpL-expressing versus LpL-deficient macrophages.²⁰ To determine if hCETP expression affected macrophage phenotype, we isolated CD68+ cells by laser capture microdissection from *LpI^{fl/fl}* and *iLpI^{-/-}* mice expressing hCETP. In line with the previous results, we did not observe significant changes in metabolism-related genes (*plin2*, *cd36*, *glut1*, *fasn*, *cpt1a*), pro-inflammatory genes (*tnfa*, *nos2*, *mcp1*), or anti-inflammatory genes (*mrc1*, *fizz1*, *il10*) (Figure 4A-C). Staining of arginase-1 (a marker of M2-like macrophages, Figure 4D-E) and iNOS (a marker of M1-like macrophages, Figure 4F-G) in atherosclerotic plaques showed no differences between *LpI^{fl/fl}* and *iLpI^{-/-}* mice expressing hCETP, further confirming transcriptomic profiling results.

Figure 4. LpL deficiency (+/- hCETP) does not affect macrophage phenotype.

Macrophage phenotype was assessed by isolating mRNA from atherosclerotic CD68+ macrophages using Laser Capture Microdissection and mRNA expression measured for (A) Metabolism-related genes (B) Inflammatory genes (C) Anti-inflammatory genes. Additionally, immunofluorescence staining of atherosclerotic arches was performed for (D-E) Arginase-1 (%cells per total cells) and (F-G) iNOS (%cells per total cells). N=5-6/group, data represented as mean $\pm$ SEM, * $P < 0.05$, (A-C) unpaired t-test, (E,G) 1-way ANOVA with Tukey's multiple comparison test

LpL deficiency-mediated HyperTG reduces HDL-C and HDL-P, but does not impair CEC

We hypothesized that regression was unchanged in our HyperTG mice because although HDL-C was reduced, CEC was maintained. To determine this, we assessed plasma levels of HDL-P and CEC. LpL deficiency leads to lower HDL-C levels in humans⁴⁸ and in animal models⁴⁹. We now show that LpL deficiency also significantly lowers circulating HDL-P levels, which were further reduced by hCETP expression (Figure 5A, $P < 0.001$). Dividing total HDL-P into their subclasses according to size showed that the reduction was in small (9.8-10nm) and medium (10.4-11.1nm), but not in large (11.9 – 12.6nm) HDL-P (Figure 5A).

We next measured CEC according the protocol developed by Rothblat and colleagues, in which radiolabeled cAMP-stimulated J774 cells are incubated with ApoB-depleted plasma for 4h, followed by quantification of labelled cholesterol in cells

and medium.⁵⁰ Total CEC and ABCA1 mediated CEC were assessed (Figure 5B). LpL deficiency decreased total CEC ($Lp^{fl/fl}$ $7.5 \pm 0.4\%$ vs. $iLp^{fl/fl}$ $5.4 \pm 0.7\%$, $P = 0.0002$), but despite the marked reduction in HDL-C, ABCA1-mediated efflux was not reduced. There was no reduction in total CEC or ABCA1-mediated efflux in mice expressing hCETP. When total CEC and ABCA1-mediated efflux on a per particle basis were calculated, there was no significant differences between $Lp^{fl/fl}$ and $iLp^{fl/fl}$ mice (Figure 5C). Thus, HyperTG did not produce dysfunctional HDL. hCETP and TG-enrichment of HDL (Figure 1G) increased total CEC per particle ($Lp^{fl/fl}$ + hCETP $1.30 \pm 0.06\%$ vs. $Lp^{fl/fl}$ $0.90 \pm 0.06\%$, $P = 0.018$) and ABCA1-mediated efflux per particle in $Lp^{fl/fl}$ mice ($Lp^{fl/fl}$ + hCETP $8.36 \pm 0.62\%$ vs. $Lp^{fl/fl}$ $7.72 \pm 0.37\%$, $P = 0.014$). Total CEC and ABCA1-mediated CEC per particle showed a trend towards an increase in $iLp^{fl/fl}$ mice expressing hCETP. These results show that despite a reduction in HDL-C and HDL-P with HyperTG, the unchanged regression correlated with maintained CEC.

Effects of LpL and CETP on the HDL proteome

We next determined how LpL deficiency expression altered the HDL proteome. Comparing HDL of $Lp^{fl/fl}$ with $iLp^{fl/fl}$ mice, of the 133 detected proteins, 50 proteins were significantly different with 18 being down- and 32 up-regulated (Figure 5D-E, Supplemental Table II-III). Differentially detected proteins were mainly associated with the apolipoprotein structural components, such as ApoA-I, ApoC-III, and ApoC-IV, but also included changes in PLTP and Angptl3 (Figure 5E). ApoA-I was reduced along with the reductions in HDL-C and HDL-P.

We next assessed how hCETP expression altered the proteome in $Lp^{fl/fl}$ and $iLp^{fl/fl}$ mice. In mice expressing hCETP, we detected a total of 97 proteins, of which 15 proteins significantly differed (1 was down- and 14 up-regulated in $iLp^{fl/fl}$ + hCETP; Figure 5F-G, Supplemental Table IV). Surprisingly, some of the proteins found in the HDL proteome from non-hCETP mice were not detected in hCETP-expressing mice, such as ApoA-V. Of the 133 detected proteins in non-hCETP expressing mice and 97 detected proteins in hCETP-expressing mice, eighty-five of the proteins were present in both groups. Of these 85 proteins, only 4 proteins (1 down- and 3 up-regulated) were significantly different with hCETP expression, showing that hCETP has modest effects on the mouse HDL proteome (Supplemental Table V).

Figure 5. LpL deficiency decreases HDL-P, changes HDL Proteome, but does not change CEC with minor effects of hCETP.

(A) Total HDL-P and its subfractions (B) Total and ABCA1-mediated HDL Cholesterol Efflux Capacity (CEC) (C) Total and ABCA1-mediated CEC per HDL-P (D) Volcano blot of HDL Proteomics in $iLpL^{-/-}$ versus $LpL^{fl/fl}$ (top panel) and $iLpL^{-/-}$ + hCETP versus $LpL^{fl/fl}$ + hCETP (bottom panel) (E) Total number of Proteins changed including the number of downregulated (green) and upregulated (red) proteins (F) Main regulated proteins are part of plasma lipoprotein (G) Proteins that significantly change in $iLpL^{-/-}$ versus $LpL^{fl/fl}$ mice due to hCETP expression. Proteomics normalized to spiked apoA1; complete list of proteins can be found in Supplemental Table II-V. N=4-24 (Proteomics: $LpL^{fl/fl}$ n=8, $iLpL^{-/-}$ n=8, $LpL^{fl/fl}$ + hCETP n=17, $iLpL^{-/-}$ + hCETP n=10), * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, 1-way ANOVA with Tukey's multiple comparison test

Subjects with HyperTG also show decreased HDL-P number, changes in the HDL proteome, and no impairment in total CEC

To extend our mouse data to humans, we measured HDL-P and CEC in HyperTG patients of which three were diagnosed with LpL deficiency (full characteristics are shown in Supplemental Table VI). Average plasma TG levels were ~750mg/dL and total cholesterol levels were ~380mg/dL (Figure 6A). HyperTG led to a reduced number of HDL-P (Figure 6B, P < 0.0001), which was mainly due to reduction in medium and large HDL-P, but not small HDL-P. This differs from the mouse data showing a

reduction in predominantly small HDL-P, but not large HDL-P, and is likely due to species differences in HDL metabolism(e.g.,⁵¹). As in the humanized mouse data (+hCETP, Figure 5B-C), total CEC did not change, meaning that CEC per HDL-P was significantly increased ($P < 0.01$), suggesting an improvement in HDL functionality. This increase was primarily due to an increase in the ABCA1-mediated efflux pathway (Figure 6C-D).

We next assessed HDL proteomics in the human samples. HDL proteomic analysis detected a total of 90 proteins (Figure 6E, Supplemental Table VII). Similar to our humanized mouse data, we found changes in HDL-associated apolipoproteins and PLTP (Figure 6F). Notably, the results of LpL deficient patients are similar to those of HyperTG patients. Taking the human and mouse results together, HyperTG leads to reduction in HDL-P without the creation of intrinsically dysfunctional HDL measured as CEC, while proteomic data mainly reflect the observed changes in HDL associated with LpL deficiency/HyperTG, such as reduced ApoA-I.

Figure 6. Subjects with HyperTG, incl. LpL deficient subjects show decreased HDL-P, but no impairment in CEC.

(A) Plasma TG and Cholesterol levels (mg/dL). (B) total HDL-P and HDL-P subsets. (C) Total and ABCA1-mediated CEC (%). (D) CEC per HDL-P (E) Volcanoplot of HDL Proteomics showing changes with HyperTG. (F) Main regulated proteins are part of plasma lipoprotein remodeling pathway. White-filled dots refer to HyperTG patients, blue filled dots refer to LpL deficient patients. Proteomics normalized to spiked ApoA-I; complete list of proteins can be found in Supplemental

Table VI. N=6 (Control), N=9 (HyperTG), * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$, students unpaired t-test; (C, D) Mann-Whitney test

Discussion

Our study shows that despite reductions in HDL-C and HDL-P, LpL deficiency-mediated HyperTG did not affect atherosclerosis resolution as assessed by the macrophage content in plaques in aortic arches, roots and BCAs. Furthermore, LpL-deficiency did not affect macrophage inflammation (as judged by M1/M2 phenotypes) or plaque stability judged indirectly by composition (necrotic core, collagen and fibrous cap) in aortic arches in mice. These results are surprising, as HDL-C and HDL-P have been shown to be inversely associated with CVD (reviewed in²). However, despite a reduction in HDL-C, HDL-P, and CEC, total CEC per particle did not change in HyperTG mice, and was increased in HyperTG humans. Thus, HDL function (CEC) was maintained in the presence of severe HyperTG. This suggests that the inverse association of HDL-C and CVD is not necessarily due to changes in CEC.

Because mice do not have CETP, the HDL-C and HDL-P reductions were likely due to reduced LpL-mediated lipid and protein transfer from TG-rich lipoproteins to HDL.⁵² LpL deficiency led to a change in the HDL-P size distribution with reduced levels of small- and medium-sized particles. In the mouse the combination of reduced lipolysis generated HDL components along with more rapid loss in the kidney might have led to this reduction in smaller HDL-P. With expression of CETP, resulting in TG-rich HDL, HDL-C and HDL-P were further reduced. Along with the reduction in HDL-C and HDL-P, ApoA-I was significantly reduced in mice and humans with HyperTG. Despite marked reductions in HDL, atherosclerosis regression proceeded normally. The reduction in lesional macrophage content was similar in the HyperTG/low HDL and control mice. Thus, despite increased circulating TG levels and marked reductions in HDL-C, atherosclerosis regression proceeded normally and is evidence that LDL-C reduction overrode changes in macrophage LpL expression and HDL, as discussed below.

Recent clinical data have implicated HDL function rather than HDL-C as a marker for CVD risk.¹⁵⁻¹⁷ Unsuccessful lowering of CVD mortality in HDL-raising trials using CETP

inhibition have been explained as a failure to increase reverse cholesterol transport, and perhaps CEC.¹³ Although HyperTG mouse plasma displayed, albeit limited, lower CEC, this reduction was less than what would be expected due to reduced HDL-P. In fact, when corrected for particle number, there was no indication that HDL was dysfunctional. HyperTG-associated reduction in CEC was lost with hCETP expression, as CEC actually increased per HDL-P. Therefore, HyperTG did not reduce HDL function and also did not affect atherosclerosis regression in mice.

In humans, HyperTG is inversely correlated with HDL-C¹. Therefore, HyperTG was expected to lead to reduced CEC. In agreement with our mouse data, however, HyperTG increased CEC per HDL-P in our human samples. These data confirm that despite the reduction in HDL-P, HDL is still functional in the HyperTG environment. Together, our results indicate that HDL enrichment with TG over cholesterol does not affect function.

In contrast to these results, increasing HDL-P in mice by transgenic overexpression of ApoA-I improved atherosclerosis regression^{6,9}. This raises the question why we do not detect impaired regression when HDL-P and ApoA-I are reduced. Perhaps ApoA-I has some function that is exclusive of its actions on HDL to affect CEC. HDL likely has multiple actions *in vivo*, such as reducing inflammation⁵³ and blood coagulation⁵⁴. These CEC-independent functions might be improved by marked increases in HDL. Such other actions of HDL rather than its effects on CEC would be consistent with its relationship to CVD and in agreement with clinical data showing similar overall cholesterol balances of humans with high and low HDL-C levels.¹⁸ Alternatively, the level of total CEC in the mice with fewer HDL-P particles may have still been sufficient to prevent a greater accumulation of cholesterol in plaque macrophages. For example, in *apoE*^{-/-} mice we have previously shown that the naturally low level of HDL-P in that model was sufficient to remove the excess free cholesterol from plaques when ACAT was inhibited.⁵⁵

It should also be considered that the effects on regression by changes in HDL-P were likely attenuated by the preservation of ABCA1-mediated cholesterol efflux that we observed. As Rothblat and colleagues have shown⁵⁶, as macrophages get

progressively loaded by cholesterol, akin to their state in the baseline atherosclerotic plaques, the majority of efflux shifts from the aqueous diffusion pathway to being mediated by ABCA1. Finally, we should also note that despite the decrease in the ABCA1-mediated ApoA-I, other mediators not captured by our HDL measurements could have contributed to preservation of ABCA1-mediated efflux. These could be, among others, pre- β HDL⁵⁷, non-HDL ApoA-IV⁵⁸, or plasminogen⁵⁹.

Part of HyperTG's atherogenicity has been postulated to depend on LpL-mediated lipolysis and the creation of atherogenic remnant particles. Mutations in the LpL gene and heterozygous LpL deficiency, resulting in elevated TG levels, associate with increased CVD risk (OR 1.51, 95% CI: 1.39-1.64 per 1-SD increase in TG).⁶⁰ Homozygous LpL-deficient patients have been viewed as atherosclerosis resistant due to the defect on atherogenic remnant production despite their TG reaching levels >1000mg/dL. However, more recently, a number of LpL deficient patients with atherosclerotic CVD have been described.^{61, 62} Because the risk of CVD with complete LpL deficiency is unclear our animal data are especially germane. Our global LpL-deficient mice had mild-to-moderate plasma TG levels (approx. 500mg/dL). This level, while lower than many LpL-deficient patients, is not dissimilar from that found in LpL-deficient patients on a very restricted diet that might be comparable to a mouse chow diet. However, on a high fat diet, TG levels of *iLpL*^{-/-} mice increased to >1,000 mg/dL. Thus, the failure to detect differences in regression in these mice is consistent with the hypothesis that nascent TG-rich lipoproteins are not atherogenic.

Regulation of lipolysis and generation of local lipolysis products depends both on LpL activity and circulating TG levels, which provide substrate for the reaction. For this reason, it has been postulated that the combination of partial LpL loss and HyperTG causes high concentrations of liberated toxic lipids or the generation of more atherogenic remnant lipoproteins. Therefore, we also created mice expressing additional LpL in arteries using the Tie2 promoter, which primarily expresses in endothelial cells. Because this transgene was bred onto the LpL knockout background, greater lipolysis along the arterial wall would be expected. This transgene also did not affect atherosclerosis regression. Therefore, neither circulating nascent TG-rich

lipoproteins nor increasing the capacity of their hydrolysis along the artery wall prevented vascular repair (i.e., regression), in the setting of a marked reduction in LDL-C.

In addition to the points discussed already, our mouse model of LpL deficiency leads to several changes exclusive of circulating HDL-C and TG that should be considered. Macrophages are the only white blood cells that express LpL and are the major source of LpL in atherosclerotic plaques. Whether LpL in atherosclerotic plaques is pro- or anti-atherogenic is still debated. *In vitro*, macrophage LpL appears to have atherogenic functions, because it creates atherogenic remnant lipoproteins, which are rapidly internalized by macrophages.⁶³ Macrophage-specific LpL knockout *in vivo* reduced atherosclerosis^{39, 40}, indicating a role of LpL produced by these cells which is exclusive of changes in circulating lipoproteins.

In contrast to these presumed inflammatory effects of macrophage LpL, fatty acids provided via lipolysis were reported to activate PPAR γ ⁶⁴ and convert macrophages to a less inflammatory phenotype.^{41, 65, 66} Our recent published data²⁰, as well as the present study, however, showed that the transcriptomic profile of regressing atherosclerotic macrophages did not differ between macrophages expressing LpL and LpL-deficient macrophages. Since macrophage LpL has been implicated in foam cell formation³⁹⁻⁴¹, loss of macrophage LpL could have opposite and counterbalancing effects on atherogenesis. To exclude the possibility that we protected our mouse model from possible atherogenic effects of HyperTG, we created HyperTG mice with macrophages expressing LpL. Again, we observed intact atherosclerosis regression. Therefore, macrophage-LpL-mediated lipolysis in atherosclerotic plaques does not impede in HyperTG mice the resolution of atherosclerotic lesions.

In conclusion, our study shows that a reduction in HDL-C and HDL-P associated with LpL-deficiency and CETP expression does not affect atherosclerosis regression. Furthermore, we show that the lipoprotein alterations caused by these interventions, including the TG-enrichment of HDL, does not affect CEC per particle. As already noted, perhaps the lack of effects on atherosclerosis is explained by the continued level of sufficient HDL function, especially through the ABCA1 pathway. Furthermore,

our data do not support a toxic effect of TG-rich lipoproteins on regression. We expect that these data reflect the human situation in which patients on high doses of LDL-lowering medications show clinical benefit, regardless of their circulating TG levels.

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Conflicts of Interest

None.

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Supplemental

A

B

Supplemental Figure I

(A) Representative pictures of the aortic arch in the donor mouse (*left*), during aortic transplantation (*middle*) and after two weeks of atherosclerosis regression (*right*). Arrows indicate atherosclerotic lesions (B) Body weight in baseline and regression groups before and after the 2 weeks atherosclerosis regression period. N= 5-24/group; data represented as mean $\pm$ SEM, **** P < 0.0001, 1-way ANOVA with Tukey's multiple comparison test

Supplemental Figure II

Correlation of plasma TG levels with (A) lesion size and (B) %CD68

Supplemental Figure III

iLpL^{-/-} with LpL expressing macrophages do not impair atherosclerosis regression.

(A) Study setup (B) Genotyping using blood DNA confirming BMT (C) body weight in baseline and regression groups before and after the 2 weeks atherosclerosis regression period (D) Plasma Cholesterol (E) Plasma TG. Cholesterol (F) and triglyceride levels (G) of isolated lipoproteins (H) CD68 staining (I) CD68 area of lesion size (%). N= 4-14/group; data represented as mean ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, 1-way ANOVA with Tukey's multiple comparison test (C,I), Kruskal-Wallis Test (D,E), students unpaired t-test (F-G).

