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CHARACTERISATION OF AUTOPHAGY PATHWAYS IN ATHEROSCLEROSIS

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To Erika and Timothy, and nanna Rosa who "does not believe in cholesterol".

ABSTRACT

Atherosclerosis is an inflammatory disease of large to intermediate-sized arteries, characterised by retention of modified low-density lipoprotein within the vessel wall; this evokes an inflammatory response. Low-density lipoprotein carries lipids such as fatty acids, triglycerides and cholesterol, from the liver to peripheral tissues. The lipids carried by lipoproteins can be mobilised for metabolic processes, or if excessive, be stored in intracellular depots called lipid droplets. When imbalances in lipoprotein transport and/or cellular lipid metabolism occur, the risk for metabolic disorders, atherosclerosis and cardiovascular disease increases. Perilipins are lipid droplet-associated proteins, which regulate lipid mobilisation and metabolism from lipid droplets by allowing lipases access to the lipids within the lipid droplet.

Macroautophagy, generally referred to as "autophagy", regulates cholesterol metabolism in macrophage foam cells of atherosclerotic plaques. This is a parallel mechanism by which cells can mobilise lipids, distinct from the traditional dogma that cytosolic lipases mobilise intracellular lipid storages. Further, it contributes to the regulation of inflammation of atherosclerosis-afflicted vessels and it has been shown that ablation of core autophagy genes exacerbates atherosclerosis in murine disease.

In this thesis we describe a protein variant in perilipin-2, which reduces plasma triglyceride levels, alters intracellular lipid metabolism and is protective of subclinical atherosclerosis. By adopting a molecular genetic approach, including a well-defined recruit-by-genotype protocol, we clearly demonstrate that perilipin-2 constitutes a hub between cholesterol metabolism and autophagy by fine-tuning liver-X-receptor activity. We also show that liver-X-receptor and autophagy are responsible for their reciprocal activation and that 27-hydroxycholesterol drives this feed-forward loop between liver-X-receptor activity autophagy – the mechanism by which the protein variant in perilipin-2 exerts its beneficial effects on subclinical atherosclerosis.

Further we determine the presence of the autophagy-related proteins ATG16L1 and MAP1LC3A in human carotid atherosclerotic plaques where they are associated to plaque inflammation and vascular smooth muscle cell phenotypic switch, respectively. Ultimately, the presence of autophagy-related proteins in human carotid atherosclerotic plaques modulates plaque stability.

Collectively, data presented herein, extend on the existing murine data and suggest that deregulated autophagy is a feature of human atherosclerosis. Treatment options targeting autophagy in the treatment of atherosclerosis are still hampered by specificity of treatment and timely intervention.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Ateroskleros, eller åderförfettning, är en inflammatorisk kärlsjukdom som karaktäriseras av att kolesterol som transporteras med lipoproteiner i blodet fastar i kärlväggen och tas upp av vita blodkroppar som initierar en inflammatorisk reaktion. Ateroskleros är den underliggande orsaken till de flesta hjärt-kärlsjukdomar, t.ex. hjärtinfarkt och stroke. Att kolesterol transporteras med hjälp av lipoproteiner beror av att kolesterol och andra lipider (fetter) inte är lösliga i blodet. Olika lipoproteiner har olika roll i transporten av kolesterol. LDL och HDL är två lipoproteiner som bär kolesterol och dessa har vitt skilda roller. LDL-buret kolesterol är traditionellt kallat ”det onda” kolesterolet, medan HDL-buret kolesterol är kallat ”det goda”. Detta beror på att LDL-kolesterol transporteras från levern till perifera vävnader, vilket möjliggör att kolesterolet fastnar i kärlväggen och resulterar i att en åderförfettning startar. HDL-kolesterol å andra sidan, transporterar kolesterol från perifera vävnader till levern, där det istället tas om hand om för att avsöndras från kroppen.

Samtliga celler i kroppen har kapacitet för att lagra fetter och kolesterol, detta i så kallade lipid-droppar och eftersom fetterna inte är vattenlösliga, måste dessa lipid-droppar stabiliseras med hjälp av proteiner som kallas perilipiner. Ett av dessa proteiner är perilipin-2. Då nivåerna av lipider ökar, ökar också nivåerna av perilipin-2 och detta protein finns det gott om i kärlväggens inflammatoriska celler vilka bidrar mest till åderförfettning. Perilipin-2 reglerar också inlagringen av lipider samt huruvida lipider mobiliseras och senare användas som energikälla för cellerna.

Autofagi, grekiska för själv-ätande, är också ett sätt för celler att mobilisera lipider, som skiljer sig från den mer traditionella väg som regleras av perilipiner.