Supplemental Figure IV. Hypertriglyceridemia and enhanced endothelial LPL expression does not alter atherosclerosis regression in a non-invasive model of regression. Atherosclerosis was created in *LpL^{fl/fl}* Tie2LPL and *iLpL^{-/-}* Tie2LPL mice with LDLR antisense oligonucleotides (ASO) and western diet feeding for 16 weeks. One set of mice were analyzed at 16 weeks as the baseline group and the rest of the mice were treated with SO to induce regression and were analyzed after 3 weeks. The mice in regression group were also treated with tamoxifen at Week 13 to induce hypertriglyceridemia in *iLpL^{-/-}* Tie2LPL mice. Plasma (A) total cholesterol (TC) and (B) triglyceride (TG) levels in baseline and regression groups. (C) Total plaque area, (D) % of macrophages (CD68+) within aortic root lesions, (E) total plaque area, (F) % of macrophage (Mac2+) in plaques within the BCA in the baseline and regression groups. n=3-5 /group, results expressed as mean ± SEM. *P < 0.05, **P < 0.01 using 1-way ANOVA with Tukey's multiple comparison test.

Supplemental Table I

List of primer used for qPCR

Gene	Forward (5' - 3')	Reverse (5' - 3')
Lpl	AGGTGGACATCGGAGAACTG	TCCCTAGCACAGAAGATGACC3
Plin2	TCTGCGGCCATGACAAGTG	GCAGGCATAGGTATTGGCAAC
Cpt1a	TGCACTACGGAGTCCTGCAA	GGACAACCTCCATGGCTCAG
Glut1	TCGTAACGAGGAGAACCG	GGCCGTGTTGACGATA
Fasn	TCTTTCTAACAACCACCCTCTGG	CTTCACGACTCCATCACGAATG
Cd36	CCTTAAAGGAATCCCCGTGT	TGCATTTGCCAATGTCTAGC
Tnf	TGGAAGTGGCAGAAGAGG	AGACAGAAGAGCGTGGTG
Mcp1	CCCAATGAGTAGGCTGGAGA	TCTGGACCCATTCTTCTTG
Nos2	CAGCTGGGCTGTACAAACCTT	CATTGGAAGTGAAGCGTTTCG
Fizz1	CCAATCCAGCTAACTATCCCTCC	AAGCCACAAGCACACCCAGT
Mrc1	TGATTACGAGCAGTGGGAAGC	G TTCACCGTA-AGCCCAATT
Il10	CTGGACAACATACTGCTAACCG	GGGCATCACTTCTACCAGGTAA

Supplemental Table II

Overview of detected Proteins

Protein.Gene	Protein.Name	Protein.Accession	Protein.Description
Actb	sp P60710 ACTB_MOUSE	P60710	Actin, cytoplasmic 1 OS=Mus musculus GN=Actb PE=1 SV=1
Agt	sp P11859 ANGT_MOUSE	P11859	Angiotensinogen OS=Mus musculus GN=Agt PE=1 SV=1
Ahsg	sp P29699 FETUA_MOUSE	P29699	Alpha-2-HS-glycoprotein OS=Mus musculus GN=Ahsg PE=1 SV=1
Alb	sp P07724 ALBU_MOUSE	P07724	Serum albumin OS=Mus musculus GN=Alb PE=1 SV=3
Angptl3	sp Q9R182 ANGL3_MOUSE	Q9R182	Angiopietin-related protein 3 OS=Mus musculus GN=Angptl3 PE=1 SV=1
Antr2	sp Q6DFX2 ANTR2_MOUSE	Q6DFX2	Anthrax toxin receptor 2 OS=Mus musculus GN=Antr2 PE=1 SV=1
Apoa2	sp P09813 APOA2_MOUSE	P09813	Apolipoprotein A-II OS=Mus musculus GN=Apoa2 PE=1 SV=2
Apoa4	sp P06728 APOA4_MOUSE	P06728	Apolipoprotein A-IV OS=Mus musculus GN=Apoa4 PE=1 SV=3
ApoB	sp E9Q414 APOB_MOUSE	E9Q414	Apolipoprotein B-100 OS=Mus musculus GN=ApoB PE=1 SV=1
Apoc1	sp P34928 APOC1_MOUSE	P34928	Apolipoprotein C-I OS=Mus musculus GN=Apoc1 PE=1 SV=1
Apoc2	sp Q05020 APOC2_MOUSE	Q05020	Apolipoprotein C-II OS=Mus musculus GN=Apoc2 PE=2 SV=1
Apoc3	sp P33622 APOC3_MOUSE	P33622	Apolipoprotein C-III OS=Mus musculus GN=Apoc3 PE=1 SV=2
Apod	sp P51910 APOD_MOUSE	P51910	Apolipoprotein D OS=Mus musculus GN=Apod PE=1 SV=1
ApoE	sp P08226 APOE_MOUSE	P08226	Apolipoprotein E OS=Mus musculus GN=ApoE PE=1 SV=2
Apon	tr G3X9D6 G3X9D6_MOUSE	G3X9D6	Apolipoprotein N OS=Mus musculus GN=Apon PE=1 SV=1
Arhgdia	GDIR1_MOUSE	sp Q09PT1 GDIR1_MOUSE	Rho GDP-dissociation inhibitor 1 OS=Mus musculus GN=Arhgdia PE=1 SV=3
B2m	sp P01887 B2MR_MOUSE	P01887	Beta-2-microglobulin OS=Mus musculus GN=B2m PE=1 SV=2
Blvrb	sp Q923D2 BLVRB_MOUSE	Q923D2	Flavin reductase (NADPH) OS=Mus musculus GN=Blvrb PE=1 SV=3
Bpifa2	sp P07743 BPIA2_MOUSE	P07743	BPI fold-containing family A member 2 OS=Mus musculus GN=Bpifa2 PE=1 SV=1
C3	sp P01027 CO3_MOUSE	P01027	Complement C3 OS=Mus musculus GN=C3 PE=1 SV=3
C4b	sp P01029 CO4B_MOUSE	P01029	Complement C4-B OS=Mus musculus GN=C4b PE=1 SV=3
Ca1	sp P13634 CAH1_MOUSE	P13634	Carbonic anhydrase 1 OS=Mus musculus GN=Ca1 PE=1 SV=4
Ca2	CAH2_MOUSE	sp P00920 CAH2_MOUSE	Carbonic anhydrase 2 OS=Mus musculus GN=Ca2 PE=1 SV=4
Cd97	sp Q9Z0M6 CD97_MOUSE	Q9Z0M6	CD97 antigen OS=Mus musculus GN=Cd97 PE=1 SV=2
Cfl1	sp P18760 COF1_MOUSE	P18760	Cofilin-1 OS=Mus musculus GN=Cfl1 PE=1 SV=3
Ciu	sp Q06890 CLUS_MOUSE	Q06890	Clusterin OS=Mus musculus GN=Ciu PE=1 SV=1
Cp	CERU_MOUSE	sp Q61147 CERU_MOUSE	Ceruloplasmin OS=Mus musculus GN=Cp PE=1 SV=2
Ctsd	sp P18242 CATD_MOUSE	P18242	Cathepsin D OS=Mus musculus GN=Ctsd PE=1 SV=1
Dbi	sp P31786 ACBP_MOUSE	P31786	Acyl-CoA-binding protein OS=Mus musculus GN=Dbi PE=1 SV=2
Dmkn	sp Q6P253 DMKN_MOUSE	Q6P253	Dermokine OS=Mus musculus GN=Dmkn PE=2 SV=2
Dsc3	sp P55850 DSC3_MOUSE	P55850	Desmocollin-3 OS=Mus musculus GN=Dsc3 PE=1 SV=3
Dsp	DESP_MOUSE	sp E9Q557 DESP_MOUSE	Desmoplakin OS=Mus musculus GN=Dsp PE=1 SV=1
Fabp4	sp P04117 FABP4_MOUSE	P04117	Fatty acid-binding protein, adipocyte OS=Mus musculus GN=Fabp4 PE=1 SV=3
Fga	sp E9PV24 FIBA_MOUSE	E9PV24	Fibrinogen alpha chain OS=Mus musculus GN=Fga PE=1 SV=1
Fgb	sp Q8K0E8 FIBB_MOUSE	Q8K0E8	Fibrinogen beta chain OS=Mus musculus GN=Fgb PE=1 SV=1
Gc	sp P21614 VTDB_MOUSE	P21614	Vitamin D-binding protein OS=Mus musculus GN=Gc PE=1 SV=2
Gm20425	tr E9Q035 E9Q035_MOUSE	E9Q035	Uncharacterized protein OS=Mus musculus GN=Gm20425 PE=4 SV=1
Gm8909	tr G3UXE9 G3UXE9_MOUSE	G3UXE9	Uncharacterized protein OS=Mus musculus GN=Gm8909 PE=1 SV=1
Gpld1	sp O70362 PHLD_MOUSE	O70362	Phosphatidylinositol-glycan-specific phospholipase D OS=Mus musculus GN=Gpld1 PE=1 SV=1
Gpx3	sp P46412 GPX3_MOUSE	P46412	Glutathione peroxidase 3 OS=Mus musculus GN=Gpx3 PE=1 SV=2
Gpx5	GPX5_MOUSE	sp P21765 GPX5_MOUSE	Epididymal secretory glutathione peroxidase OS=Mus musculus GN=Gpx5 PE=2 SV=3
H2-L	sp P01897 HA1L_MOUSE	P01897	H-2 class I histocompatibility antigen, L-D alpha chain OS=Mus musculus GN=H2-L PE=1 SV=2
H2-Q10	sp P01898 HA1O_MOUSE	P01898	H-2 class I histocompatibility antigen, Q10 alpha chain OS=Mus musculus GN=H2-Q10 PE=1 SV=3
H2-Q4	tr Q8HWB2 Q8HWB2_MOUSE	Q8HWB2	Histocompatibility 2, Q region locus 4 OS=Mus musculus GN=H2-Q4 PE=1 SV=1
H2-Q7	sp P14429 HA17_MOUSE	P14429	H-2 class I histocompatibility antigen, Q7 alpha chain OS=Mus musculus GN=H2-Q7 PE=1 SV=1
Hba	sp P01942 HBA_MOUSE	P01942	Hemoglobin subunit alpha OS=Mus musculus GN=Hba PE=1 SV=2
Hbbt1	tr A8DUK4 A8DUK4_MOUSE	A8DUK4	Beta-globin OS=Mus musculus GN=Hbbt1 PE=1 SV=1
Icam1	sp P13597 ICAM1_MOUSE	P13597	Intercellular adhesion molecule 1 OS=Mus musculus GN=Icam1 PE=1 SV=1
Igfbf	sp P70389 ALS_MOUSE	P70389	Insulin-like growth factor-binding protein complex acid labile subunit OS=Mus musculus GN=Igfbf PE=1 SV=1
Ighm	sp P01872 IGHM_MOUSE	P01872	Ig mu chain C region OS=Mus musculus GN=Ighm PE=1 SV=2
IGKC	IGKC_MOUSE	sp P01837 IGKC_MOUSE	Ig kappa chain C region OS=Mus musculus PE=1 SV=1
Igkv14-126	tr AA0A075B5K0 AA0A075B5K0_MOUSE	AA0A075B5K0	Uncharacterized protein (Fragment) OS=Mus musculus GN=Igkv14-126 PE=4 SV=7
Itih4	sp A6X935 ITIH4_MOUSE	A6X935	Inter alpha-trypsin inhibitor, heavy chain 4 OS=Mus musculus GN=Itih4 PE=1 SV=2
K1C10_HUMAN	sp K1C10_HUMAN	K1C10_HUMAN	#N/A
K22E_HUMAN	sp K22E_HUMAN	K22E_HUMAN	#N/A
Kng1	sp O08677 KNG1_MOUSE	O08677	Kininogen-1 OS=Mus musculus GN=Kng1 PE=1 SV=1
Krt1	sp P04104 K2C1_MOUSE	P04104	Keratin, type II cytoskeletal 1 OS=Mus musculus GN=Krt1 PE=1 SV=4
Krt10	sp P02535 K1C10_MOUSE	P02535	Keratin, type I cytoskeletal 10 OS=Mus musculus GN=Krt10 PE=1 SV=3
Krt13	sp P08730 K1C13_MOUSE	P08730	Keratin, type I cytoskeletal 13 OS=Mus musculus GN=Krt13 PE=1 SV=2
Krt14	sp Q61781 K1C14_MOUSE	Q61781	Keratin, type I cytoskeletal 14 OS=Mus musculus GN=Krt14 PE=1 SV=2
Krt5	sp Q922U2 K2C5_MOUSE	Q922U2	Keratin, type II cytoskeletal 5 OS=Mus musculus GN=Krt5 PE=1 SV=1
Krt72	K2C72_MOUSE	sp Q6IMES9 K2C72_MOUSE	Keratin, type II cytoskeletal 72 OS=Mus musculus GN=Krt72 PE=3 SV=1
KRT9	sp K1C9_HUMAN	P35527	Keratin, type I cytoskeletal 9 (Cyokeratin-9) (CK-9) (Keratin-9) (K9)
Krt90	tr E9Q1Z0 E9Q1Z0_MOUSE	E9Q1Z0	Keratin 90 OS=Mus musculus GN=Krt90 PE=1 SV=1
Lcat	sp P16301 LCAT_MOUSE	P16301	Phosphatidylcholine-sterol acyltransferase OS=Mus musculus GN=Lcat PE=1 SV=2
Mb	sp P04247 MYG_MOUSE	P04247	Myoglobin OS=Mus musculus GN=Mb PE=1 SV=3
Mug1	sp P28665 MUG1_MOUSE	P28665	Murineoglobulin-1 OS=Mus musculus GN=Mug1 PE=1 SV=3
Mup18	sp A2BIM8 MUP18_MOUSE	A2BIM8	Major urinary protein 18 OS=Mus musculus GN=Mup18 PE=3 SV=1
Mup20	sp Q5FW60 MUP20_MOUSE	Q5FW60	Major urinary protein 20 OS=Mus musculus GN=Mup20 PE=1 SV=1
Napsa	sp O09043 NAPSA_MOUSE	O09043	Napsin-A OS=Mus musculus GN=Napsa PE=1 SV=1
Pcyox1	sp Q9CQF9 PCYOX_MOUSE	Q9CQF9	Prenylcysteine oxidase OS=Mus musculus GN=Pcyox1 PE=1 SV=1
Pfn1	sp P62962 PROF1_MOUSE	P62962	Profilin-1 OS=Mus musculus GN=Pfn1 PE=1 SV=2
Pla2g7	sp Q60963 PAFA_MOUSE	Q60963	Platelet-activating factor acetylhydrolase OS=Mus musculus GN=Pla2g7 PE=2 SV=2
Pltp	sp P55065 PLTP_MOUSE	P55065	Phospholipid transfer protein OS=Mus musculus GN=Pltp PE=1 SV=1
Pm20d1	sp Q8C165 P20D1_MOUSE	Q8C165	N-fatty-acyl-amine acid synthase/hydrolase PM20D1 OS=Mus musculus GN=Pm20d1 PE=1 SV=1
Pon1	sp P52430 PON1_MOUSE	P52430	Serum paraoxonase/arylesterase 1 OS=Mus musculus GN=Pon1 PE=1 SV=2
Ppia	PPIA_MOUSE	sp P17742 PPIA_MOUSE	Peptidyl-prolyl cis-trans isomerase A OS=Mus musculus GN=Ppia PE=1 SV=2
Ppic	sp P30412 PPIC_MOUSE	P30412	Peptidyl-prolyl cis-trans isomerase C OS=Mus musculus GN=Ppic PE=1 SV=1
Prdx2	sp Q61171 PRDX2_MOUSE	Q61171	Peroxioredoxin-2 OS=Mus musculus GN=Prdx2 PE=1 SV=3
Psap	sp Q61207 SAP_MOUSE	Q61207	Prosaoposin OS=Mus musculus GN=Psap PE=1 SV=2
Pzp	sp Q61838 PZP_MOUSE	Q61838	Pregnancy zone protein OS=Mus musculus GN=Pzp PE=1 SV=3
Qsok1	sp Q8BND5 QSOX1_MOUSE	Q8BND5	Sulphydryl oxidase 1 OS=Mus musculus GN=Qsok1 PE=1 SV=1
Rbp4	sp Q00724 RET4_MOUSE	Q00724	Retinol-binding protein 4 OS=Mus musculus GN=Rbp4 PE=1 SV=2
Saa1	sp P05366 SAA1_MOUSE	P05366	Serum amyloid A-1 protein OS=Mus musculus GN=Saa1 PE=1 SV=2
Saa2	sp P05367 SAA2_MOUSE	P05367	Serum amyloid A-2 protein OS=Mus musculus GN=Saa2 PE=1 SV=1
Saa4	sp P31532 SAA4_MOUSE	P31532	Serum amyloid A-4 protein OS=Mus musculus GN=Saa4 PE=1 SV=2
Sell	sp P18337 LYAM1_MOUSE	P18337	L-selectin OS=Mus musculus GN=Sell PE=1 SV=1
Serpina1a	sp P07758 A1AT1_MOUSE	P07758	Alpha-1-antitrypsin 1-1 OS=Mus musculus GN=Serpina1a PE=1 SV=4
Serpina1b	sp P22599 A1AT2_MOUSE	P22599	Alpha-1-antitrypsin 1-2 OS=Mus musculus GN=Serpina1b PE=1 SV=2
Serpina1e	sp Q00898 A1AT5_MOUSE	Q00898	Alpha-1-antitrypsin 1-5 OS=Mus musculus GN=Serpina1e PE=1 SV=1
Serpina3k	sp P07759 SPA3K_MOUSE	P07759	Serine protease inhibitor A3K OS=Mus musculus GN=Serpina3k PE=1 SV=2
Serpinf2	A2AP_MOUSE	sp Q61247 A2AP_MOUSE	Alpha-2-antiplasmin OS=Mus musculus GN=Serpinf2 PE=1 SV=1
Tfrc	sp Q62351 TFR1_MOUSE	Q62351	Transferrin receptor protein 1 OS=Mus musculus GN=Tfrc PE=1 SV=1
Thbs1	sp P35441 TSP1_MOUSE	P35441	Thrombospondin-1 OS=Mus musculus GN=Thbs1 PE=1 SV=1
Ttr	sp P07309 TTHY_MOUSE	P07309	Transthyretin OS=Mus musculus GN=Ttr PE=1 SV=1
Vcam1	sp P29533 VCAM1_MOUSE	P29533	Vascular cell adhesion protein 1 OS=Mus musculus GN=Vcam1 PE=1 SV=1
Vtn	sp P29788 VTNC_MOUSE	P29788	Vitronectin OS=Mus musculus GN=Vtn PE=1 SV=2