Eftersom perilipin-2 spelar en sådan central roll i att stabilisera lipid-droppar i celler som bidrar till åderförfettning, så ställde vi hypotesen att en vanlig förändring i strukturen av perilipin-2 påverkar inlagringen av det onda kolesterolet i dessa celler. Individer rekryterades till vår studie med avseende på deras variant av perilipin-2 och vi visar att en vanlig proteinförändring i perilipin-2 påverkade inte bara inlagringen av kolesterol i inflammatoriska celler, utan ökade även nivåerna av autofagi, vilket resulterade i att transport av kolesterol till HDL ökade. Parallellt med ökad kolsterol-transport mot HDL, som anses vara skyddande för åderförfettning, så minskade kärlväggens tjocklek (ett mått av åderförfettning) i en europeisk population med hög risk för hjärt-kärlsjukdom hos de individer som bar på proteinförändringen. Vidare visar vi att autofagi ingår i ett intrikat molekylärt förhållande med det maskineri som förser HDL-partiklarna med kolesterol, något som aldrig tidigare visats. Sammantaget, pekar dessa data på att en vanlig variant av perilipin-2 är skyddande mot åderförfettning.

I avhandlingens två sista delarbeten visar vi att två autofagi-relaterade proteiner är vanligt förekommande i åderförfettning av halspulsådern (också kallat plack) hos svenskar som genomgått kirurgi för att ta bort dessa plack. Om plack spricker, kan detta innebära att en hjärtinfarkt eller stroke uppstår. Deras risk för att spricka har namngivits termen ”stabilitet”. Dessa autofagi-relaterade proteiner påverkar plackens stabilitet genom att reglera inflammation samt funktionen av muskelceller som finns i kärlväggen. Funktionen hos dessa muskel-

celler bidrar till kärlens elasticitet/styvhet. Mer inflammation och en styvare kärlvägg är karaktärsdrag hos ett mindre stabilt plack vilket har hög risk för spricka.
Avhandlingen i sin helhet pekar på att autofagi spelar en central roll i åderförfettning genom att reglera både själva inlagringen av kolesterol i kärlväggen, inflammationens utsträckning och till slut kärlväggens muskelcellers funktion. Sammantaget påverkar detta stabiliteten av de så kallade placken som åderförfettningen utgör, vilket påverkar en individs risk att råka ut för en hjärtinfarkt eller stroke. Behandling av åderförfettning med avseende på autofagi, kan komma att vara invecklad då tidsramen för behandlingen med största sannolikhet är snäv och måste definieras väl.

POPULAR SCIENCE SUMMARY

Atherosclerosis is an inflammatory disease of blood vessels that is driven by the retention of cholesterol within the vessel wall. Atherosclerosis is the underlying cause of most cardiovascular diseases, including heart attack and stroke. Retained cholesterol evokes an inflammatory response by white blood cells that are present within the vessel wall. Since cholesterol and lipids (fats) are not soluble in water, they are carried by lipoproteins. Different lipoproteins serve different functions. For example, cholesterol carried by low-density lipoprotein, also referred to as the "bad cholesterol", is delivered to peripheral tissues from the liver. This facilitates the retention of cholesterol at sites where it should not be, i.e. the vessel wall. Cholesterol carried by high-density lipoprotein on the other hand, transports cholesterol in the reverse direction, and reduces the risk of cholesterol retention in vessel walls. This is why it is considered to be the "good cholesterol".

Virtually any cell (our body's smallest single unit making up all our organs) has the capability of storing lipids and cholesterol. Cells store lipids in lipid droplets, and since lipids are not soluble in water, these droplets are stabilised by proteins called perilipins. Perilipin-2 is such a protein. Perilipin-2 plays a central role in stabilising the lipid droplet and is abundant in the white blood cells, which contribute to the development of atherosclerosis. As lipid levels increase in a cell, so do the levels of perilipin-2. This in turn regulates the mobilisation or usage of lipids as an energy source for the cell.

Autophagy, Greek for self-eating, is yet another mechanism by which a cell can regulate its fat metabolism, independent of perilipins.

Since perilipin-2 plays such a central role in lipid metabolism and is abundant in cells contributing most to atherosclerosis, we hypothesised that a common structural variation in this protein alters lipid metabolism, atherosclerosis and risk of cardiovascular disease. Individuals were recruited to our study based on their structure of perilipin-2. We were able to show that an altered structure of perilipin-2 brings about a reduced cholesterol accumulation in white blood cells and increased autophagy coupled to cholesterol transport to high-density lipoprotein. In parallel, a European population at high risk of cardiovascular disease had their vessel walls measured by ultrasound. The vessel walls' thickness was reduced in those individuals carrying the altered structure of perilipin-2, which indicates a lower risk of cardiovascular disease. Further, we were able to demonstrate that autophagy is interconnected with the machinery that loads cholesterol onto the high-density lipoprotein particle in an intricate molecular fashion, something that has never been revealed before. Collectively, data suggest that a structural variant in perilipin-2 and the increase in autophagy that it brings about, is protective of atherosclerosis.

In the two subsequent studies of this thesis, two autophagy-related proteins were abundant in atherosclerotic lesions (also called "plaques") of human carotid arteries that were surgically removed from patients suffering from atherosclerosis in the attempt to prevent cardiovascular disease. A plaque may rupture if it grows too much. This will result in a heart attack or stroke. A plaque's inherent risk of rupturing has been termed "stability". The presence of these autophagy-related proteins modifies the stability of the plaques by altering the inflam-

matory responses and the function of the muscle cells of the vessel wall that contribute to its elasticity/stiffness. More inflamed and stiffer vessel walls are characteristics of a more vulnerable or less stable plaque with high risk of rupturing.

The thesis in its entirety emphasises the role of autophagy in atherosclerosis by regulating the retention of cholesterol, extent of inflammation within the vessel wall and the function of muscle cells in the vessel wall. Taken together, all this influences the stability of the atherosclerotic plaques, which alters an individual's risk of suffering a heart attack or stroke. Treatment of atherosclerosis which targets autophagy may be challenging, since timely treatment is essential and the optimal time-frame for treatment is in all likelihood slim and yet to be defined.

LIST OF SCIENTIFIC PAPERS

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II. ***Subclinical atherosclerosis and its progression are modulated by PLIN2 through a feed-forward loop between LXR and autophagy.***
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* Equal contribution
Eur Heart J., under review.

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Paloschi V*, Gonçalves I*, **Saliba-Gustafsson P***, Werngren O, Perisic Matic L, Skogsberg J, Razuvaev A, Mitsios N, Mulder J, Janas V, Bengtsson E, Jin H, Li Y, F.J. Ketelhuth D, Maegdefessel L, Hedin U, Eriksson P, Ehrenborg E*, Magné J*
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SCIENTIFIC CONTRIBUTIONS NOT INCLUDED IN THE THESIS

I. ***Identification of a novel proinsulin-associated SNP and demonstration that proinsulin is unlikely to be a causal factor in subclinical vascular remodelling using Mendelian randomisation.***
Strawbridge RJ, Silveira A, Hoed MD, Gustafsson S, Luan J, Rybin D, Dupuis J, Li-Gao R, Kavousi M, Dehghan A, Haljas K, Lahti J, Gådin JR, Bäcklund A, de Faire U, Gertow K, Giral P, Goel A, Humphries SE, Kurl S, Langenberg C, Lannfelt LL, Lind L, Lindgren CCM, Mannarino E, Mook-Kanamori DO, Morris AP, de Mutsert R, Rauramaa R, **Saliba-Gustafsson P**, Sennblad B, Smit AJ, Syvänen AC, Tremoli E, Veglia F, Zethelius B, Björck HM, Eriksson JG, Hofman A, Franco OH, Watkins H, Jukema JW, Florez JC, Wareham NJ, Meigs JB, Ingelsson E, Baldassarre D, Hamsten A; IMPROVE study group.
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* Equal contribution
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LIST OF ABBREVIATIONS

27HC	27-hydroxycholesterol (27OH-cholesterol)
3MA	3-methyladenine
ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette transporter G1
ACAT1	Acetyl-coenzyme A acetyltransferase 1
ACTA2	Alpha-actin 2
AMPK	Adenosine monophosphate-activated protein kinase
ASAP	Advanced Study of Aortic Pathology
ATG(s)	Autophagy-related gene(s)
ATG3	Autophagy-related 3
ATG4	Autophagy-related 4
ATG5	Autophagy-related 5
ATG7	Autophagy-related 7
ATG10	Autophagy-related 10
ATG12	Autophagy-related 12
ATG13	Autophagy-related 13
ATG16L1	Autophagy-related 16L1
BECN1	Beclin 1
BiKE	Biobank of Karolinska Endarterectomies
BMP2	Bone morphogenetic protein 2
BSA	Bovine serum albumin
CAD	Coronary artery disease
CaPi	Calcification medium
cIMT	Carotid Intima-media thickness
CMA	Chaperone-mediated autophagy
CPIP	Carotid Plaque Imaging Project
CVD	Cardiovascular disease
DAPI	4',6-diamidino-2-phenylindole
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmatic reticulum
FA(s)	Fatty acid(s)
FBS	Foetal bovine serum