Supplemental Table III

Detected Proteins in $iLpl^{-/-}$ versus $Lpl^{fl/fl}$ $iLpl^{-/-}$ vs. $Lpl^{fl/fl}$

Protein	Fold Change	Pvalue
Dmkn	0.299	<0.001
Apoc3	0.207	<0.001
Apoc2	0.176	<0.001
Apoc4	0.129	0.001
Rbp4	1.588	0.001
Camp	0.281	0.001
Hpx	4.842	0.001
Apoc1	0.125	0.001
Pfn1	2.52	0.001
Ctsd	1677	0.001
ApoE	0.362	0.001
F6Y6L6	1.753	0.001
Lrg1	2.055	0.001
Gm5160	3.343	0.002
Psap	1.392	0.002
Cst6	0.221	0.002
Apoa2	0.700	0.002
Clec3b	4.941	0.002
Apoa5	3.663	0.002
Apod	0.744	0.002
Gm	1.771	0.003
Ag1	2.336	0.003
Blvrb	3.109	0.003
Tmsb4x	3.142	0.004
Ppic	0.717	0.004
Apoa1	0.797	0.004
Ttr	1.681	0.004
Apom	0.703	0.007
Fabp5	3.580	0.007
Gc	1.694	0.008
Serpina1b	1.607	0.009
Pltp	0.771	0.009
Serpina1a	1.467	0.011
Clf1	2.214	0.011
Cs1	3.043	0.012
Igfals	1.627	0.013
Vnn1	2.693	0.013
Pm20d1	0.372	0.015
Gm20425	4.585	0.017
Itih4	1.963	0.017
Serpina1d	1.523	0.018
Sncs	3.082	0.018
Cd97	1.791	0.019
Prdx2	2.318	0.019
Tfhs1	2.974	0.021
Ces1b	4.959	0.025
Antxr2	0.684	0.026
Ahsg	1.359	0.029
Ithh	0.783	0.031
Pycn1	0.825	0.032
Angptl3	0.383	0.035
Pla2g7	0.643	0.038
Kng1	1.775	0.040
H2-Q1	0.809	0.049
H2-Q10	0.810	0.052
C7	1.533	0.055
Saa1	0.503	0.061
Krt10	2.176	0.064
Pzp	2.033	0.064
Saa2	0.446	0.064
Ambp	1.506	0.066
Fga	1.917	0.067
Tfpi	0.833	0.068
Krt13	2.159	0.078
H2-L	0.822	0.082
B2m	0.827	0.083
K22E	2.505	0.089
Cst3	1.735	0.090
Apcs	1.751	0.094
Saa3	0.513	0.094
Hbex1	4.038	0.100
Napsa	0.802	0.104
Hba	4.064	0.114
Bpifa2	1.390	0.115
hHBA	3.786	0.130
Gm8909	0.847	0.140
Dn	2.087	0.140
Scgb1a1	1.767	0.147
K1C10	1.786	0.149
Gpx3	1.757	0.151
Actb	4.394	0.171
Igkv14-126	0.333	0.175
Fabp4	2.193	0.179
Apon	1.135	0.182
Fgb	1.582	0.207
Dc3	1.521	0.222
Saa4	0.809	0.239
H2-K1	0.852	0.241
Mup15	1.592	0.258
Fgg	1.474	0.263
Gpld1	1.226	0.297
Qsrx1	1.304	0.302
H2-Q7	0.877	0.334
Mb	1.566	0.348
Ighm	0.574	0.381
Mup18	1.551	0.392
Apoa4	1.128	0.447
KRT9	0.320	0.471
Serpina3k	1.229	0.480
Hamp	1.817	0.482
Krt2	1.472	0.535
Tfrc	1.144	0.545
C4b	1.140	0.560
Vcam1	0.869	0.562
Mup17	1.560	0.592
H2-Q4	0.946	0.594
Mup3	1.222	0.596
Mup2	1.252	0.604
K2C1	0.582	0.625
P01837	1.185	0.625
Clu	1.061	0.638
Krt90	0.647	0.659
CSN1S1	1.047	0.665
Arsg	0.902	0.688
Pon1	1.036	0.710
Icam1	1.113	0.728
Krt78	0.743	0.740
Engg7	1.040	0.776
Lcat	0.975	0.800
Anxa2	1.129	0.801
Krt1	0.813	0.809
Serpina1e	0.887	0.839
Apdh	1.043	0.840
Emc9	0.964	0.846
Vtn	1.043	0.855
Krt5	1.125	0.860
Fabp3	1.052	0.867
Mup20	0.909	0.884
TRV1	0.987	0.901
Krt42	0.944	0.926
Mug1	1.013	0.955
Sell	0.993	0.961
Gm7298	1.003	0.990

Supplemental Table IVDetected Proteins in $iLpl^{-/-}$ +hCETP versus $Lpl^{fl/fl}$ +hCETP

Protein	Fold Change	P value
Pfn1	2.096	0.009
Dbi	1.926	0.010
Arhgdia	2.725	0.013
Fga	1.908	0.020
Dsc3	2.094	0.024
Hbbt1	3.166	0.024
Kng1	3.343	0.025
Blvrb	2.449	0.032
Serpinf2	2.864	0.032
Prdx2	2.220	0.032
Fabp4	2.209	0.033
Ca1	2.462	0.040
Pzp	4.669	0.043
Apoa2	0.819	0.044
Itih4	2.670	0.045
Thbs1	3.245	0.056
Ppic	1.415	0.061
Cfl1	2.862	0.065
Serpina3k	2.132	0.077
Serpina1b	1.588	0.078
IGKC	2.200	0.082
Hba	2.408	0.088
Apoa4	1.383	0.095
Gpld1	0.584	0.104
Apoa1	0.706	0.107
Bpifa2	0.324	0.110
Ppia	2.599	0.117
Ttr	1.405	0.120
Tfrc	0.177	0.122
Qsox1	0.173	0.122
Igfals	0.125	0.129
Pla2g7	0.288	0.130
Fgb	1.617	0.134
Gm20425	2.948	0.134
Vcam1	0.272	0.148
Serpina1a	1.469	0.152
Ca2	2.596	0.159
Cd97	0.168	0.187
Pon1	0.658	0.192
Gpx5	1.632	0.197
Gpx3	1.581	0.201
Mb	1.693	0.222
Cp	2.750	0.235
C4b	0.554	0.237
Apoc2	0.670	0.243
Pm20d1	2.010	0.253
Gc	1.460	0.257
Antxr2	0.494	0.310
ApoE	1.353	0.322
B2m	1.214	0.329
Mup18	1.697	0.339
Saa2	1.635	0.346
Mup20	1.813	0.362
Ighm	1.564	0.370
Apoc3	0.807	0.375
Saa1	1.439	0.384
Agt	1.205	0.393
Lcat	0.793	0.409
Dmkn	0.713	0.427
H2-Q7	1.388	0.430
KRT9	1.307	0.438
Napsa	0.859	0.447
Dsp	0.000	0.451
C3	0.805	0.459
Icam1	0.477	0.474
Krt14	0.310	0.479
Serpina1e	1.253	0.504
Clu	0.775	0.513
Actb	0.224	0.520
H2-L	1.179	0.569
Pltp	1.544	0.587
Apon	1.284	0.590
Ahsg	1.193	0.592
H2-Q4	0.900	0.604
Igkv14-126	1.357	0.605
Gm8909	1.164	0.620
Apod	0.849	0.635
Krt13	0.644	0.637
Apoc1	1.110	0.640
Pcyox1	0.908	0.641
Krt72	0.650	0.650
Krt5	0.603	0.651
Mug1	1.226	0.719
H2-Q10	1.102	0.730
K1C10_HUMAN	0.771	0.753
Krt90	0.822	0.766
Krt1	0.830	0.781
Psap	0.759	0.804
TRYP5	1.012	0.821
Krt10	0.827	0.822
Angptl3	1.156	0.826
Rbp4	1.128	0.853
Saa4	1.054	0.876
K22E_HUMAN	0.922	0.887
Vtn	0.939	0.932
Sell	0.953	0.936
Ctsd	1.025	0.947

Supplemental Table V

Overlapping Proteins Supplemental Table II and III

Total of 85 proteins were detected in both proteomic data sets. 9 of them were significantly downregulated, 52 upregulated. They were associated with lipoprotein remodeling.

	Cre vs Flox	Cre vs flox hCETP	
Protein	Fold Change	Fold Change	P value
Qsox1	1.304	0.172	0.001
Cd97	1.791	0.168	0.002
Apoc1	0.125	1.110	0.019
Thbs1	2.974	3.245	0.032
Ppic	0.717	1.414	0.047
Apoc3	0.206	0.806	0.058
Tfrc	1.143	0.176	0.064
H2-L	0.821	1.178	0.088
Serpina3k	1.228	2.132	0.121
Apon	1.135	1.284	0.149
Pltp	0.771	1.544	0.163
Serpina1a	1.466	1.468	0.183
Actb	4.334	0.223	0.188
Dmkn	0.298	0.713	0.195
Fgb	1.581	1.616	0.197
H2-Q10	0.810	1.102	0.198
H2-Q4	0.946	0.899	0.200
Gm8909	0.847	1.164	0.210
Apoc2	0.176	0.669	0.211
Rbp4	1.587	1.128	0.214
Ahsg	1.358	1.192	0.215
Sell	0.992	0.952	0.223
Apod	0.744	0.849	0.238
Krt5	1.124	0.602	0.238
Gc	1.693	1.459	0.252
Saa2	0.446	1.634	0.253
Mup20	0.909	1.812	0.256
Cf1l	2.214	2.861	0.274
Bpifa2	1.389	0.324	0.286
Dbi	2.087	1.925	0.295
Apoa4	1.128	1.383	0.300
Krt10	2.175	0.826	0.303
Saa1	0.503	1.438	0.304
Mb	1.566	1.692	0.305
Pfn1	2.352	2.095	0.305
ApoE	0.362	1.352	0.326
B2m	0.827	1.214	0.328
Ca1	3.042	2.462	0.328
KRT9	0.320	1.306	0.330
Mug1	1.012	1.226	0.334
H2-Q7	0.876	1.388	0.338
Serpina1e	0.887	1.253	0.352
Vtn	1.043	0.939	0.359
Napsa	0.801	0.859	0.368
Pzp	2.033	4.668	0.383
Krt1	0.813	0.829	0.397
Dsc3	1.521	2.094	0.401
Hba	4.063	2.407	0.405
Fga	1.916	1.907	0.415
Serpina1b	1.607	1.587	0.426
Gpld1	1.226	0.583	0.434
Psap	1.392	0.759	0.437
Krt90	0.646	0.822	0.449
AgT	2.336	1.204	0.454
Antxr2	0.684	0.494	0.456
Blvrb	3.108	2.448	0.463
C3	1.533	0.805	0.466
Igfals	1.626	0.124	0.469
Ctsd	1.677	1.025	0.472
Gm20425	4.584	2.947	0.486
Kng1	1.774	3.343	0.488
Krt13	2.158	0.644	0.504
Pm20d1	0.372	2.010	0.507
Vcam1	0.869	0.271	0.508
Pcyox1	0.824	0.908	0.508
Fabp4	2.192	2.208	0.515
C4b	1.140	0.554	0.526
Apoa2	0.700	0.818	0.553
Saa4	0.809	1.054	0.571
Itih4	1.963	2.669	0.587
Angptl3	0.383	1.155	0.629
Icam1	1.112	0.476	0.642
Ttr	1.681	1.404	0.673
Mup18	1.551	1.697	0.716
Clu	1.060	0.775	0.716
Lcat	0.974	0.792	0.752
Gpx3	1.756	1.580	0.783
Prdx2	2.318	2.220	0.802
Hbbt1	4.038	3.166	0.820
Igkv14-126	0.333	1.357	0.854
Pon1	1.036	0.658	0.900
Apoa1	0.797	0.705	0.914
Pla2g7	0.643	0.288	0.925
Ighm	0.574	1.564	0.989

Supplemental Table VI

Patient characteristics

Patient	Diagnosis	LpL deficient
1	HyperTG, Pancreatitis	
2	LCAT Deficient	
3	HyperTG	
4	HyperTG	X
5	Hyperlipidemia	X
6	HyperTG, Pancreatitis, T1D, Ketoacidosis	
7	No Pancreatitis, Good TGs, On Meds	X
8	HyperTG	
9	Recurring Pancreatitis, DM	

Supplemental Table VII**HyperTG versus Control**

90 detected proteins in human samples, 6 being significantly downregulated, 14 upregulated with HyperTG.

HyperTG vs.
normal TG

Protein	Fold change	P-value
ANTXR2	0.336	<0.001
ORM1	2.940	0.003
GC	4.023	0.003
FGG	3.300	0.004
FGB	3.164	0.004
SERPINA1	3.128	0.007
APOA1	0.586	0.007
APOC2	2.509	0.008
FGA	3.798	0.009
MENT	0.476	0.010
CD44	0.519	0.010
PLTP	0.494	0.011
A1BG	3.403	0.013
SERPINF1	4.354	0.014
APOH	1.718	0.019
AHSG	3.104	0.021
AZGP1	2.171	0.023
ALB	4.670	0.039
APOC3	2.666	0.045
PPBP	2.769	0.046
LPA	0.065	0.048
APOA2	0.669	0.063
C1R	4.764	0.064
DEFA1	4.460	0.073
APOC4	2.399	0.080
IGHA1	1.696	0.080
TTR	1.927	0.083
IGHG1	4.492	0.084
IGKC	3.779	0.091
PLA2G7	3.085	0.095
S100A8	2.797	0.098
CST3	3.877	0.116
APOE	2.382	0.134
RBP4	3.897	0.143
FGFBP2	0.638	0.147
APOD	0.831	0.152
VTN	0.761	0.162
APOM	0.798	0.164
CA1	74.468	0.166
CLU	0.745	0.171
APOA4	2.524	0.172
KRT10	2.325	0.172
APOA5	7.754	0.173
C4BPA	13.435	0.173
SAA4	0.707	0.174
APOL1	1.436	0.175
APMAP	1.608	0.192
CA2	34.141	0.204
A2M	6.285	0.211
KRT2	2.327	0.224
SAA1	41.561	0.234
CAMP	1.832	0.249
AMBP	6.197	0.249
KRT5	1.876	0.254
KRT1	2.102	0.256
IGHM	8.624	0.257
B2M	7.417	0.262
LCAT	1.455	0.263
KRT14	1.901	0.267
APOC1	1.288	0.288
TF	9.556	0.294
PCYOX1	0.847	0.301
CD99	0.769	0.306
MADCAM1	0.732	0.316
S100A7	1.533	0.318
SHH	0.566	0.327
PSAP	0.697	0.335
C3	1.170	0.335
PON3	0.827	0.342
IGHG2	2.638	0.374
KRT9	1.953	0.384
HBA1	175.992	0.415
HBB	365.166	0.417
HBD	119.098	0.420
PON1	0.832	0.421
HBZ	58.339	0.422
ANXA2P2	1.663	0.423
KRT75	1.534	0.428
HP	1.392	0.456
KRT6A	1.532	0.470
HLA-A	2.551	0.473
DCD	1.321	0.486
KRT16	1.724	0.510
C4A	1.251	0.528
HPR	1.282	0.549
LTF	0.806	0.643
SFTPB	0.768	0.657
POTEF	0.934	0.818
KRT17	0.936	0.916
PFN1	1.011	0.986
C3orf85	0.998	0.996

Part 2

Hyperglycemia

**Neutrophil Extracellular Traps (NETs) Promote
Macrophage Inflammation and Impair Atherosclerosis
Resolution in Diabetic Mice**

Tatjana Josefs*, Tessa J. Barrett*, Emily Brown, Alexandra Quezada, Xiaoyun Wu, Maud Voisin,
Jaume Amengual, Edward A. Fisher

* Equal contribution

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Abstract

Neutrophil extracellular traps (NETs) promote inflammation and atherosclerosis progression. NETs are increased in diabetes and impair the resolution of inflammation during wound healing. Atherosclerosis resolution, a process resembling wound healing, is also impaired in diabetes. Thus, we hypothesized that NETs impede atherosclerosis resolution in diabetes by increasing plaque inflammation. Indeed, transcriptomic profiling of plaque macrophages from NET positive and negative areas in low-density lipoprotein receptor-deficient (*Ldlr*^{-/-}) mice revealed inflammasome and glycolysis pathway upregulation, indicating a heightened inflammatory phenotype. We found that NETs decline during atherosclerosis resolution, which was induced by reducing hyperlipidemia in non-diabetic mice, but they persist in diabetes, exacerbating macrophage inflammation and impairing resolution. In diabetic mice deoxyribonuclease 1 (DNase1) treatment reduced plaque NETs content and macrophage inflammation, promoting atherosclerosis resolution after lipid-lowering. Given that humans with diabetes also exhibit impaired atherosclerosis resolution with lipid-lowering, these data suggest that NETs contribute to the increased cardiovascular disease risk in this population and are a potential therapeutic target.

Introduction

Cardiovascular disease (CVD) and diabetes are both associated with leukocytosis (1, 2). We and others have previously reported that hyperglycemia in mice impaired the resolution of atherosclerosis after lipid-lowering (3, 4). Hyperglycemia also stimulated myelopoiesis in the mice, which increased circulating levels of monocytes and neutrophils and their entry into plaques (5-7). While the contribution of monocyte-derived macrophages to atherogenesis has been extensively studied, less is known about neutrophils. Accumulating evidence indicates that neutrophils are also important protagonists in plaque progression (8). Their ability to form neutrophil extracellular traps (NETs) (9), structures found in human and mouse plaques (10-12), is of increasing interest in the context of atherogenesis due to the proinflammatory nature of NETs (13).

First reported in 2004 (14), NETosis is a process in which, in response to pathogens and sterile inflammatory stimuli, neutrophils release cytosolic and nuclear material forming a net-like extracellular structure (15). Relevant to atherogenesis, stimulators of NETosis include cholesterol crystals, oxidized low-density lipoprotein, oxysterols, platelets, and various chemokines (9). NETosis is not only stimulated by inflammation but can also promote it (9), for example, by increasing monocyte recruitment to inflamed sites and triggering macrophages to release reactive oxygen species and proinflammatory cytokines (16). The effects of NETs on plaque macrophages are thought to involve activation of their inflammasomes, but direct evidence *in vivo* to support this is scant (17).

In mice, genetic and pharmacological inhibition of NETosis reduces plaque size, inflammation, and features of instability (10, 18). In humans, circulating NET biomarkers positively correlate with atherosclerotic plaque size, and are independently associated with clinical severity of CVD and the incidence of major adverse cardiac events (19, 20). One clinical population in which NETs are hypothesized to be particularly atherogenic is those with diabetes. Compared to people without diabetes, those with diabetes exhibit increased CVD risk and impaired atherosclerosis resolution, or plaque regression, after lipid-lowering therapy (21, 22). That NETs could be an important basis of these findings is supported by studies in which circulating

NET biomarkers are correlated with both CVD and diabetes severity (23). Factors promoting NETosis in diabetes include both neutrophilia, and hyperglycemia, which primes neutrophils for NET formation (24) and provides an increased substrate for NETosis, which is glucose dependent (25).

To date, CVD clinical studies have addressed the role of NETs only in the progression of atherosclerosis. Given that most patients, including those with diabetes, commence primary or secondary CVD prevention (typically lipid-lowering) after the formation of plaques, resolution of atherosclerosis is an important, and largely unmet, clinical goal. The information presented above suggested to us that the failure of lipid-lowering therapies to fully reduce CVD risk in patients with diabetes, or to maximally resolve atherosclerosis in mice and patients with diabetes (reviewed in (26-28)), represented adverse consequences of the increased plaque level of NETs in this metabolic setting (25). This phenomenon would be similar to NETs impairing wound healing in diabetes (24).

Thus, we have investigated the effect of NETs on atherosclerosis resolution in mice with and without diabetes, using deoxyribonuclease 1 (DNase1) as a tool to deplete NETs from plaques (as in (10)). NETs had a direct proinflammatory influence on plaque macrophages, while DNase1-mediated depletion was associated with dampening of macrophage inflammation and improved atherosclerosis resolution after lipid-lowering despite ongoing hyperglycemia. Given that DNase1 has approved applications in other human diseases (e.g., cystic fibrosis), the results also suggest a new mechanistic approach to improving atherosclerosis resolution in high-risk patients with diabetes, which remains an important clinical goal.

Materials and Methods

Animals & Atherosclerosis Regression Study: *Ldlr*^{-/-} mice (Jackson Laboratory) were fed western diet (0.3% cholesterol; Dyets Inc. D101977Gi) for 16 weeks (progression), followed by 4-weeks (regression) on chow diet. Streptozotocin (STZ; Sigma Aldrich, cat. S0130-500MG; 0.05mg/g body weight) or sodium citrate (control) was injected daily for 5 days intraperitoneal (i.p.) just prior to regression to induce diabetes, as described previously (7). During the four-week regression period, DNase1

(62.5µg/mouse Dornase Alfa, Genentech, Inc.) in 0.9% NaCl or 0.9% NaCl only (control) was injected i.p. every other day. At the day of sacrifice, mice were anesthetized via intraperitoneal (i.p.) injection of ketamine (100mg/kg) and xylazine (10mg/kg). Blood was collected via cardiac puncture in EDTA-containing tubes and mice perfused with 10% sucrose in saline solution (0.9% NaCl). Collected aortic roots were embedded in optimal cutting temperature (OCT) compound and frozen at -80°C. All procedures were conducted under NYU Animal Care and Use Committee approved protocol #160725 (Creating glucose-responsive cardiovascular complications in the mouse).

Plasma Lipid and Glucose Measurements: Total cholesterol was measured using Total Cholesterol E Kit (Wako Life Science, cat. NC9138103). Plasma glucose levels were determined using the Contour Blood Glucose Monitoring System (Bayer).

Flow Cytometry: Total white blood cell counts were measured from freshly collected mouse blood using a hematology cell counter (Oxford Science Inc.). Red blood cells were lysed with buffer (Sigma-Aldrich), and cells stained with CD45 Pe/Cy7 (BioLegend, Cat. 103114), CD115 PE (BioLegend, Cat. 135505), and APC Ly-6G/Ly-6C (Gr-1, BioLegend, Cat. 108412). Neutrophils were identified by flow cytometry using LSRII analyzer and analyzed using FlowJo v10.

Atherosclerotic Plaque Assessment: Serial sections (6µm thick) were stained for CD68 (rat anti-mouse CD68, Bio-Rad, Cat. MCA1957), as done before (29). Briefly, sections were fixed and permeabilized with 100% ice-cold acetone, blocked, and stained with a rat anti-mouse CD68 antibody (Bio-Rad, cat. MCA1957) followed by an incubation with a biotinylated rabbit anti-rat IgG secondary antibody (Vector Laboratories, cat. BA4000), and visualized using the Vectastain ABC kit (Fisher Scientific, Cat. NC9313719). Slides were counterstained with hematoxylin/eosin (Sigma), dehydrated in an ethanol gradient and xylene (Fisher Scientific, NH), and mounted with coverslips using Permount (Fisher Scientific). NETs were identified using colocalization immunofluorescence staining of the lymphocyte antigen 6 complex locus G6D (Ly6G; BD Bioscience, cat. 551459), myeloperoxidase (MPO) (Abcam, cat. ab90812), and citrullinated H3 (H3Cit; Abcam, cat. ab5103). Tissue was fixed in

formalin, permeabilized using 0.5% Triton-X, followed by incubation of primary Ab o/n at 4°C and stained with the secondary antibodies for 2h (Thermo Fisher Scientific, cat. A21434 & A21245). The same protocol was used to stain for NLR family pyrin domain-containing protein 3 (NLRP3; Abcam, cat. ab4207) and Caspase-1 (Millipore, cat. AB1871; secondary antibodies: Thermo Fisher Scientific, cat. A-11055 & A-31573). Inducible nitric oxide synthase (iNOS) staining was done on formalin-fixed tissue, permeabilized using 0.05% Tween and 1% Triton-X, followed by incubation with a conjugated iNOS antibody (Abcam, cat. Ab209027) incubated o/n at 4°C. Collagen content was determined by Picosirius Red staining as done before (30). NETs and iNOS stainings were imaged using Hamatsu NanoZoomer 2.0; a Leica SP5Confocal Microscope was used to obtain images of NLRP3 and Caspase1 staining. Collagen was visualized using polarized light microscopy (Zeiss Axio Observer). ImagePro Plus 7.0 software was used to determine plaque, CD68⁺, collagen and necrotic core areas. NET⁺ area, NLRP3⁺, Caspase1⁺, and iNOS⁺ cells were determined by Image J 1.51r. These data were then divided by the plaque area of the section to get the % positive for NETs or CD68. NET⁺ macrophages were calculated by dividing %CD68 by %NETs. NLRP3⁺, Caspase-1⁺ and iNOS⁺ cells were divided by total cells (DAPI nuclear staining) to get %total cells.

Cholesterol crystal images were acquired using an Axioscan microscope (Carl Zeiss, Jena, Germany) using a 10x air objective. Cholesterol crystal images were captured using polarized light and represented as % plaque area.

Laser Capture Microscopy and RNA-seq: CD68⁺ cells were selected from plaques by laser capture microdissection (LCM) as previously described (31), and all LCM procedures were performed under RNase-free conditions. Briefly, aortic root sections were stained with hematoxylin-eosin, and foam cells identified under a microscope and verified by positive CD68 staining. Following LCM, RNA was isolated using the PicoPure Kit (Molecular Devices, Inc., Sunnyvale, CA), treated with DNase and the quality and quantity determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA was amplified (Automated Nugen Ovation Trio Low Input RNA (500pg)), sequenced (HiSeq 4000 Paired-End, Illumina), and sequencing results analyzed using Ingenuity Pathway Analysis (IPA; Qiagen). Cutoffs

of -0.5 to 0.5 log₂ fold change yielded 7337 analyses-ready molecules of which 4046 were down- and 3291 up-regulated in NET+ versus NET- areas. The data discussed in this report have been deposited in NCBI's Gene Expression Omnibus (32) and are accessible through GEO Series accession number GSE145200 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE145200>).

To obtain transcripts of bone-marrow derived macrophages (BMDM), bone marrow cells were isolated by flushing cells from the femurs and tibiae of wild-type C57BL6 mice. Cells were differentiated into BMDMs in 4.5 g/L glucose DMEM (Lonza) with 20% fetal bovine serum (FBS), 1% penicillin/streptomycin, and murine M-CSF (10 ng/mL; #315-02, PeproTech) at 37 °C and 5% CO₂ for 7 days. Cells were lysed with Buffer RLT (Qiagen) containing 10 % betamercaptoethanol and the Norgen Animal Tissue RNA Purification Kit (#25700, Norgen Biotek) was used to isolate RNA from cells. The Dynabeads mRNA Direct Purification Kit (#61011, ThermoFisher Scientific) was used to isolate mRNA transcripts using Poly-A selection, and cDNA synthesis was performed using SuperScript II Reverse Transcriptase (#18064014, ThermoFisher Scientific). Libraries were prepared from 50ng of each cDNA sample using the Nextera DNA library prep kit (#FC-121-1031, Illumina). Samples were sequenced on the Illumina HiSeq 2000 using 50 paired-end reads.

In the case of vascular smooth muscle cells (VSMC), cells were prepared from wild-type C57BL6 mice as in (33), and the RNA isolated and sequenced as described above.

Statistics: Data are expressed as mean $\pm$ SEM. Data were tested for normality and equal variance and analyzed by the appropriate parametric or non-parametric test in GraphPad Prism 7 as stated in each figure. $P \leq 0.05$ was considered significant.

Results

NET formation is associated with a proinflammatory macrophage phenotype

Although NETs have been associated with macrophage activation and potentiating plaque formation (10), their molecular effects on plaque macrophage phenotype *in vivo* have not been directly determined. To address this gap, we collected macrophages

(CD68+ cells) in NET positive (NET+) and NET negative (NET-) atherosclerotic plaque areas (Figure 1A, Supplemental Figure 1A) by laser capture microdissection (LCM) in *Ldlr*^{-/-} mice after 16 weeks of western diet feeding. Collected macrophages provided material for profiling by RNA sequencing to characterize the phenotypical features of macrophages in NET+ areas in atherosclerotic plaques (Figure 1B, C).

Consistent with a proinflammatory phenotype, glycolysis and inflammasome pathways were enriched in macrophages isolated from NET+ areas relative to those in NET- regions (Figure 1D, E). Confirmation of inflammasome activation was achieved by immunohistochemical staining for NLRP3 and Caspase-1, and quantification of their localization in NET+ versus NET- macrophage regions (Figure 1F). Inflammasome activation was found to be significantly associated with macrophages present in NET+ areas ($P = 0.03$, Figure 1G). In addition to the enrichment in inflammasome-related genes in the NET+ areas, the enrichment of glycolytic markers also indicates a switch to a proinflammatory M1-like macrophage phenotype (34). Indeed, based on standard markers, the CD68+ cells from NET+ vs. NET- areas were enriched in M1 (3.6-fold increased, *inos*), and suppression of M2 (2-fold decrease (*Cd206*)) characteristics. Further, immunofluorescence staining of iNOS confirmed increased proinflammatory macrophages in NET+ plaque areas ($P=0.055$, Figure 1H).

Figure 1. Neutrophil extracellular traps (NETs) in atherosclerotic plaques skew macrophages to a proinflammatory phenotype.

(A) Representative atherosclerotic plaque section stained for neutrophil extracellular traps (NETs) as determined by colocalization of myeloperoxidase (MPO), the lymphocyte antigen 6 complex locus G6D (Ly6G), citrullinated histone H3 (H3Cit) and macrophages (CD68). CD68⁺ cells were collected via laser capture microdissection (LCM) in NET positive (NET⁺) and NET negative (NET⁻) areas and sequenced. Scale bar 100 μ m. (B) Heatmap and (C) volcano plot showing differential gene expression in NET⁺ versus NET⁻ macrophages. (D) Upregulation of the glycolysis and (E) inflammasome pathways in NET⁺ compared to NET⁻ macrophages determined using Ingenuity Pathway Analysis. (F) Immunofluorescence staining and (G) quantification confirming the inflammasome pathway activation (NLR family pyrin domain containing protein 3 (NLRP3⁺), caspase1⁺) in NETs⁺ area (bottom), scale bar 50 μ m. (H) Quantification of iNOS immunofluorescence staining showing an increase in iNOS in NET⁺ versus NET⁻ Area (%DAPI). N=3-5/group, *P<0.05 using unpaired t-test.

To exclude the possibility that the RNA from CD68⁺ cells selected from NET⁺ areas was contaminated by neutrophil RNA, we compared the reads for CD68 to that of the neutrophil markers *Ly6g*, *Mpo* and *neutrophil elastase* (*Ne*). Counts for Cd68 were 7500-fold greater than that of neutrophil transcripts, confirming the specificity of our approach (Supplemental Figure 1B). Additionally, to exclude the possibility of significant contamination with smooth muscle cells (SMC), which can take on characteristics of macrophages when cholesterol-loaded *in vitro* (35) or in atherosclerotic plaques (36-38), we compared the transcript expression of *Cd68* to the myeloid-specific transcript *Ptpc* (*Cd45*) and the smooth muscle cell markers myosin heavy chain-11 (*Myh11*) and calponin 1 (*Cnn1*) using the expression values from RNA seq data obtained in the present study, and in primary murine bone marrow-derived macrophage (BMDMs) and murine vascular SMCs. As shown in Supplemental Figure 1C, the *Cd45/Cd68* ratio was comparable between the laser captured CD68⁺ cells and BMDMs, whereas *Cd45* was undetectable in murine vascular SMCs. Further, compared to laser captured CD68⁺ cells, the *Myh11/Cd68* and *Cnn1/CD68* ratios are approximately 12-fold higher in vascular SMCs. Overall, these data show that the data in Figure 1 are not confounded to a significant extent by contamination by other cell types and provide further support that NETs skew macrophages towards a proinflammatory phenotype in atherosclerotic plaques.

Clearing NETs can overcome impaired atherosclerosis resolution in diabetic mice

Neutrophils isolated from diabetic mice and humans are more prone to form NETs, with NET formation leading to impaired wound healing (24). We have also shown that even with lipid-lowering, maximal atherosclerosis resolution requires reduced macrophage inflammation (29). Thus, we hypothesized that the expected increase in plaque NETs in diabetic mice would impair atherosclerosis resolution due to their proinflammatory effects on macrophages (Figure 1), and that resolution in diabetic mice would improve by reducing NETs.

To test this, *Ldlr*^{-/-} mice were fed a Western diet for 16 weeks to generate advanced atherosclerotic lesions, and atherosclerosis resolution was induced, as in a previous study (5), by feeding the mice a chow diet for four weeks, which resulted in decreased plasma lipid levels (Figure 2A; Table 1). A subset of mice was made diabetic by

injection of streptozotocin. Since NETs are predominantly degraded by exonucleases (39), mice received DNase1 or vehicle injections every other day during the resolution period. In total, we compared baseline (mice sacrificed after 16 weeks on a western diet) and four regression groups: control, control + DNase1, STZ-induced diabetes, and diabetes + DNase1.

Table 1. Total plasma cholesterol and blood glucose levels

	Cholesterol (mg/dL)	Blood Glucose (mg/dL)
Baseline	1000 ± 327.7	108.6 ± 32.3°
Regression	221.6 ± 71.1*	123.5 ± 31.3°
Regression + DNase1	216.4 ± 28.5*	175.0 ± 26.7°
Diabetes	325.1 ± 83.4*	450.5 ± 79.5
Diabetes + DNase1	323.3 ± 96.4*	453.8 ± 99.1

Data shown as mean ± SD, n=5-13/group, 1-way ANOVA, *P<0.0001 compared to Baseline; ° P<0.0001 compared to Diabetes

Neither STZ nor DNase1 affected total plasma cholesterol levels in the four resolution groups, with the levels in each significantly reduced when compared to baseline mice (Table 1). Plasma glucose levels were increased in the STZ groups only (Table 1). To evaluate NET content, we performed standard NET staining (MPO, H3Cit and the neutrophil marker Ly6G; Figure 2B) and quantified NETs as % plaque area (Figure 2C). Strikingly, in the non-diabetic mice, we observed a decrease in total plaque NETs area in the control and DNase1 resolution groups versus baseline (5.7% vs. ~1.5% of plaque area), showing that NETs can resolve spontaneously in a non-hyperlipidemic environment. As expected, NETs did not resolve spontaneously in hyperglycemic conditions, but, notably, we observed a substantial decrease in NET content in the diabetes + DNase1 group (7.0% vs. 2.3% of plaque area, or a 68% reduction).

Figure 2. DNase1 treatment promotes atherosclerosis regression in diabetic mice.

(A) Male *Ldlr*^{-/-} mice were fed a western diet for 16 weeks to develop baseline plaques. At week 15 a subset of mice received streptozocin injections to induce diabetes. At week 16 a subset of mice was harvested for baseline measures, and all other mice were switched to a chow diet to induce resolution of plaques. These mice were then split into 2 groups, and over a 4-week period received either DNase1 (62.5ug/mouse) or vehicle injections every other day. At week 20, mouse tissues were harvested for plaque analyses. (B) NETs staining and (C) quantification in aortic roots, as determined by composite staining of myeloperoxidase (MPO), the lymphocyte antigen 6 complex locus G6D (Ly6G) and citrullinated histone H3 (H3Cit); scale bar 100 μ m. (D) Representative pictures and (E) quantification of CD68 staining as a marker of plaque macrophage content. Scale bar 100 μ m. Data are shown as mean $\pm$ SEM, N=7-12/group *P<0.05, 1-way ANOVA with Tukey's multiple comparison test.

To determine if NETs content correlated negatively with atherosclerosis resolution, CD68+ macrophages were quantified and represented as the percentage of plaque area (%CD68; Figure 2D-E). As expected, we found a significant decrease in lesion %CD68 in control mice compared to the baseline group (43.6 % vs. 24.1 %). Atherosclerosis resolution was unchanged in control mice receiving DNase1, consistent with the spontaneous loss of NETs in those mice. In diabetic mice, the reduction of %CD68 immunostaining was impaired (43.6 % vs. 35.2 %), as expected (3). Notably, DNase1 treatment in diabetic mice showed significantly enhanced atherosclerosis resolution (35.2 % vs. 23.0 %), which was comparable to that of control mice.

To determine the relationships among the relative contributions to plaque characteristics by macrophages (above), extracellular matrix (as assessed by collagen) and necrotic core, additional plaque analyses were performed. As shown in Figure 3A, there was a significant reduction in plaque size in all of the resolution groups (Figure 3A) compared to baseline. Collagen content tended to increase relative to baseline, with statistical significance achieved in the resolution control group (Figure 3B). The necrotic core plaque areas were found to be similar in the baseline and the diabetes resolution groups, and significantly lower in the other resolution groups, including DNase1 treated diabetic mice (Figure 3C).

Figure 3. Effects of DNase1 treatment on plaque size, collagen content, and necrotic area in atherosclerotic plaques.

More analyses of mice described in Figure 2: Quantification of (A) plaque size, (B) Sirius red staining to determine collagen content and (C) plaque necrotic area. Data are shown as mean $\pm$ SEM, N=4-12/group *P<0.05, ** P<0.005, *** P<0.001 1-way ANOVA with Dunnett's multiple comparison test compared to the baseline (A,B) or diabetes (C) group.