GWAS	Genome-wide association studies
HDL	High-density lipoprotein
HEK293	Human embryonic kidney 293
Hsc70	Heat shock protein family A 70
IMPROVE	Carotid Intima-Media Thickness and IMT-Progression as predictors of Vascular Events
IMT	Intima-media thickness
IR	Insulin resistance
KLF-4	Kruppel-like factor 4
LAMP2A	Lysosome-associated membrane protein type 2A
LC3(-I/II)	Microtubule-associated protein 1 light chain 3
LD(s)	Lipid droplet(s)
LDL	Low-density lipoprotein
LPL	Lipoprotein lipase
LXR	Liver-X-receptor
MAP1LC3A	Microtubule Associated Protein 1 Light Chain 3 Alpha
MAP1LC3B	Microtubule Associated Protein 1 Light Chain 3 Beta
M-CSF	Macrophage colony stimulating factor
MI	Myocardial infarction
MMP(s)	Matrix metalloproteinase(s)
MSD	Meso-Scale Discovery
mTOR	Mammalian target of rapamycin
MYH11	Smooth muscle cell myosin heavy-chain
OA	Oleic acid
OrO	Oil red O
oxLDL	Oxidised low-density lipoprotein
p62	Sequestosome 1 (*also refer to SQSTM1*)
PBMC(s)	Peripheral blood mononuclear cell(s)
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PE	Phosphatidylethanolamine
PEST	Penicillin-Streptomycin
PLIN2	Perilipin-2
PLIN3	Perilipin-3
PVDF	Polyvinylidene difluoride

RPMI	Roswell Park Memorial Institute medium
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SM22α	Smooth muscle protein 22-alpha (*also refer to TAGLN*)
SMC	Smooth muscle cell
SMTN	Smoothelin
SNP	Single nucleotide polymorphism
SQSTM1	Sequestosome 1 *(also refer to p62)*
T2D	Type II diabetes
TAGLN	Transgelin (*also refer to SM22α*)
TC	Total cholesterol
TG(s)	Trigylceride(s)
TGF-β1	Transforming growth factor beta 1
UC	Unesterified cholesterol
ULK1	Unc-51-like kinase 1
VSMC(s)	Vascular smooth muscle cell(s)
VLDL	Very low-density lipoprotein

1 INTRODUCTION

1.1 CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD), including conditions such as coronary artery disease (CAD), peripheral vessel disease and stroke, remain the major causes of mortality and morbidity, accounting for around one third of all deaths worldwide. The underlying cause of the vast majority of CVD is atherosclerosis [(1)].

1.1.1 Risk factors

Risk factors of atherosclerosis and overt CVD include age, male sex, obesity, insulin resistance (IR), type II diabetes (T2D), dyslipidaemia, smoking, socio-economic status, hypertension and family history of CVD. Many risk factors are modifiable and/or preventable; for example controlling IR, T2D, obesity and hyperlipidaemia reduce CVD risk [(2)].

1.1.2 Public health burden

Not only is CVD the most common cause of death worldwide, with over 17 million people dying from CVD in 2015 [(1)], but additional estimates suggest that more than 1.9 billion adults were overweight and more than 10% of the world's adult population was obese in 2014 [(2)]. With these numbers in mind and a dramatic increase in IR and T2D, in concert with the increase in incidence of other CVD risk factors, the incidence of CVD is expected to rise over the next decade. This will have devastating consequences on public health.

1.1.3 Genetics

Although most CVD risk factors are modifiable, we all carry an intrinsic risk of developing CVD within our genome. Genome-wide association studies (GWAS) have unravelled well over 100 loci robustly associated with cardiovascular risk factors including T2D, IR, hyperlipidaemia as well as overt CVD [(3)]. GWAS comprise a completely unbiased scan of the genome, aiming at identifying loci associated to essentially any trait. Traditionally, GWAS were advantageous in identifying rather common loci associated with common diseases. A candidate gene approach is in complete contrast to GWAS in that a biological rationale supports the study of a particular locus in relation to a trait; this kind of approach can also be applied on common traits. The latter approach has been adopted in the two first constituent papers of this thesis.

1.2 ATHEROSCLEROSIS

Atherosclerosis involves lesion formation in intermediate-size and large arteries, characterised by lipid retention in the vessel walls, inflammation, cell death and fibrosis [4]. A maladaptive immune response to modified lipids retained in the vessel wall engages innate as well as adaptive immune mechanisms, initiating the formation of an atherosclerotic plaque [4-6]. As the disease progresses, flow-limiting stenosis may develop, clinically manifesting as angina pectoris in the event that coronary arteries are afflicted (*Figure 1A*). Modulation of the nature of shear stress and its magnitude on the shoulder-region of the plaque may contribute to plaque rupture, exposing pro-thrombotic material from the highly inflammatory core of the plaque. The exposure of pro-thrombotic material initiates the activation of the coagulation cascade, resulting in clot formation and potential complete occlusion of the vessel. If located in a coronary artery, this gives rise to a myocardial infarction (MI) [4, 5]. Many factors contribute to the risk of plaque rupture. Generally large necrotic cores and thin smooth muscle cell-rich fibrous caps are features of a prone-to-rupture or unstable plaque, also called thin-cap fibroatheroma [7, 8]. In some instances, the clot dislodges from the vessel wall, enters the circulation as an embolus, adheres at another location and occludes a distant vessel. This is a frequent mechanism of ischaemic stroke.