DNase1 treatment reduces NET-induced plaque macrophage inflammation and promotes atherosclerosis resolution in diabetic mice

The results in Figure 2 showed DNase1 treatment was able to overcome the impairment of atherosclerosis resolution by diabetes. Because a prominent feature of macrophages in NET+ areas is the induction of the inflammasome and other metabolic and transcriptomic changes characteristic of inflammation (Figure 1), we hypothesized that the benefits of NET clearance in diabetic mice (Figure 2) were associated with a reduction in the inflammatory phenotype of plaque macrophages. Consistent with this, the percentage of macrophages associated with plaque NETs was significantly decreased by 90%, from 2.7 to 0.3% in the DNase1 treated diabetic mice compared to diabetic mice with no treatment (Figure 4A-B). To support this hypothesis more directly, we performed immunofluorescence staining for the inflammasome markers NLRP3 and Caspase-1 (Figure 4C). Indeed, NLRP3 and Caspase-1 positive cells were significantly increased in diabetic mice, compared to the control group, and decreased with DNase1 treatment. Additionally, the increase in plaques of diabetic mice of the inflammatory macrophage marker iNOS was reversed with DNase1 treatment (Figure 4D). As we previously reported (6, 7), there was a significant increase in circulating neutrophils in diabetes. There was also a trend towards increased plaque neutrophils (Supplemental Figure 2A, B), with a suggestion that DNase1 reduced both increases.

Figure 4. DNase1 treatment promotes atherosclerosis resolution in diabetic mice by reducing NETs-induced plaque macrophage inflammation.

More analyses of mice described in Figure 2: **(A)** Overlapping NET (*left panel*) and CD68 staining (*right panel*) in diabetic mice versus diabetic mice treated with DNase1, scale bar 50 μ m. **(B)** Amount of macrophages (%) co-localized with NETs. **(C)** Quantification of NLRP3⁺, caspase-1⁺ cells, and, **(D)** iNos⁺ cells, all as % DAPI staining of plaques. Data are shown as mean $\pm$ SEM, N=3-5/group, *P<0.05, 1-Way ANOVA with Tukey's multiple comparison test.

Cholesterol crystals have been shown to be an inflammasome activator in atherosclerosis (40). Thus, we examined whether they were associated with the macrophage inflammation we observed and if DNase1 treatment affected their plaque content. Cholesterol crystals in atherosclerotic plaques were detected using polarized light (Figure 5A). As shown in Figure 5B, in the resolution groups, the cholesterol crystal content was ~70% greater (P = 0.01) in the diabetes (2.2%) vs. control mice (1.3%). Interestingly, cholesterol crystals tended to be decreased in the diabetes + DNase1 compared to diabetes group (P = 0.06).

Figure 5. Effects of diabetes and DNase1 treatment on cholesterol crystal content in resolving plaques.

More analyses of mice described in Figure 2: **(A)** Cholesterol crystal visualization (white), and **(B)** quantification (as % plaque area) in atherosclerotic plaques with DAPI (blue) as counterstain; scale bar 100 μ m. Data are shown as mean $\pm$ SEM, N=5-8/group, *P<0.05, 1-Way ANOVA with Tukey's multiple comparison test.

Discussion

NETs are found in human and mouse plaques, and in mice are associated with increased atherosclerosis progression (10-12). They have also been shown to impair wound healing in diabetic mice (24). Though considered to be generally inflammatory, how NETs contribute to macrophage activation is incompletely understood (17). The present study addresses this gap in knowledge. By isolation of macrophages from NET positive and negative plaque areas, we identify that NETs induce a proinflammatory "M1-like" macrophage phenotype, as evidenced at the molecular level by enriched transcripts characteristic of glycolysis and inflammasome activation and at the protein level by increases in relevant immunohistochemical markers. We also detected enhanced NET presence in diabetic plaques, consistent with a study in non-atherosclerotic mice that diabetes primes neutrophils for NET production (24).

The NET-macrophage interaction is a likely contributing factor to persistent macrophage inflammation in the plaques of diabetic mice even after lipid-lowering. This is supported by our current study, which finds a reduction in plaque inflammation and proinflammatory macrophages in lesions of diabetic mice following the degradation of

NETs with DNase1 in conjunction with lipid-lowering. Further, we provide evidence that the resolution of plaque NETs in diabetic mice promotes a more stable appearance, as noted by a decrease in plaque necrotic area following DNase1 treatment. These data are consistent with the association between NET formation and human plaque instability (12, 41).

DNase1 reduces NETs by degradation of the chromatin fibers that comprise their backbone (14), which usually, but not always, reduces inflammation and disease severity in several models (42). Based on the results in the present study, we can add the resolution of atherosclerosis in diabetes to the list of conditions that are improved by reducing NET content. In addition to the direct effects in plaques by the reduction of NETs (e.g., reduced inflammasome activation in plaque macrophages), there are probably indirect benefits as well. For example, inflammation in plaques also increases neutrophil recruitment, further amplifying inflammation (43). This reasoning likely provides the logical link between the reduction in plaque inflammation in diabetic mice (Figure 4) with the data suggesting that the increased circulating and plaque neutrophil levels in the diabetic mice were partially suppressed by DNase1 treatment (Supplemental Figure 2).

The present results showing a powerful effect of NETs on macrophage inflammasome activation led us to consider this finding in the context of another mediator of inflammasome activation in atherosclerosis, namely cholesterol crystals (40). The current understanding is that the inflammasome pathway requires two signals for full activation. Warnatsch et al. (10) have shown that NETs are a priming cue, with the second signal being cholesterol crystals (9). Furthermore, crystal formation and inflammasome activation were recently shown by Westerterp et al. to be promoted by reduced cholesterol efflux via ATP-binding cassette transporter A1/G1 (ABCA1/ABCG1) (44). Additionally, cholesterol crystals are not only the second signal for inflammasome activation, but they also stimulate NETosis (10).

As shown in Figure 5, cholesterol crystals were increased in diabetic mice relative to the other resolution groups, which is consistent with hyperglycemia reducing cholesterol efflux in macrophages and their precursors by decreasing ABCA1/ABCG1

expression (e.g., (6, 7)). Taken together, our and the published data imply that cholesterol crystals could play a dual role in atherosclerosis, namely to promote NETosis, which provides multiple priming cues (first signals) for macrophage inflammasome activation, and also to serve as the second signal to complete the activation process in the primed macrophages. DNase1 treatment, by clearance of NETs, would reduce both types of macrophage inflammasome signals. This results in less macrophage inflammation, which we have shown is required to promote maximal atherosclerosis resolution after lipid-lowering (29).

In summary, we have shown for the first time the molecular impact of NETs on plaque macrophages, especially on their inflammasome and glycolytic pathways. We also show that NETs formed during atherosclerosis progression in non-diabetic mice can spontaneously resolve upon the correction of hyperlipidemia. In contrast, they persist in the setting of diabetes and impair atherosclerosis resolution, likely due to their activation of macrophages and exacerbation of the general inflammatory environment in the plaque. Notably, DNase1 was an effective treatment to improve atherosclerosis resolution in the face of ongoing hyperglycemia. Because the rate of CV disease remains higher in people with versus without diabetes after similar lipid-lowering, the results suggest not only a basis for this but also the therapeutic potential of NET reduction, especially in those whose diabetes is not well controlled.

Author Contributions

T.J., T.J.B. and E.A.F. designed experiments. T.J. and T.J.B performed the experiments and analyses. E.B. assisted with the RNA seq analyses. A.Q. helped with the analysis performed in Figure 2. X.W. and J.A. performed cholesterol crystal visualization and quantification. M.V. added BMDM sequencing data. T.J., T.J.B., J.A., E.A.F wrote and edited the manuscript.

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Conflicts of Interest

Based on a review of the criteria of JCI Insight, the only author with a disclosure is Edward A. Fisher, who reports receiving income >\$10,000 from being an expert witness on behalf of Amgen (PCSK9 antibodies) and Dr. Reddy's Laboratories (eicosapentaenoic acid).

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Supplemental

Supplemental Figure 1

(A) Atherosclerotic plaque section stained for neutrophil extracellular traps (NETs) as determined by colocalization of MPO (left), Ly6G (middle), H3Cit (right). **(B)** Macrophage (*Cd68*) and neutrophil (*Ly6g*, *Mpo*, neutrophil elastase (*Ne*)) markers detected with RNA sequencing (counts/million). **(C)** Expression of macrophage (*Cd45*) and smooth muscle cell (Myosin Heavy Chain 11, *Myh11*; Calponin 1, *Cnn1*) transcripts in isolated LCM macrophages, murine bone marrow-derived macrophages (BMDM)s and murine vascular smooth muscle cells (SMC)s, as determined by RNAseq and represented as transcript/CD68 ratio. ** $P < 0.005$, **** $P < 0.0001$ 1-way ANOVA with Dunnett's multiple comparison test, $n = 3$ /cell type.

Supplemental Figure 2

(A) Circulating neutrophil counts and **(B)** plaque neutrophil contents. Data are shown as mean $\pm$ SEM, N=4-15/group *P<0.05, *** P<0.001 1-way ANOVA with Dunnett's multiple comparison test.

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Loss of DNase1, DNase1L3 or both does not significantly change atherosclerotic neutrophil extracellular traps (NETs) content, or affect atherosclerosis

Tatjana Josefs, Tarik Zahr, Lee Serpas, Kristiaan Wouters, Boris Reizis, Edward A. Fisher

Pilot Study

Abstract

Rationale: Neutrophil extracellular traps (NETs) are a pro-inflammatory component of atherosclerotic plaques. NETs can be cleared by endogenous endonucleases. Until now, it remains unclear whether the endonucleases deoxyribonuclease 1 (DNase1) and DNase1 Like 3 (DNase1L3) affect NET formation in atherosclerotic plaques and influence plaque progression and regression. We hypothesized that 1) single-deficiency of these DNases increases NET formation, with the most prominent effects in the double-deficient mice, and, 2) the increase in NETs enhances atherosclerosis progression and impairs regression.

Objective: To investigate the role of DNase1 and DNase1L3 in atherosclerosis progression and regression

Approach & Results: After the progression period of 20 weeks on western diet, plasma cholesterol levels were relatively low (~600mg/dL), especially in the *DNase1L3*^{-/-} mice (~470mg/dL). Compared to control (1.5 ± 1.0 % plaque area) animals, NET formation tended to be increased in the double-deficient mice (3.3 ± 1.4 % plaque area) and was unaffected in *DNase1*^{-/-} mice (0.7 ± 0.3 % plaque area). NET formation was absent in *DNase1L3*^{-/-} mice (0.0 ± 0.0 % plaque area). After the regression period of 4 weeks on chow diet, plasma cholesterol dropped to ~100mg/dL without affecting NET area and plaque size. Macrophage content (%plaque area) significantly decreased during regression, but we did not find differences between single- or double-deficient mice compared to control.

Conclusion: Loss of DNase1, DNase1L3 or both does not significantly change NET content, worsen atherosclerosis progression or impairs regression. Notably, NET formation was limited likely due to rather low cholesterol level leading to insignificant results. Future studies need to be performed using a more severe atherosclerosis model and higher n.

Introduction

Two major endonucleases are present in the circulation and are members of the deoxyribonuclease 1 (DNase1) protein family, *i.e.* DNase1 and DNase1 Like 3 (DNase1L3). DNase1 is mainly expressed by exocrine cells of salivary glands, kidney and gut.¹ DNase1L3 is expressed in immune cells, specifically in dendritic cells and macrophages, but also in Kupffer and gastrointestinal cells.^{2,3} DNases are critical for DNA degradation and are required for healthy development and function. The role of these endonucleases in autoimmune and inflammatory diseases has been intensively studied, given their ability to degrade neutrophil extracellular traps (NETs).

NETs are web-like structures expelled by neutrophils upon activation by pathogens or sterile inflammatory signals (e.g. autoantibodies, oxLDL).⁴⁻⁷ These NETs consist of cytosolic and nuclear material including histones, neutrophil elastase (NE), myeloperoxidase (MPO), proteinase 3 (PR3), cathepsin and gelatinase and are part of the hosts' immune defense.⁴ However, NETs are implicated in several noninfectious diseases, such as Lupus or atherosclerosis and therefore, clearance by endonucleases might prevent inadvertent effects of prolonged NETs exposure. *In vitro* experiments showed that DNase1 or DNase1L3 can degrade NETs.^{8,9} Given the NETs composition, DNase1 seems to be more likely the main endonuclease for NET degradation as DNase1 only works in combination with proteases (e.g. neutrophil elastase), while DNase1L3 is inhibited by proteases. Additionally, DNase1 preferentially cleaves free DNA, while DNase1L3 targets DNA-protein complexes, e.g. nucleosomes. In line, DNase1 has been reported to be important for NET degradation in lupus erythematosus (SLE) patients and is linked to its pathogenesis.⁹ Further, mutations in DNase1, leading to decreased DNase1 activity in the circulation, are associated with SLE severity.^{10,11}

Next to autoimmune diseases, increasing evidence showed that NETs also play a pro-inflammatory role in atherosclerosis.^{12,13} Atherosclerosis is a lipid-driven chronic inflammatory disease and the underlying pathology of cardiovascular diseases (CVDs) causing the most deaths worldwide. Administering DNase1 in atherosclerotic *apoE*^{-/-} mice fed a high-fat¹⁴ or a high cholesterol¹⁵ diet was reported to lower atherosclerosis plaque size. In contrast, another study showed no benefit of DNase1 administration on atherosclerosis progression in *apoE*^{-/-} mice fed a high-fat diet for 4 months.¹⁶ In a recent single-cell RNA seq study of atherosclerotic macrophages, Dnase1L3 was

present in progressing, but not regressing plaques. Unexpectedly, DNase1L3 was associated with a proinflammatory interferon response, suggesting a harmful role of DNase1L3 in atherosclerosis¹⁷, instead of the anti-inflammatory role via clearing NETs. However, a study by Jiménez-Alcázar et al. using *DNase1*^{-/-} and *DNase1L3*^{-/-} single as well as double KO (DKO) mice showed that only the double-deficiency led to vascular occlusion while both single KO were able to prevent it.¹⁸ This was in line with increased NET formation in the double compared to the single KO, indicating a detrimental role for NETs in atherothrombotic disease and further, shows that DNase1 as well as DNase1L3 can clear NETs.

Current treatment of atherosclerosis involves lipid-lowering therapy, but is only preventing a fraction of recurrent events in survivors of myocardial infarction.^{19,20} It is thought that inflammation resolution is needed to get the most benefit of lipid-lowering therapies.^{21,22} Hence, degradation of NETs using endonucleases is a potential therapeutic target to decrease inflammation and thereby overcome the residual risk of CVD after lipid-lowering therapy. DNase1 is clinically already used to treat cystic fibrosis²³ and shows no toxicity, and thus represents a possible therapeutic approach. However, as noted above, current published results on DNases in atherosclerosis are controversial and still scant, and further research is needed to elucidate the role of the two endonucleases in atherosclerosis.

To assess which endonuclease plays the main role in clearing NETs in atherosclerotic plaques, we used three different knock-outs – DNase1, DNase1L3 and a DKO. We hypothesized that knocking out either endonuclease would increase NET formation, with the most prominent effect in the double-deficient mice, and thereby enhance atherosclerosis progression and impair regression. Surprisingly, we did not find differences in NET content between the sufficient and single-deficiency models, but detected a trend towards increased NET formation in the double-deficient model. In line with no significant differences in NET content, loss of DNase1, DNase1L3 or both did not lead to increased progression or impaired regression measured as plaque area and macrophage content. Notably, NET formation was limited likely due to rather low cholesterol levels leading to insignificant results. These outcomes suggest that a stronger or an additional stimulus, such as higher cholesterol levels than we achieved or hyperglycemia, is needed for enhanced NET formation leading to adverse effects on atherosclerosis. Nonetheless, this pilot-study gives first hints on the role of different

endonucleases in atherosclerosis and an increase in n is needed to draw final conclusions.

Materials and Methods

Animals and Atherosclerosis Studies: The *Dnase1L3*^{-/-} mouse model on C57BL/6 (B6) background [*Dnase1L3*^{LacZ/LacZ}] has been described previously.²⁴ Mice carrying a targeted allele of *Dnase1* (*Dnase1*^{tm1.1(KOMP)Vlcr}) on B6 background were obtained from the Knockout Mouse Project and crossed with WT B6 and *Dnase1L3*^{-/-} mice to obtain *Dnase1*^{-/-} and *Dnase1*^{-/-}/*Dnase1L3*^{-/-} mice, respectively. C57BL/6J (WT; Jackson Laboratory, stock No: 000664).

Mice of both gender were injected with a gain of function mutant of proprotein convertase subtilisin/kexin type 9 (PCSK9)-encoding adeno-associated vector (AAVmPCSK9; AAV.8TBGmPCSK9D377Y) as done before²⁵ and fed a western diet (WD; 0.3% cholesterol, Dyets Inc. D101977Gi) for 20 weeks (progression). To induce regression, mice were injected with apolipoprotein B antisense oligo nucleotides (ApoB ASO, Ionis Pharmaceuticals) and fed a chow diet for 4 weeks. AAV PCSK9 was injected 1x at a concentration of 1*10¹² viral particles/mouse. ApoB ASO was injected 1x at a concentration of 50µg/g body weight (BW).

At day of sacrifice, mice were anesthetized via intraperitoneal (i.p.) injection of ketamine (100mg/kg) and xylazine (10mg/kg). Blood was collected via cardiac puncture in EDTA-containing tubes and mice perfused with 10% sucrose in saline solution (0.9% NaCl). Collected aortic roots were embedded in optimal cutting temperature (OCT) compound and frozen at -80°C.

Plasma Lipid Measurement: Total Cholesterol was measured using Total Cholesterol E Kit (Wako Life Science, cat # NC9138103).

Atherosclerotic plaque assessment: Serial sections (6µm thick) of OCT embedded roots were stained for CD68 (rat anti-mouse CD68, Bio-Rad, Cat. MCA1957) as done before²⁶. Briefly, sections were fixed and permeabilized with 100% ice-cold acetone, blocked, and stained with anti-CD68 antibody followed by an incubation with biotinylated anti-rat IgG secondary antibody (Vector Laboratories, CA), and visualized using the Vectastain ABC kit (Vector Laboratories). Slides were counterstained with

hematoxylin/eosin (Sigma), dehydrated in an ethanol gradient and xylene (Fisher Scientific, NH), and mounted with coverslips using Permount (Fisher Scientific). NETs were identified using colocalization immunofluorescence staining of Ly6G (BD Bioscience, Cat.551459), MPO (Abcam, Cat. ab90812), and citrullinated H3 (H3Cit; Abcam, Cat. ab5103). Tissue was fixed in Formalin, permeabilized using 0.5% Triton-X, followed by incubation of primary Ab o/n at 4°C and stained with the secondary antibodies for 2h (Thermo Fisher Scientific, Cat. A21434 & A21245). Stained lesions were imaged (Nikon Eclipse) and lesion area and CD68⁺ areas determined using ImagePro Plus software (Micro Optical Solutions). NETs staining was imaged using Hamatsu NanoZoomer 2.0 and NET⁺ area determined using ImageJ 1.51r.

Statistics: Data are expressed as mean $\pm$ SEM. Data were tested on normality and equal variance and analyzed by appropriate parametric or non-parametric test as stated in each figure, $P \leq 0.05$ was considered significant (GraphPad Prism 7).

No gender differences were observed.

Results and Discussion

Loss of DNase1, DNase1L3 or both does not significantly change atherosclerotic NET content

DNase1 has been shown to efficiently clear NETs and its activity inversely associates with SLE severity⁹⁻¹¹, but how DNases affect atherosclerosis still needs to be determined. *DNase1^{-/-}/DNase1L3^{-/-}* mice have been shown to have a higher NET content compared to single-deficient models in atherothrombosis¹⁸, but no data on atherosclerosis are available. To address this gap, we used WT (control), *DNase1^{-/-}*, *DNase1L3^{-/-}*, and *DNase1^{-/-}/DNase1L3^{-/-}* mice injected with 10^{12} viral particles/mouse PCSK9 to increase plasma cholesterol levels (Study Setup: Figure 1A). After an atherosclerosis progression period for 20 weeks on western diet (WD, 0.3% cholesterol), we induced atherosclerosis regression by injecting 50 μ g/g (of body weight; BW) apoB ASO and placed the mice on chow diet for 4 weeks. BW did not differ between groups and compared to control, neither during progression or regression (Figure 1B, Supplemental Table1). Cholesterol levels during atherosclerosis progression were lower in the *DNase1L3^{-/-}* mice (468.2 ± 39.61 mg/dL)

compared to the other groups (WT 638.2 ± 51.5 , *DNase1*^{-/-} 721.3 ± 87.2 , *DNase1*^{-/-}/*DNase1L3*^{-/-} 618.1 ± 64.8 ; mg/dL). We saw the same pattern during regression (WT 115.8 ± 20.0 , *DNase1*^{-/-} 114.1 ± 18.7 , *DNase1L3*^{-/-} 50.4 ± 6.8 , *DNase1*^{-/-}/*DNase1L3*^{-/-} 101.8 ± 11.7 ; mg/dL).

To evaluate NET content, we performed NETs staining (MPO, Ly6G, H3Cit; Figure 1D) and quantified NETs as % of total plaque area (Figure 1E). NETs did not significantly differ between groups, neither during progression or regression. *DNase1L3* deficient mice did not develop NETs during progression, while *DNase1* deficient mice (0.7 ± 0.3 % plaque area) as well as the double-deficient mice (3.3 ± 1.4 % plaque area) did. At first glance, these results could be interpreted in the sense that *DNase1* dominates over *DNase1L3* in clearing NETs and that *DNase1L3* might even promote NET formation. However, control mice expressing both endonucleases - *DNase1* and *DNase1L3* - had the same amount of NETs as *DNase1* deficient mice. With *DNase1* clearing NETs and *DNase1L3* promoting NETs, we would have expected to see decreased NET formation in control compared to *DNase1* deficient mice. Further, the trend towards increased NET formation in the double-deficient model compared to control (3.3 ± 1.4 versus 1.5 ± 1.0 % plaque area), suggests that both DNases can clear NETs. This is in contrast to *DNase1L3* promoting NET formation and would assume NET formation to be higher than 0 in *DNase1L3*^{-/-} mice. Overall, interpretation of the data should be done with caution, as our study has rather low cholesterol levels (WT ~600mg/dL), especially in the *DNase1L3*^{-/-} mice (~470 mg/dL). This was unexpected since we did observe higher cholesterol levels (~1200mg/dL) using PCSK9 before²⁵, and could be the result of a change in animal facility and staff. Therefore, the lack of NETs in *DNase1L3*^{-/-} mice could be due the relatively low cholesterol levels rather than the higher efficiency of *DNase1* or *DNase1L3* promoting NETs. The less severe mouse model might have also led to not being able to recapitulate the NETs resolving effect during regression as shown before using *Ldlr*^{-/-} mice on WD for 16 weeks. The NET content after the progression period in those two studies differed by ~4-fold (WT + AAV PCSK9 20wks WD: 1.4%plaque area; *Ldlr*^{-/-} 16wks WD: 5.7%plaque area). Hence, NET formation was likely not high enough to measure differences in NET content between progression and regression. In contrast, another study showed NET formation <1% in *Ldlr*^{-/-} mice after 12 weeks on WD

despite cholesterol levels $\sim 1000\text{mg/dL}$, suggesting that NETs formation is independent of cholesterol levels.²⁷

Our results propose that neither endonuclease – DNase1 nor DNase1L3 – dominates over the other in clearing NETs in atherosclerotic plaques. Further, enhanced NET formation and stronger differences might have been observed with a more severe atherosclerosis model and increased n numbers are necessary for definitive determination.

Figure 1. Loss of DNase1, DNase1L3 or both does not significantly change atherosclerotic NET content

(A) WT, $DNase1^{-/-}$, $DNase1L3^{-/-}$, $DNase1^{-/-}/DNase1L3^{-/-}$ were injected with PCSK9 and fed a western diet for 20 weeks to develop plaques. At week 20, a subset of mice was harvested for the progression group, all other mice received 1x ApoB ASO injection and were fed a chow diet for 4 weeks to induce plaque regression. (B) Body weight (g) and (C) Plasma Cholesterol Levels (mg/dL) at time of harvest. (D) NETs staining (scale bar 100μm) and (E) quantification in aortic roots as

determined by composite staining of myeloperoxidase (MPO), Ly6G and citrullinated Histone 3 (H3Cit). Data is presented as mean $\pm$ SEM, N=4-6/group *P<0.05, 1-way ANOVA with Tukey's multiple comparison test (C), Kruskal-Wallis test (E).

Loss of DNase1, DNase1L3 or both does not affect atherosclerosis

To determine if NET content and the slight increase in the DKO mice had an impact on atherosclerosis, we measured plaque area (μm^2 , Figure 2A-B) and macrophage content represented as percentage of plaque area (%CD68; Figure 2C). Plaque area was similar in each group during progression and did not decrease during regression (Figure 2B). Plaque area in *DNase1L3^{-/-}* mice was significantly lower compared to control and *DNase1^{-/-}* mice, likely due to lower cholesterol levels (Figure 1C). As expected, macrophage content (%CD68) significantly decreased during atherosclerosis regression (Figure 2C). We did not observe significant differences between groups, but a trend towards impaired regression in the DKO (~49% reduction) compared to the single KO (~60% *DNase1^{-/-}*, ~80% *DNase1L3^{-/-}*), which is in line with NET content. However, the control group also showed a ~44% reduction in macrophage content and we did not observe a correlation between %CD68 and NETs (data not shown).

Figure 2. Loss of DNase1, DNase1L3 or both does not affect atherosclerosis

(A) Representative pictures and quantification of (B) plaque area (μ m²) and (C) CD68⁺ area (%plaque area). Scale bar 100 μ m. Data is shown as mean $\pm$ SEM, N=5-6/group *P<0.05, 1-way ANOVA with Tukey's multiple comparison test.

In conclusion, this pilot study shows that loss of DNase1, DNase1L3 or both does not significantly change NET content, worsen atherosclerosis progression or impair atherosclerosis regression. The trend towards higher NET formation in the double-deficient model suggests that with a stronger stimulus, atherosclerosis will be more likely affected compared to the fully sufficient and single-deficient models. To confirm the results of our study, n number should be increased. Further, adding an additional stimulus, such as hyperglycemia, which is known to increase NET formation and impair atherosclerosis regression, is of interest.

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Supplemental**Supplemental Table I.** Body weight (g) at harvest

	WT	<i>DNase1</i> ^{-/-}	<i>DNase1L3</i> ^{-/-}	<i>DNase1</i> ^{-/-} / <i>DNase1L3</i> ^{-/-}
Progression	30.5 ± 4.8	25.3 ± 0.7	31.0 ± 2.3	27.7 ± 2.0
Regression	28.8 ± 2.5	29.2 ± 1.8	28.5 ± 1.6	27.9 ± 1.5

7

Summary & Discussion

The main goal of this thesis was to investigate two different aspects of the 2 to 4-fold increased CVD risk in individuals with diabetes. Diabetes is an independent CVD risk factor, even after lipid-lowering therapy and further, impairs regression of atherosclerosis. A study by Nicholls *et al* in 2008 showed that lowering LDL-C to desired levels of <80mg/dL in individuals with no diabetes promotes atherosclerosis regression in almost 25% of them, measured as reduction in lesion size by intravascular ultrasound (Figure 1).¹ In individuals with diabetes, lowering LDL-C levels to <80mg/dL did only promote regression in ~17% individuals. Strikingly, the number of individuals with regression in non-diabetics with elevated plasma LDL-C and diabetics with desired LDL-C is similar. Our lab has been able to replicate findings consistent with these in mice, showing that diabetic mice have about 50% the regression of non-diabetic mice.²

Figure 1. Comparison of atherosclerosis regression with and without diabetes in humans and mice. Human data (*left*) are based on the results published by Nicholls *et al*.¹ Mouse data (*right*) are based on our published study by Parathath *et al*.²

In this thesis, two probable causes for residual CVD risk, which are involved in high risk populations such as patients with diabetes, have been discussed:

- Effect of elevated plasma TG levels and low HDL (**Chapters 2-4**)
- Effect of hyperglycemia-associated circulating neutrophils and NET formation (**Chapters 5 & 6**)

Triglycerides and their role in atherosclerosis

Main findings

Chapter 2 describes an inverse association of HDL characteristics, such as HDL-C, apo-AI, HDL size and HDL-P, with pre-clinical atherosclerosis and CVD/CVE in a human cohort. Surprisingly, CEC was not associated with atherosclerosis surrogates or CVD/CVE. Stratifying the population in subjects with NGM and (pre)diabetes, revealed that associations of HDL-size and HDL-P with prevalence of CVD are lost. **Chapter 3** shows that the role of LpL in macrophage polarization *in vitro* does not resemble the *in vivo* situation. While LpL regulates lipid uptake, as well as pro- and anti-inflammatory gene expression in macrophages *in vitro*, myeloid LpL deficiency *in vivo* is dispensable for lipid accumulation and macrophage polarization. Global LpL deficiency *in vivo* reduces lipid content in adipose tissue macrophages (ATM) and the number of induced peritoneal macrophages. Further, global LpL deficiency does affect the phenotype of circulating monocytes and peritoneal macrophages, but does not affect macrophages in regressing atherosclerotic plaques. **Chapter 4** reports that LpL-induced HyperTG causes reduced HDL-C and HDL-P in mice and humans, but no dysfunctional HDL measured as CEC. Despite the reduction in HDL-C and HDL-P, LpL-deficiency-induced HyperTG did not impair atherosclerosis regression, neither in aortic arches, roots or BCAs, likely because HDL function (CEC) was maintained.

Discussion

The connection between low HDL-C and elevated TG with cardiovascular risk has been shown in observational studies, but how HyperTG affects other HDL characteristics is unknown and pre-clinical trials showing benefits of TG reduction are scant. While there are limited experimental models linking HyperTG and atherosclerosis, a large series of experimental studies have documented benefits of raising HDL levels as reviewed in ^{3,4}. One postulated reason for these benefits is an increase in CEC or reverse cholesterol transport (RCT).⁵ However, interventions in humans that increased HDL-C levels using CETP inhibition did not show clinical benefits^{6,7}, indicating that an increase of HDL-C does not necessarily reflect an increase in HDL function (CEC or RCT). In line, although CETP is considered pro-atherogenic due to its contribution to an atherogenic lipid phenotype, including lowering HDL-C, CETP has also been shown to increase RCT⁸, which is considered anti-

atherogenic. We showed that although inducing hCETP expression in mice lowers HDL-C and HDL-P, the ABCA1-mediated efflux per HDL particle was increased. Overall these effects resulted in atherosclerosis regression not being impaired (**Chapter 4**). Instead of solely measuring HDL-C, focus in the field of research has shifted to determining HDL functions. Therefore, we assessed HDLs' considered key-antiatherogenic function, *i.e.* CEC, and other characteristics such as apoA-I, HDL-P, and HDL size (**Chapter 2 & 4**). As the metabolic milieu may affect various properties of HDL, we looked into how diabetes (**Chapter 2**) and HyperTG alone (**Chapter 4**) impact HDL. To further determine how changes in HDL affect CVD, we associated HDL characteristics with pre-clinical atherosclerosis (endothelial dysfunction; EnD) and with CVD/CVE and were the first ones to do so in (pre)diabetic individuals (**Chapter 2**). Of note, we also associated HDL characteristics with another atherosclerosis surrogate – cIMT – but due to lack of associations, likely due to sensibility of the cIMT measurement itself, these results are not discussed here, but in **chapter 2**.

The (pre)diabetic individuals of the CODAM Cohort displayed typical features of diabetic dyslipidemia, *i.e.* reduced HDL-C and increased TG levels. As expected, HDL-C was inversely associated with EnD and CVE in the total cohort and in individuals with (pre)diabetes. Other HDL characteristics, such as apoA-I, HDL size and HDL-P were also inversely associated, with associations of HDL size and HDL-P being lost in individuals with (pre)diabetes, but not in healthy subjects. Notably, while previous studies have reported HDL-P and HDL-size to decrease in diabetes^{9,10}, we report an increase of both. With HDL-P and size being increased in (pre)diabetics, but lack of its association with atherosclerosis, this suggests that in individuals with NGM, the protective effect of a certain number of HDL particles against development of atherosclerosis is better than for that same number of HDL particles in (pre)diabetes. To determine if observed associations of HDL characteristics with atherosclerosis and CVE in (pre)diabetics, or the lack thereof, were dependent on TG levels, we performed TG adjusted analyses. While all HDL characteristics were still inversely correlated with atherosclerosis in healthy subjects after adjusting for TG, the association of HDL-C with atherosclerosis was lost specifically in (pre)diabetic patients. This suggests that the inverse relationship of HDL-C with atherosclerosis in (pre)diabetes is dependent on TG thereby confirming the intimate relationship of HDL-C and TG.

To allow a closer comparison of human data from the CODAM study with human and mouse data from **chapter 4**, we stratified the CODAM cohort into TG<150mg/dL (N=363; control) and >150mg/dL (N=167; HyperTG). Thereby, 66% of HyperTG patients were (pre)diabetic and had Hb1Ac levels of $6 \pm 0.9\%$. Next to reduced HDL-C, HyperTG individuals from the CODAM Cohort also displayed decreased HDL-P, but not change in CEC, compared to control (Figure 2). This is in line with results from **chapter 4**, showing that despite reduction of HyperTG-induced HDL-C and HDL-P in mice and human, overall CEC is not impaired. Interestingly, individuals with (pre)diabetes have increased HDL-P, but also no change in CEC (**Chapter 2**). This suggests that HDL functionality can be preserved despite changes in HDL-P number. Thus, composition of HDL is of particular interest regarding its atheroprotective properties. Nonetheless, if preserved CEC is the reason for not observing impaired regression in HyperTG mice despite a reduction in HDL-C and HDL-P (**Chapter 4**), we would expect to see an association of CEC with atherosclerosis and CVD/CVE in the CODAM study (**Chapter 2**) as it has been shown in other studies.¹¹⁻¹⁴ So far, CEC measurements are not standardized and although we employed a well validated CEC assay, different protocols could have led to discrepancies between studies. Another limitation is the cross-sectional approach of our study, which hampers a direct causal interpretation of the observed associations. A better comparison of **chapter 2** and **chapter 4** would have been measuring associations of HDL-characteristics with the change in cIMT over the study period (Δ cIMT) as surrogate for atherosclerosis regression. However, as mentioned above, we did not find any significant associations of HDL characteristics with cIMT.

Figure 2. Comparison of HDL-P (*left*) and CEC (*right*) between Control (TG<150mg/dL, N=363) and HyperTG (TG>150mg/dL, N=167) in the CODAM study.

HyperTG has been shown to be associated with CVD in a number of studies and is not in line with our results from **chapter 4**. However, next to CEC, HDL has other functions as well as including antithrombotic functions.¹⁵ Interestingly, proteomic analysis of HDL revealed an upregulation of proteins that are associated with platelet activation, signaling and degranulation in mice and in humans (data now shown), suggesting a role of HyperTG-reduced HDL in atherothrombosis. Current publications have reported that acute HyperTG induces platelet hyperactivity¹⁶ and contributes to resistance of antiplatelet therapy such as aspirin and clopidogrel.^{17,18} Additionally, platelet activity was also increased by TG-rich lipoproteins from T2DM patients.¹⁹ Meta-regression analyses of 64 randomized control trials showed that TG levels were associated with stroke risk, but not cIMT measures. However, TG lowering did only result in a trend towards reduced stroke incidence.²⁰ Therefore, the association of HyperTG with increased CVD risk could be explained by loss of HDLs antithrombotic function, but future studies are needed.

Another aspect of the atherogenic potential of HyperTG is the lipolysis of TGs. LpL hydrolyzes TGs either at the vessel wall or within the atherosclerotic plaque, resulting in remnants and other lipolysis products (FFA, mono- diglyceride and lysolecithin). TG lipolysis at the vessel wall leads to atherogenic remnants that can enter the arterial wall and deposit lipids, while lipolysis products (FFA, mono- diglyceride and lysolecithin) can result in increased inflammation, monocyte adhesion and coagulation. In contrast, we did not find impaired vascular regression despite vascular lipolysis (**Chapter 4**). As macrophage LpL has been implicated in foam cell formation²¹⁻²³, not observing HyperTG-impaired atherosclerosis regression could also be due to counterbalancing effects of LpL deficiency in macrophages. Thus, macrophage LpL deficiency could prevent lipoprotein uptake by macrophages, masking possible atherogenic effects of HyperTG. Macrophages are the major source of LpL in atherosclerotic plaques²⁴ and whether LpL in atherosclerotic plaques acts pro- or antiatherogenic is still debated. Interestingly, macrophage specific LpL knockout *in vivo* reduced atherosclerosis.^{21,22} *In vitro*, macrophage LpL appears to have atherogenic functions, because it causes accumulation of atherogenic lipoproteins and their rapid uptake by macrophages²⁵, which we recapitulated in **chapter 3**. It is therefore feasible that LpL deficiency in macrophages overcomes the detrimental

effects of HyperTG in LpL-deficient mice. However, whether or not LpL was present in macrophages, we did not observe differences in atherosclerosis regression in mice (**Chapter 4**). The potential antiatherogenic effects of LpL are thought to rely on the fatty acids provided by lipolysis, which were reported to convert macrophages to a more anti-inflammatory phenotype via peroxisome proliferator-activated receptors (PPARs).^{23,26,27} We showed that LpL *in vitro* changes both, pro- and anti-inflammatory markers, despite a decrease in FFA and increase in glycolysis suggestive of an increase in pro- and decrease of anti-inflammatory markers (**Chapter 3**). Further, transcriptomic profiling of macrophages in atherosclerosis regressing plaques *in vivo* did not reveal differences between macrophages expressing LpL and LpL deficient macrophages (**Chapters 3 & 4**).