A plethora of immune and non-immune cells contribute to atherosclerosis development and progression. The macrophage foam cell is perhaps the cell that traditionally has defined atherosclerosis, and vascular smooth muscle cells are considered to modulate plaque stability. This thesis will therefore focus on these two cell types in atherosclerosis and how autophagy in these cells regulates their phenotype, atherosclerosis development and progression as well as cardiovascular risk.

1.2.1 The macrophage foam cell in atherosclerosis

The subendothelial retention of lipoproteins, constituting the very initiation of atherosclerosis, triggers a maladaptive immune response, resulting in a non-resolving inflammatory process driving disease progression [4, 9, 10]. Lipoproteins are susceptible to modification (e.g. aggregation, acetylation or oxidation) when within the vessel wall, and this induces the overlying endothelial cells to become activated. Once activated, the endothelium mediates the recruitment of monocytes, which differentiate to macrophages in the subendothelial space [11]. Within the atherosclerotic plaque, macrophages are the predominating immune cells [12]. A set of receptors, called scavenger receptors, internalise the modified lipoproteins, rendering macrophages, which are turning into foam cells, massively engorged with lipids. Different scavenger receptors may have different specificities for different kinds of low-density lipoprotein (LDL) modifications. Scavenger receptors AI and AII account for the vast majority of internalisation of acetylated LDL and have some specificity for extensively oxidised LDL (oxLDL) [13-16]. Contrasting, CD36 binds moderately oxLDL with high affinity, whereas it does not bind with acetylated LDL or extensively oxidised LDL [14, 17]. The LDL-receptor-independent uptake of atherogenic lipoproteins is devastating since it is not subjected to negative feedback, allowing enormous amounts of lipids to enter the progressively growing population of macrophage foam cells [18]. The substantial accumulation of cholesterol within the

macrophage foam cell has not only repercussions on cholesterol metabolism, but also on activation of innate immunity, cell death and efferocytosis [19-22] (*Figure 1B*).
Despite macrophages in atherosclerosis being described as rather deleterious, not all macrophages may be. The secretion of cytokines accompanies the retention of monocytic cells in the vessel wall and their subsequent differentiation to macrophages. The cytokine profile of monocyte-derived macrophages within the vessel wall, in concert with their cholesterol accumulation and gene expression profile, may describe the macrophage's role or subtype. Crudely, and probably quite controversially, one can divide macrophages into two subtypes; M1 (pro-inflammatory) and M2 (pro-resolving) macrophages. These two macrophage subtypes localise to different areas of the atherosclerotic plaque, and display distinct roles in the atherosclerotic process [23]. M1 and M2 macrophages can be polarised *in vitro* and, studies on these *in vitro* systems together with mouse models of atherosclerosis, have created a simplified dogma that M1 macrophages promote plaque inflammation and M2 macrophages resolve plaque inflammation. For example, removing the transcriptional programme resulting in the differentiation of M2 macrophages, potentiates the differentiation of M1 macrophages [24]. Ultimately, forcing macrophages into the inflammatory M1 phenotypes exacerbates atherosclerosis [25, 26]. Conversely, promoting an M2 phenotype results in a halted atherogenesis, and it has been shown that M2 macrophages are enriched in regressing plaques of atherosclerotic mice receiving an intense regimen of lipid-lowering drugs [27, 28]. Naturally, the assortment of macrophage phenotypes *in vivo* is likely to be much more complex than this binary model, not at least since macrophages encounter an array of microenvironment signals that may both coerce a specific phenotype, but also sometimes oppose each other.