In conclusion, in a human cohort we have shown that HDL-C, but not CEC, is associated with atherosclerosis and CVE, also in patients with diabetes. The found association of atherosclerosis with HDL-P is, however, no longer observed in diabetes patients. Experimentally reducing HDL-C and HDL-P by induction of HyperTG did not impair atherosclerosis regression, likely because CEC was unchanged. Further, lipolysis of circulating TG-enriched lipoproteins at the vessel wall or at atherosclerotic macrophages does not impair regression either. HDL proteins that changed with HyperTG were associated with lipoprotein remodeling (e.g. reduced ApoA-I) and platelet activation, signaling and degranulation. Overall, our results indicate that HyperTG does not impair atherosclerosis regression, but might be of importance for atherothrombosis via decreased antithrombotic functions of HDL and should be addressed in future studies.

Neutrophils and their role in atherosclerosis

Main Findings

Chapter 5 shows that NETs lead to inflammasome activation and inflammatory macrophages in atherosclerotic plaques. Our results illustrate that NETs can resolve during atherosclerosis regression. In a hyperglycemic environment however, NETs persisted thereby exacerbating macrophage inflammation and impairing atherosclerosis resolution. DNase1 treatment reduced plaque NETs content and macrophage inflammation, promoting atherosclerosis resolution after lipid-lowering in diabetic mice. **Chapter 6** describes a pilot study about the potential role of two different

endonucleases – DNase1 and DNase1L3 – in atherosclerosis. The data show that double-deficiency tends to increase NET formation compared to single-deficient mice and control with no change in NET content during regression. Further, NET content did not associate with atherosclerosis severity.

Discussion

The possible implication of neutrophils in the pathophysiology of CVD has long been overshadowed by other leukocyte subtypes, such as monocytes and macrophages, partly due to neutrophil's short life span in the circulation of <24h in mice²⁸ and human²⁹⁻³¹. However, the life span of neutrophils within different tissues as well as under inflammatory conditions is not well defined. Therefore, the discovery of neutrophils ability to form NETs³² and the presence of both in human³³⁻³⁷ and mouse³⁸⁻⁴⁴ atherosclerotic plaques has sparked new interest in neutrophils. It has been shown that inflammation can increase longevity of neutrophils, in part via regulation of anti-apoptotic factors, such as Mcl-1.^{45,46} While survival of neutrophils could be beneficial in acute inflammation, it might be detrimental in a chronic inflammatory state due to increased NETs release as senescent neutrophils have a higher potential for NET formation.⁴⁷ NETs' proinflammatory role and contribution to chronic inflammatory disease has been postulated to trigger crosstalk of neutrophils with other cells.^{48,49} Warnatsch et al. suggested that the interaction of neutrophils and macrophages leads to inflammasome activation in plaque macrophages and enhanced atherosclerosis progression.³⁸ In agreement, using transcriptomic profiling of atherosclerotic macrophages, we prove that macrophages in a NET+ area possess indeed a enhanced proinflammatory phenotype compared to macrophages outside of a NETs area (**Chapter 5**). Since CVD as well as diabetes are associated with neutrophilia in mice^{50,51} and human⁵² and hyperglycemia has been shown to prime neutrophils for NET production⁵³, diabetics are one clinical population in which NETs may be particularly harmful in with respect to cardiovascular risk. Further, plasma NETs markers are not only correlated with severity of CAD, but also with severity of T2DM as judged by correlation of nucleosomes (NETosis marker) with HemoglobinA1c levels (average blood glucose levels over a period of time) in humans.⁵⁴ Studying atherosclerosis regression in diabetic mice, we found impaired atherosclerosis

regression (Chapter 5), as we have shown before^{2,55,56}. Thereby, macrophage content was positively associated with NET content (**Chapter 5**).

While current studies of NETs focused on atherosclerosis progression, we are the first ones reporting on atherosclerosis regression. Our novel results showed that NETs can spontaneously resolve in a non-hyperlipidemic environment, but not in hyperglycemic conditions, even with non-hyperlipidemia. Thus, presence of NETs could be one reason for the increased CVD risk in diabetic patients, even when they have desired LDL-C levels. One way to clear NETs is treatment with DNase1, as NETs are thought to be mainly degraded by endonucleases⁵⁷ and have been reported to be cleared by DNase1 in atherosclerosis in mice^{38,58}. Given published studies and existent clinical use of DNase1 (e.g. cystic fibrosis⁵⁹), we treated mice with DNase1. As expected, DNase1 treatment led to reduced NET content and excitingly, promoted atherosclerosis regression despite ongoing hyperglycemia by reducing plaque inflammation (**Chapter 5**). The pro-inflammatory nature of NETs are thought to increase monocyte and neutrophil recruitment, further enhancing plaque inflammation^{38,60}, leading to the hypothesis that DNase1 treatment lowers leukocyte recruitment. We do find increased monocytosis (data not shown) as well as neutrophilia in diabetic mice (**Chapter 5**), as previously reported by our lab.^{50,51} In line, we also found increased plaque neutrophils in diabetic mice and a trend towards decreased circulating and plaque neutrophil levels upon DNase1 treatment. This is in agreement with a study by Wong et al. showing no difference in neutrophil recruitment in diabetic *Padi4*^{-/-} (NETs abrogated) mice compared to diabetic WT mice⁵³, but in contrast to other studies showing that plaque neutrophil content is reduced upon the ablation of the inflammasome³⁹ or NETs abrogation³⁸. Notably, not all neutrophils detected in plaques do undergo NETosis, which has been shown before in other *in vitro* and *in vivo* studies.⁶¹ Thus, our results indicate that activation of neutrophils rather than neutrophil levels per se are of relevance for NET formation and its effect on plaque composition.

We have seen that restoring normal circulating cholesterol levels after a period of elevated cholesterol levels, led to spontaneous resolution of NETs without additional DNase1 treatment (**Chapter 5**), indicating that physiological amounts of endonucleases are sufficient for NETs clearance in healthy conditions. Therefore, we

were interested to know if *DNase1*^{-/-} mice have higher NET content in atherosclerotic plaques and if atherosclerosis progression was worse or atherosclerosis regression impaired with DNase1 deficiency. Since it has been shown before that serum from DNase1 KO mice show some residual KO activity that displayed characteristics of DNase1L3⁶², we decided to include DNase1L3 deficient and double-deficient mice. We found that double-deficient mice showed a trend towards increased NET content, which is in line with a study by Jimenez-Alcazar et al., showing that especially the double-deficient mice have more NETs than single-deficient mice leading to occlusion of vascular vessels.⁶³ In contrast to our previous results (**Chapter 5**), the trend towards increased NET content in double-deficient mice did not result in changes in atherosclerosis progression or regression and further, NETs content did not decrease with regression (**Chapter 6**). However, we did not observe significant differences in NET content between groups. Further, NET content in progressing mice on WT background was about 4-fold lower compared to NET content we observed in the LDLR-deficient mice and is likely due to circulating plasma cholesterol levels. In our study in **chapter 5** using *Ldlr*^{-/-} fed a WD, we reached cholesterol levels of ~1000mg/dL. In our study in **chapter 6**, we switched from *Ldlr*^{-/-} to WT mice injected with PCSK9 fed a WD to avoid crossbreeding of DNaseKO mice onto *Ldlr*^{-/-} background. However, WT mice injected with PCSK9 only reached levels of about ~60% from those of *Ldlr*^{-/-} mice. We did observe higher cholesterol levels (~1200mg/dL) using PCSK9 before⁶⁴ and the rather low cholesterol levels could be the results of a change in animal facility and staff. In line with lower cholesterol levels, we also observed lower atherosclerotic plaque sizes. Hence, low NET content could be due to lack of stimulus. If NET formation requires these high levels of cholesterol and can resolve spontaneously by lowering cholesterol levels, clinical relevance of NETs in hyperlipidemia is questionable, since patients with cholesterol levels of >200mg/dL are already put on lipid-lowering therapy. However, NETs do not resolve under hyperglycemic conditions, not even after lipid-lowering. Hence, population to benefit from DNase1 treatment are patients suffering from diabetes, especially those with not well-controlled blood glucose levels. Clinical use of DNase1 (Pulmozyme®) has been approved by the FDA (Federal Drug Administration) in 1993 and since then is on the market in >65 countries with no signs of major toxicity or detrimental long-term effects. Pulmozyme® has been shown to be effective in treatment of cystic fibrosis by

increasing lung function and reducing exacerbation of cystic fibrosis. This beneficial effect has been proposed to be partly due to NETs degradation by DNase1.⁶⁵ Thus, DNase1 treatment to lower plaque inflammation and to induce atherosclerosis resolution would be a novel therapeutic approach to overcome increased CVD risk in diabetic patients. We (**Chapter 5**) and others^{38,53,58} have shown that NETs degradation via DNase1 is beneficial in the context of CVD in mice. Notably, there is one contradictory study showing no effect of DNase1 treatment on atherosclerosis⁶⁶, including the thought that DNase1 has little effect on other NET components, such as neutrophil elastase, that might continue to cause inflammation at the vessel wall.⁶⁷ NETs from isolated human neutrophils can also be degraded by DNase1.⁶⁸ Interestingly, it has been shown that an increase in circulating NETs is counterbalanced by an increase in DNase activity in healthy individuals.⁶⁹ Further, mutations in DNase1 gene has been associated with disease states such as myocardial infarction⁷⁰ or SLE^{71,72}. Thus, DNase1 activity might be impaired in individuals suffering from diabetes. In contrast, one study shows that DNase1 activity is actually increased in diabetes⁷³ and further research is needed for clarification. So far, there are no reports on CVD risk in individuals treated with Pulmozyme®, however whether DNase1 reaches atherosclerotic plaques by delivery via a nebulizer is questionable. DNase1 administration in current mouse atherosclerosis studies is done via intraperitoneal injections, but in humans this may compromise immunoprotective functions of NETs e.g. antimicrobial activity. A better way to administrate DNase1 might be DNase1-coated nanoparticles, which were shown to reduce size of lung metastases in mice⁷⁴, but this way of administration needs further exploration in atherosclerosis. Although evidence for the detrimental effects of NETs in several diseases such as diabetes and atherosclerosis is raising, current research is still in its infancies. Further research is needed to deepen the knowledge about NETs in CVD and the exact mechanisms of DNase1 treatment to translate those findings into a novel therapy strategy.

Future Directions

Several questions have been raised by the results of this thesis and need to be addressed in future studies.

The effect modifications by the insulin resistant state in the CODAM study are highly interesting and are worth to further investigate in a larger cohort with sufficient power for more extensive subgroup analyses.

Interestingly, HDL proteomics revealed increased platelet associated markers on TG-enriched HDL and future experiments should focus on the role of HyperTG in thrombosis. Therefore, platelet aggregation assays using platelet-rich plasma of HyperTG patients and LpL deficient patients should be tested against control plasma to see if published results of HyperTG inducing platelet hyperactivity can be replicated. If that is the case, aggregation assays should be repeated using incubation of isolated HDL from healthy and HyperTG (LpL deficient) subjects. To determine the effect on atherothrombosis, experimental studies should be performed using a mouse model of thrombosis such as ligation of the inferior vena cava.⁷⁵ Aggregation assays should also be done using platelet-rich plasma from LpL deficient and control mice and further, flow cytometry assays using platelet markers (e.g. P-selectin, JON/A) can give additional insights on platelet activation.

Our novel results showed the therapeutic potential of NET reduction in individuals with diabetes, especially in those whose diabetes is not well controlled. Future studies should focus on understanding the mechanisms by which DNase1 leads to improved atherosclerosis regression in a hyperglycemic condition. We have shown that DNase1 treatment reduced inflammation in atherosclerotic plaques, thereby improving atherosclerosis regression. Additional inflammatory markers in the circulation are of interest. Since we were not able to detect IL1 β in the circulation using ELISA and CBA assays, other markers, such as IL-17, IL-18, IL-6, can be tested. Decreased inflammation and improved atherosclerosis regression also lead to the hypothesis that fewer monocytes were recruited, macrophage retention is improved and/or proliferation is reduced. To test this, monocyte and macrophage trafficking studies should be performed. Preliminary results showed reduced circulating Ly6C^{high} monocytes with DNase1 treatment, but no differences in monocyte trafficking (data not shown) and completed study results need to be waited for to draw final conclusions.

Another aspect to consider is the possible effect of hypercholesterolemia and/or hyperglycemia on DNase1 activity. NETs have been shown to be involved in the pathogenesis of systemic lupus erythematosus (SLE)⁶⁸, partly due to reduced DNase1

activity^{71,76}. This raises the question if hypercholesterolemia/hyperglycemia may have similar effects, hence the increased NET formation and inflammation. In contrast, one study reported increased DNase1 activity in serum of individuals with diabetes and of STZ-injected rats.⁷³ For clarification, DNase activity of hypercholesterolemic and hyperglycemic mice should be compared to control and DNase1 treated mice.

As physiological amounts of endonucleases are sufficient in clearing NETs and results of DNase deficient mouse models were modest under hypercholesterolemic conditions, future studies should focus on hyperglycemia. Given that we see positive effects on DNase1 treatment in diabetic mice and increased NET formation with the double-deficient mouse model, the impact of double-deficient diabetic mice on atherosclerosis is of interest.

Main conclusion & clinical recommendation

HyperTG and hyperglycemia have similar effects on HDL, but while HyperTG alone did not impair regression, hyperglycemia does. We observed reduced HDL-C (HyperTG and (pre)diabetics) and HDL-P (HyperTG only), but no differences in CEC (HyperTG and (pre)diabetes) in mice and humans. Experimental studies showed no impairment of atherosclerosis regression with HyperTG, but with hyperglycemia. Thereby, circulating neutrophil levels were increased along with NET content in atherosclerotic plaques and macrophage inflammation. Of note, we did not observe NETs in HyperTG mice (data not shown). Excitingly, clearing NETs using DNase1 lowers plaque inflammation and can overcome diabetes-impaired regression, despite ongoing hyperglycemia.

These results suggest that treating hyperglycemia-associated inflammation is prominent over treating HyperTG in the residual CVD risk in individuals with diabetes. Future studies should focus on establishing the mechanisms of DNase1 treatment and on evaluating the possible effect of HyperTG on other HDL characteristics such as its antithrombotic function.

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Valorisation

Valorisation

Type1 & Type2 Diabetes Mellitus (T1DM, T2DM) are common diseases with increasing prevalence worldwide.¹ Globally, 425 million people are diagnosed with diabetes with an estimated cost of ~\$727 billion.² Global incidence for diabetes is projected to rise to \$629 million by 2045. In the United States alone, with >30 million diagnosed patients, diabetes is the most expensive chronic disease, reaching an economic burden of \$327 billion.³ In the Netherlands, about 6.5% of the Dutch population has been diagnosed with diabetes⁴ with projection to rise to 8% by 2025⁵ and yearly costs of €6.8 billion⁴. These costs not only include direct costs of treating the disease, e.g. medication, but also costs of treating its complications, such as cardiovascular diseases (CVDs). Subjects suffering from diabetes have a significantly increased risk of atherosclerosis and associated CV events.⁶ In the US, cost of treatment of CV-related in diabetics is estimated at \$37.3 billion.⁷ In the Netherlands, about 30% of the people with diabetes are suffering from CVD, and the treatment of CVD in those diabetes patients makes up ~ €1 billion of the total economic burden of diabetes. CVD is , after end-stage renal disease, the most costly complications of diabetics in the Netherlands.⁴

CVD is the deadliest disease worldwide. Despite a steady decline in death from CVDs due to advances in scientific discoveries, clinical cardiology, as well as public health, it is still responsible for 30% of deaths.⁸ The global cost is estimated to be \$863 billion and is predicted to increase to \$1044 billion until 2030⁹ due to a rising and aging population and the high incidence of secondary complications after survival of a primary CVD incident. The increased risk of patients with diabetes to suffer from CVD is due to the fact that metabolic risk factors for atherosclerotic CVD are commonly found in subjects with diabetes. Hyperglycemia is an independent risk factor for CVD, next to genetic predisposition, hypertension and diabetic dyslipidemia. The adjusted relative risk for T2DM compared to non-T2DM patients is 1.7 for cardiovascular death, 1.8 for myocardial infarction, and 1.5 for stroke.¹⁰

Strikingly, individuals suffering from diabetes die mostly from CVD complications rather than diabetes itself. In the US, a total of 277,000 individuals with diabetes died in 2017 of which for 111,000 CVD was the primary cause of death.⁷ In the Netherlands, mortality rates of CVD have halved over the past two decades and with 26%, it holds

second place of the most non-communicable diseases, right after cancer (32%).^{11,12} Interestingly, diabetes takes with 2% the 4th place, and although CVD mortality has declined, the increase in diabetes has reversed positive trends and represents a major challenge to further prevent CVD.

The underlying mechanisms of increased CVD risk in individuals with diabetes is still unclear and current treatment focuses on treating hyperglycemia in diabetic patients, followed by statins in case of elevated LDL cholesterol (LDL-C) levels. Nonetheless, even if both are treated, diabetes patients still have a ~2 to 4-fold increased CVD risk compared to non-diabetics. Other components that potentially lead to the increased risk, such as elevated triglycerides (TG) levels and low HDL cholesterol (HDL-C) levels, are not being treated due to lack of evidence. While experimental studies of raising HDL documented benefits^{13,14}, clinical trials raising HDL-C have mostly failed to show beneficial CVD outcome^{15,16} and other HDL functions are currently explored. Experimental studies focusing on hypertriglyceridemia (HyperTG) and atherosclerosis are limited, and clinical trials of TG-lowering drugs and CVD risk are currently ongoing.

Our research has focused on the two comorbidities of diabetes, *i.e.* elevated plasma TG levels accompanied by low HDL-C and hyperglycemia-related neutrophilia/Neutrophil extracellular traps (NET) formation. The aim was to elucidate the importance of both in atherosclerosis regression to shed light into possible new treatment options and thereby lower the residual CVD risk in diabetics.