1.2.2 Smooth muscle cells in atherosclerosis

Vascular smooth muscle cells (VSMCs) are very plastic, and in part, their phenotype depends on their origin. Lineage tracing studies have determined numerous developmental origins, which give rise to VSMCs [29]. Although lineage-specific VSMCs present distinct phenotypes, there are still considerable phenotypic similarities [30]. Considering these phenotypic differences between different VSMC lineages, care has to be taken when choosing an *in vitro* VSMC model system, as well as translating results obtained *in vitro* to clinical observations.
Normal, arterial VSMCs express an array of smooth muscle cell (SMC) markers. Conventionally, SMC myosin heavy-chain (*MYH11*), transgelin (*TAGLN* or *SM22α*), alpha-actin 2 (*ACTA2*) and smoothelin (*SMTN*) amongst others, have been considered markers of VSMCs. In atherosclerosis, as well as *in vitro*, VSMCs lose their expression of these markers and acquire capacities such as cytokine production, proliferation, migration and extracellular matrix (ECM) protein secretion [30-36]. This phenomenon has recently been termed, *phenotypic switching* of VSMCs. Phenotypic switching causes VSMCs to lose their contractility and this phenomenon has a significant impact on atherosclerosis development and plaque stability. Loss of contractile genes has proven to increase atherosclerosis, inflammation and neointimal macrophage infiltration *in vivo* [37].
Exposing VSMCs to cholesterol *in vitro* drives a phenotypic switch towards a macrophage-like phenotype; this also induces an increased expression of macrophage markers and down-

regulation of contractile VSMC genes [38]. Shankman et al. showed that these events were dependent on Kruppel-like factor 4 (*KLF-4*), which can be used as a marker for phenotypic switching of VSMCs. Significantly, macrophage-like VSMCs are distinct from macrophages in that macrophages are professional phagocytes, and thus have a decreased phagocytic activity as compared to macrophages [39].

By modulating VSMC phenotypes, autophagy is an important determinant of atherosclerosis progression. Transmission electron microscopy has generated ultrastructural data supporting the notion that the autophagic process is engaged in dying VSMCs of both human and rabbit atherosclerotic plaques [40, 41]. Upon vascular injury, VSMCs modify their phenotype from a contractile to a proliferative one, a feature stimulated by growth factors such as platelet-derived growth factor (PDGF). By treating VSMCs with PDGF, one is able to induce autophagy through what seems to be an AMPK- and mTOR-independent mechanism [42]. Conversely, inhibition of autophagy by 3-methyladenine (3MA) incapacitates phenotypic switching [42]. Recently, autophagy has been identified as a novel mechanism that protects against phosphate-induced VSMC calcification [43]. Drugs activating autophagy have been shown to protect VSMCs from becoming calcified through a transforming growth factor beta 1 (TGF-β1)-dependent process [44] as well as foam cell formation [45]. Defective autophagy in VSMCs affects their contractile capacity by altering Ca^{2+} homeostasis and mobilisation, resulting in vascular reactivity and alterations of the contractile apparatus [46]. Taken together, these data demonstrate that modulation of autophagy plays a pivotal role in VSMC plasticity and phenotypic switching.

Figure 1

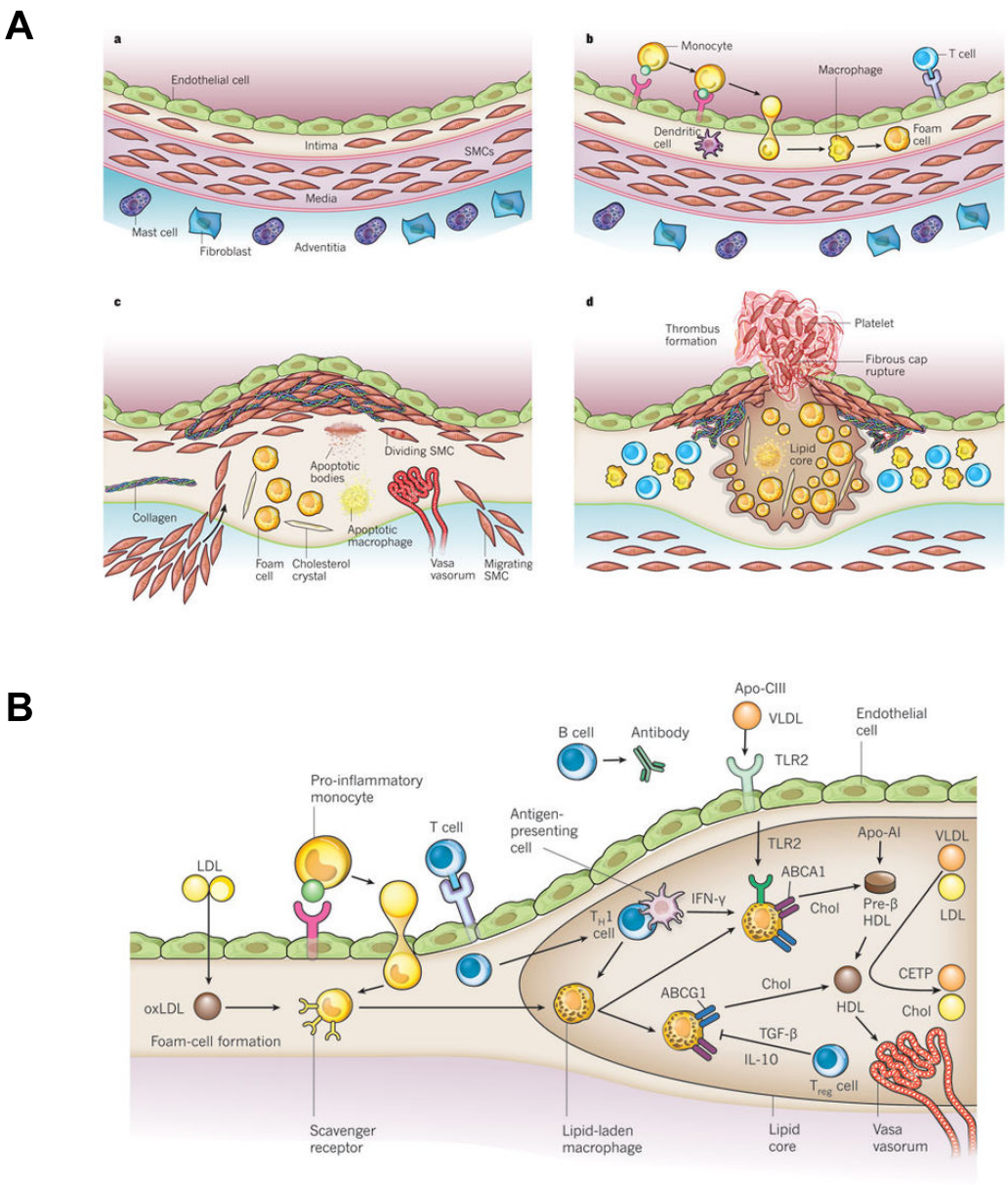

Figure 1 A) Illustration of the progression from a healthy vessel (upper left panel) to a fatty streak (upper right panel) to an atherosclerotic plaque (lower left panel) and finally plaque rupture (lower right panel). **B)** Exemplifies the events taking place within the plaques leading to the uptake of modified lipids and the foam cell formation.

1.3 LIPID METABOLISM AND CARDIOVASCULAR DISEASE

Lipids such as triglycerides (TGs), cholesterol esters and fatty acids, are important sources of energy. Lipids may be acquired and released into the circulation by dietary intake or endogenous production (release from storages). In the circulation, lipids are carried by lipoproteins which solubilise neutral lipids such as cholesterol esters and/or triglycerides so that they can be transported to tissues [47]. Lipids are transported to peripheral tissues by two lipoproteins; chylomicrons in the case of dietary lipids or very low-density lipoprotein (VLDL) in the case of endogenous mobilisation from the liver. When at their destination (e.g. adipose tissue or

skeletal muscles), the lipoproteins interact with lipoprotein lipase (LPL), which hydrolyses triglycerides to free fatty acids (FAs) and glycerol for uptake by the tissue.

1.3.1 Lipoprotein metabolism

As the lipoprotein particles interact with LPL, neutral lipids are removed and the composition of the lipoprotein particle changes. In the case of VLDL, this particle forms the highly atherogenic LDL particle [48, 49]. Modified LDL particles contribute to foam cell formation and the build-up of an atherosclerotic plaque. In addition to the uptake of FAs and glycerol by peripheral tissue, the whole lipoprotein particle can also be taken up through, for example, the LDL-receptor.

1.3.2 Intracellular lipid metabolism

Once in the cell, FAs may be re-esterified to form triglycerides, and stored within lipid droplets. Alternatively, FAs may form acetyl coenzyme A through the process of beta-oxidation and participate in the citric acid cycle, yielding large quantities of energy. From here the triglyceride building blocks may end up in cholesterol production used for membrane formation, for example. The modified lipoproteins subsequently end up in the endolysosomal compartment, where cholesteryl esters of the lipoproteins are hydrolysed to free cholesterol and fatty acids. Free cholesterol may undergo re-esterification by acetyl-coenzyme A acetyltransferase 1 (ACAT1) to generate cytoplasmic cholesteryl esters, which creates the foamy appearance of the macrophage foam cell [18, 50]. The liver-X-receptor (LXR) is a transcription factor, which controls numerous genes involved in lipid and cholesterol metabolism. LXR-dependent upregulation of cholesterol transporters such as ATP-binding cassette transporter A1 (ABCA1) and ATP-binding cassette transporter G1 (ABCG1) results in efflux of cholesterol to extracellular acceptors. This is an important process through which macrophages expel their excess cholesterol [51]. Mouse model studies have proposed that LXR-dependent cholesterol efflux from macrophages is a major determinant of susceptibility to atherosclerosis [52, 53]. Corroborating these data, the ability of plasma to accept cholesterol expelled from macrophages via, for example, the ABCA1-mediated pathway, has been shown to be inversely associated with the incidence of cardiovascular events [54]. This ability has been designated the term “cholesterol efflux capacity”.