Valorisation of key findings of this thesis

The results from our pre-clinical models showed that elevated TG levels do not lead to impaired atherosclerosis regression after LDL-C lowering, despite significantly reduced HDL-C and HDL particles (HDL-Ps). This might be due maintained cholesterol efflux capacity (CEC) - the considered key-antiatherogenic HDL function.

Further, we found that clearance of NETs using DNase1 can overcome diabetes-impaired regression despite ongoing hyperglycemia, due to reduction in inflammation. This suggested that NETs contribute to the increased CVD risk in this population. Further, given that DNase1 (Pulmozyme®) is already clinically used for patients with cystic fibrosis¹⁷, these results also suggest a potential treatment for NET reduction.

Potential of TG lowering drugs to reduce residual CVD risk of diabetics

Effective TG-lowering drugs are available and are used in patients with TG levels >500 mg/dl to reduce the risk of pancreatitis.¹⁸⁻²¹ However, guidelines on using TG-lowering drugs to reduce CVD risk are not clearly defined due to lack of evidence. European Guidelines (ESC/EAS guidelines for the management of dyslipidemias²² and European guidelines on CVD prevention in clinical practice²³) recommend that individuals with TG levels >1.7 mmol/l (>150 mg/dl) should be considered at increased risk of CVD, but did not include risk estimation of TG or recommended target values. Earlier guidelines from the adult treatment panel are in line with the European guidelines²⁴, but more recent guidelines from the ACC/AHA on the assessment of CVD risk do not mention TGs at all.²⁵ There is evidence suggesting that reducing TG could be beneficial for CVD, especially in individuals with preexisting coronary heart disease and diabetes.^{24,26-28} But one of the key problems is that clinical trials that appear to confirm CVD benefits of TG-lowering therapy rely on subgroup analyses, and trials designed to focus on subjects with hyperTG only were missing. Thus, these results are non-conclusive.

The REDUCE-IT trial was the first published intervention trial to determine the relationship between TG and atherosclerosis in a population with elevated plasma TG levels. Patients at high risk for CVD and TG levels ≥ 135 mg/dL and < 500 mg/dL were prescribed icosapent-ethyl (a highly purified eicosapentaenoic acid; EPA) over a median time period of 4.9 years and showed reduced CVD risk including first, subsequent, and total ischemic events.^{29,30} However, it is still unclear if the reduction in ischemic events was due to lowering TG or due to a combination of effects involving plaque instability reduction and inflammation. Notably, results were consistent among diabetic patients. Another trial looking at the effect of icosapent ethyl on progression of coronary atherosclerosis in patients with elevated TG levels on Statin therapy – EVAPORATE – might help to explain the cardiovascular benefit noted in the REDUCE-IT trial, and the 9 months interim results show reduction in total plaque volume measured as coronary CTA (computed tomography angiogram), but no reduction in low attenuation plaque volume.^{31,32} Further, intense research is being done on the mechanisms of action for omega-3 fatty acids (reviewed in ³³).

Less exciting was the STRENGTH Trial, which studied the effect of *Epanova* (omega-3 carboxylic acids) on CVD risk reduction in ~13.000 patients with mixed

dyslipidemia (increased TG, low HDL-C) and increased risk of CVD.³⁴ The Trial has been discontinued earlier this year (January 2020) due to the unlikelihood of demonstrating a benefit to patients.³⁵

There are currently further clinical trials ongoing that are looking at the effect of TG-lowering therapy in high-risk CVD subjects, including the PROMINENT Trial (Pemafibrate to reduce CV outcomes by reducing TG in patients with diabetes), with results being expected over the next 1-2 years (reviewed in³⁶).

Potential of targeting NETs

Two possible therapeutic targets to reduce NETs in atherosclerosis have been studied in mice – 1. PAD4 inhibition and 2. DNase1 treatment.³⁷ In our studies, we focused on DNase1, because it is clinically already used to treat cystic fibrosis. DNase1 (Pulmozyme®) has been approved by the FDA (Federal Drug Administration) >25 years ago, is on the market in >65 countries and did not lead to any signs of major toxicity or detrimental long-term effects. Excitingly, the positive effects of DNase1 in treating cystic fibrosis has been proposed to be partly due to NETs degradation.³⁸ Our, as well as other studies³⁹⁻⁴¹ showed that NETs degradation via additional intraperitoneal (i.p.) DNase1 administration is beneficial in the context of CVD in mice. In humans, however, i.p. administrations of DNase1 solely may compromise immunoprotective functions of NETs, e.g. antimicrobial activity. Cystic fibrosis patients take DNase1 via a nebulizer, but whether an effective dose of DNase1 would also reach atherosclerotic plaques, is questionable and rather unlikely. A better way to administrate DNase1 without compromising immunoprotective functions might be the use of DNase1-coated nanoparticles, which were shown to reduce size of lung metastases in mice via NETs degradation.⁴² Nano-based delivery systems have been proven to be safer and more effective than delivering a drug solely, with targeted delivery to site of disease, increased efficacy per drug dose and avoidance of off-target effects⁴³. Nanodrugs are clinically already used and are administered orally or intravenously⁴⁴ to e.g. treat cancer, and have been shown to be beneficial for CVD in preclinical models (reviewed in⁴⁵). Various types of nanoparticles are available (e.g. type IV collagen, cyclodextrin-based polysaccharides, lipid nanoparticles) and the correct therapeutic strategy with DNase1 as therapeutic agent needs to be tested in atherosclerotic mouse models.

Despite the exciting potential of DNase1 treatment in (diabetic) atherosclerosis, NET research is still in its infancies and we are lacking enough evidence as well mechanistic details before therapeutically targeting NETs to lower CVD risk is clinically transferable.

Translational capacity of the animal models

To study the effect of elevated TG levels on atherosclerosis, we used an inducible lipoprotein lipase deficient mouse model (*iLpL^{-/-}*). Our mouse model had mild to moderate hyperTG with TG levels of ~500mg/dL, which is significantly lower than that of LpL deficient patients whose TG levels reach > 1000 mg/dL. While our mouse model also has TG levels >1000mg/dL when fed a high-fat diet, we chose to use a western diet resulting in mild-to-moderate hyperTG (~500mg/dL). These levels reflect LpL deficient patients on treatment and restricted diet as well as diabetics suffering from diabetic dyslipidemia. Further, subjects with genetic LpL deficiency are rare and not likely to impact genetic analysis of overall CVD risk associated with plasma TG levels. One limitation of our mouse model is the lack of lipolysis, which has been postulated together with hyperTG to be the cause for CVD risk by creating high concentrations of toxic lipids and remnant lipoproteins. To overcome this limitation, we have included mice with LpL expression in macrophages or endothelial cells. These studies led to the same results, showing that hyperTG does not impair atherosclerosis regression after LDL-C lowering.

Another limitation of our mouse model is the lack of cholesterol ester transfer protein (CETP) expression in mice. To humanize the lipoprotein metabolism in mice, we have introduced hCETP and again, confirmed that hyperTG + hCETP does not impair atherosclerosis regression after lipid lowering. Overall, we think our mouse model can be translated to the human condition, which we underline with the human data.

To study the effect of hyperglycemia-induced neutrophilia and NET formation, we used *Ldlr^{-/-}* mice injected with streptozotocin (STZ). STZ is a glucosamine-nitrosourea compound (glucose analogue) that is preferentially toxic towards insulin-producing beta cells.⁴⁶ We used a low dose (0.05 mg/g BW) over five consecutive days leading to cytotoxic effects in the pancreas resulting in pancreatic islet inflammation, insulin deficiency and hyperglycemia.^{46,47} Thereby, this method mimics human T1DM, while

the majority of individuals with diabetes are diagnosed with T2DM⁴⁸. Further, compared to severe systemic inflammation in mice, human leukocytosis is almost always mild. Therefore, translation from the animal model to human situation has to be done with caution.

To further study the role of endonucleases in atherosclerotic plaques, we used different DNase1 knock-out (KO) mouse models - DNase1 and DNase1L3 single KO, and DNase1/L3 double KO mice. DNase1 and DNase1L3 single KO mouse models are commonly used to study systemic lupus erythematosus (SLE). For *DNase1*^{-/-} mice, it takes 9 months to develop the SLE phenotype (increase in circulating Anti-Nuclear Antibodies (ANA), as well as antibodies against double-stranded DNA, histones, and Smith proteins).⁴⁹ At 12 month of age, these mice develop severe nephritis and kidney damage. *DNase1L3*^{-/-} can develop SLE as early as 5 weeks of age.⁵⁰ The double KO mice were created by our collaborators and not much is published about their phenotype. Therefore, *DNase1*^{-/-} mice are suitable for non-SLE experiments shorter than 9 months, while results from *DNase1L3*^{-/-} and the double KO mice might be confounded. In our performed 20 to 24 weeks long atherosclerotic study, we did not observe significant differences between our mouse models regarding NET formation, atherosclerosis progression and regression. Further, we did not detect any physical or behavioral abnormalities as well as no signs for major inflammation or tissue damage. Notably, we did not test for SLE markers.

Using administration of additional DNase1 results in beneficial effects on NET formation and atherosclerosis regression in especially diabetic mice and it is of interest to determine if the opposite effect can be observed using the various DNase KO mouse models. However, a study reported that double KO mice die within days after induction of severe neutrophilia.⁵¹ Thus, using the double KO mice in a diabetic atherosclerosis study (shown to induce neutrophilia) might not be feasible. In conclusion, the translation of results from the various atherosclerotic DNase KO mouse models to humans is challenging.

Conclusion

Overall, our results show that treatment of hyperglycemia-associated inflammation seems to be superior over treating hyperTG, especially in individuals whose diabetes is not well controlled.

Although effective TG-reducing treatments are available, our results do not suggest benefits in CVD prevention. In agreement, the results of two recent interventional trials – REDUCE-IT & STRENGTH, studying the potential benefits of TG-lowering therapy in high CVD-risk patients, were rather disappointing. With research on the mechanisms behind EPA treatment as well as results from ongoing TG-lowering trials being on their way, we will hopefully get an answer to decide if lowering elevated TGs should be included in the CVDs treatment guidelines.

While relative lack of evidence and knowledge does not allow clinical translation of NET targeting therapeutics in CVD as of yet, rapidly evolving research holds promise for their clinical translation in the near future.

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Curriculum Vitae

Tatjana Josefs was born on the 6th of June, 1990 in Bad Friedrichshall, Germany. She graduated from secondary school (Eckenberg-Gymnasium, Adelsheim, Germany) in 2009 followed by 9 months in New Zealand, where she worked as an au-pair and spent time travelling. In 2013, she received her bachelor's degree in nutritional sciences with focus on research from the Friedrich-Schiller University Jena, Germany. This was followed by a master's degree in biomedical sciences at the Maastricht University, the Netherlands. She performed internships in the department of Internal Medicine at Maastricht University, Maastricht, the Netherlands supervised by Dr. Kristiaan Wouters, and in the department of Cardiology at NYU School of Medicine, New York, USA, supervised by Prof. Dr. Edward A. Fisher. The master thesis was conducted in the Fisher lab entitled: ApoB100 on hepatic lipid droplets is associated with the autophagy factor p62, and was financially supported by a scholarship from the Dutch Heart Foundation (Hartstichting). In 2015, she received her master's degree with distinction and continued her work in the Fisher lab as a PhD student. The PhD project was conducted under the supervision of Prof Dr. Casper Schalkwijk (Maastricht University), Prof Dr. Edward A. Fisher (NYU School of Medicine), and Dr. Kristiaan Wouters (Maastricht University). During this time, she studied the role of diabetes-related factors, such as elevated circulating triglyceride levels, in atherosclerosis regression. This work was funded by a predoctoral fellowship from the American Heart Association and the results of her research are presented in this thesis.

Scientific Output

List of Publications

Josefs T*, Barrett TJ*, Brown EJ, Quezada A, Wu X, Voisin M, Amengual J, Fisher EA. Neutrophil Extracellular Traps (NETs) promote macrophage inflammation and impair atherosclerosis resolution in diabetic mice. *JCI Insight*. 2020;5(7):e134796. (IF:6.014)

Josefs T, Wouters K, Tietge UJF, Annema W, Dullaart RPF, Vaisar T, Arts ICW, van der Kallen CJH, Stehouwer CDA, Schalkwijk CG, Goldberg IJ, Fisher EA, van Greevenbroek MJ. High-density lipoprotein cholesterol efflux capacity is not associated with atherosclerosis and prevalence of cardiovascular outcome: The CODAM Study. *J Clin Lipidol*. 2020 Jan-Feb;14(1):122-132.e4. (IF: 3.860)

Chang HR*, **Josefs T***, Scerbo D, Gumaste N, Hu Y, Huggins LA, Barrett TJ, Chiang SS, Grossman J, Bagdasarov S, Fisher EA, Goldberg IJ. Role of LpL (Lipoprotein lipase) in Macrophage Polarization In Vitro and In Vivo. *Arterioscler Thromb Vasc Biol*. 2019 Oct;39(10):1967-1985. (IF: 6.607)

Bijnen M, **Josefs T**, Cuijpers I, Maalsen CJ, van de Gaar J, Vroomen M, Wijnands E, Rensen SS, Greve JWM, Hofker MH, Biessen EAL, Stehouwer CDA, Schalkwijk CG, Wouters K. (2017). Adipose tissue macrophages induce hepatic neutrophil recruitment and macrophage accumulation in mice. *Gut*. 2018 Jul;67(7):1317-1327. (IF: 16.658)

Additional Manuscripts

Josefs T, Basu D, Vaisar T, Arets B, Kanter JE, Huggins LA, Hu Y, Liu J, Heinecke JW, Bornfeldt KE, Goldberg IJ*, Fisher EA*. Atherosclerosis regression and its relationship to cholesterol efflux capacity in hypertriglyceridemic mice.
In Revision

Voisin M, Shrestha E, Rollet C, **Josefs T**, Barrett TJ, Chang HR, Ruoff R, Nikain CA, Garabedian ML, Brown EJ, Goldberg IJ, Pineda-Torra I, Fisher EA, Garabedian MJ. Inhibiting bone marrow LXRα phosphorylation controls inflammation, atherosclerosis & obesity.
In Revision

Presentations

2019 American Heart Association's Scientific Sessions; Philadelphia, PA, United States

- **Oral presentation** "Neutrophil Extracellular Traps (NETs) promote macrophage inflammation and contribute to diabetes-impaired atherosclerosis regression"
- **Mediated Poster Presentation** "Reduced HDL due to hypertriglyceridemia does not affect atherosclerosis regression"
- **Invited Poster Presentation** ATVB Early Career Networking Reception "Reduced HDL due to hypertriglyceridemia does not affect atherosclerosis regression"

2019 Gordon Research Conference Atherosclerosis; Newry, ME, United States

- **Poster Presentation** "Neutrophil Extracellular Traps (NETs) promote macrophage inflammation and contribute to diabetes-impaired atherosclerosis regression"

2018 Department of Medicine Research Day; New York, NY, United States

- **Poster Presentation** "High-density Lipoprotein Cholesterol Efflux Capacity is not associated with Atherosclerosis and Cardiovascular Events: The CODAM Study"

2018 Vascular Discovery: From Genes to Medicine 2018 Scientific Sessions (ATVB/PVD); San Francisco, CA, United States

- **Poster Presentation** "High-density Lipoprotein Cholesterol Efflux Capacity is not associated with Atherosclerosis and Cardiovascular Events: The CODAM Study"

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Abbreviations

Abbreviation	Meaning
AAM ϕ	alternatively activated macrophage
AAV	adeno-associated vector
ABCA1/ABCG1	ATP-binding cassette transporter A1/G1
AHA	American Heart Association
apoA-I	apolipoprotein A-I
apoB	apolipoprotein B
<i>ApoE</i> ^{-/-}	apolipoprotein E deficient
ASO	antisense oligonucleotides
AT	adipose tissue
ATM	adipose tissue macrophage
BMDM	bone marrow-derived macrophage
BMI	body mass index
BP	blood pressure
CAD	Coronary Artery Disease
CAM ϕ	classically activated macrophages
CD36	cluster of differentiation 36
CE	cholesteryl ester
CEC	cholesterol efflux capacity
CETP	cholesteryl ester transfer protein
CI	confidence interval
cIMT	carotid intima-media thickness
CODAM	Cohort on Diabetes and Atherosclerosis Maastricht
CRAMP	cathelicidin-related antimicrobial peptide
CVD	Cardiovascular Disease
CVE	Cardiovascular Event
DNase1	deoxyribonuclease 1
DNase1L3	DNase1 like 3
ECAR	extracellular acidification rate
EnD	endothelial dysfunction
FA	fatty acid
FAO	fatty acid oxidation
FFA	free fatty acids

FPG	fasting plasma glucose
H3Cit	citrullinated H3
Hb1Ac	Hemoglobin A1c
hCETP	human CETP
HDL	high-density lipoprotein
HDL-C	HDL cholesterol
HDL-P	HDL particle number
HL	hepatic lipase
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HyperTG	hypertriglyceridemia
IDLs	intermediate-density lipoproteins
IGM	impaired glucose metabolism, prediabetes
IL	interleukin
iNOS	inducible nitric oxide synthase
IQR	Interquartile Range
LDL	low-density lipoprotein
LDL-C	LDL-Cholesterol
LDLR	low-density lipoprotein receptor
<i>Ldlr^{-/-}</i>	low-density lipoprotein receptor deficient
LpL	lipoprotein lipase
LPM	large peritoneal macrophage
Ly6G	lymphocyte antigen 6 complex locus G6D
MET	Metabolic Equivalent of Task
MI	myocardial infarction
MPO	myeloperoxidase
Myh11	myosin heavy chain-11
M ϕ	macrophage
NADPH	nicotinamide adenine dinucleotide phosphatase
NE	neutrophil elastase
NETs	neutrophil extracellular traps
NGM	normal glucose metabolism
NLRP3	NLR family pyrin domain-containing protein 3
OA	oleic acid

OCR	oxygen consumption rate
OR	odds ratio
oxLDL	oxidized LDL
PAD4	peptidylarginine deiminase 4
PCSK9	proprotein convertase subtilisin/kexin type 9
pDCs	plasmacytoid dendritic cells
PMA	phorbol myristate acetate
PPAR	peroxisome proliferator-activated receptor
PR3	proteinase 3
RCT	reverse cholesterol transport
ROS	reactive oxygen species
sdLDL,	small dense LDL
SLE	lupus erythematosus
SO	sense oligonucleotide
SPM	small peritoneal macrophages
STZ	Streptozotocin
T1DM, T2DM	Type1 & Type2 Diabetes Mellitus
TG	triglyceride
TRL	TG-rich lipoproteins
VLDL	very low-density lipoprotein
VSMC	vascular smooth muscle cells
WD	western diet
WT	wild-type; C57BL/6