1.3.3 Implication for cardiovascular disease

As plasma levels of atherogenic lipoproteins rise, so does the risk of retention of these particles within the vessel wall, and consequently also the risk for atherosclerotic plaque build-up. Deregulated intracellular lipid and cholesterol metabolism may result in the inability of macrophage foam cells to expel their excessive cholesterol, ultimately initiating a vicious cycle where excessive amounts of cholesterol are taken up, but none expelled. Finally, cholesterol levels may rise to cytotoxic levels, exacerbating plaque formation by activation of maladaptive innate and adaptive immune responses as well as efferocytosis in macrophage foam cells [19-22]. This gives rise to larger lipid-rich, inflamed necrotic cores, which aggravate the risk for plaque rupture, resulting in a cardiovascular event.

1.4 AUTOPHAGY

Autophagy, Greek for self-eating, was first described in the 1960's and is considered to be a cell survival mechanism. Macroautophagy, henceforth simply referred to as autophagy, involves the formation of a double membrane vesicle, sequestering parts of the cytoplasm and/or damaged organelles for degradation through the later fusion with the lysosome [55].

The process of autophagy has been implicated in numerous physiological as well as pathophysiological processes. Traditionally, autophagy has been considered a cell survival mechanism, protecting the cell from a plethora of stressful stimuli [56, 57]. Under normal conditions, autophagy may serve as means by which misfolded proteins or damaged organelles are disposed of [55]. Apart from being involved in the removal of intracellular bacteria [55], autophagy has been implicated in chronic inflammation, cancer, diabetes, cardiovascular disease, neurodegenerative diseases like Huntington's and Parkinson's disease, as well as ageing [58-60]. It is not surprising that over-stimulated autophagy may be overwhelming for the cell, and ultimately result in cell death [56, 61-63].

Early studies in yeast unravelled a complex autophagy molecular machinery involving modifications and interactions of numerous proteins, which are homologues of products of human autophagy-related genes (ATGs). Since it was first described, the molecular machinery of autophagy has been well characterised and now we know that numerous ATGs are well conserved from yeast to human and absolutely pivotal for functional autophagy [60, 64-67] (*Figure 2*). Ohsumi and colleagues worked extensively to map the autophagy machinery, which started in yeast. Ohsumi was awarded the Nobel Prize in 2016 for his great contributions to the field. Despite almost 60 years of autophagy research, its pleotropic effects in cellular homeostasis and metabolism, and by extension human disease, is still partly eluding us. Although autophagy is being targeted in a number of conditions, timely treatment is essential and frequently disregarded, potentially resulting in overlooked adverse effects.

Figure 2

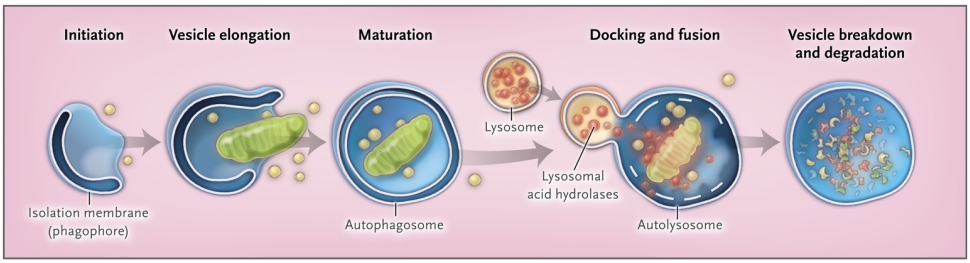

Figure 2. Visualisation of the autophagy process; from the initiation of the isolation membrane to the vesicle breakdown taking place in the autolysosome.

1.4.1 Induction of autophagy

The autophagy process consists of five distinct stages, initiated by the nucleation of the autophagosomal membrane, involving ATGs such as the unc-51-like kinase 1 (ULK1) and beclin 1 (BECN1) complexes. The location of the ULK1 complex determines the source of the

autophagosomal membrane, which has been shown to be either the endoplasmatic reticulum (ER), mitochondria or cell membrane [68].

The master gatekeeper of autophagy is the mammalian target of rapamycin (mTOR), which constitutively inhibits the activation of the ULK1 complex. This is accomplished by the phosphorylation of ULK1 itself as well as ATG13. Inhibition of mTOR, and thus dephosphorylation and consequential disinhibition of the ULK1 complex, readily induces vesicle nucleation and activation of the autophagic machinery [69, 70]. This can be accomplished by physiological cues such as nutrient deprivation or hypoxia, or pharmacologically by, for example, everolimus or rapamycin [71, 72]. Pharmacological inducers of autophagy have lately been given immense attention since inducing autophagy may be beneficial in several disease states. Metformin, a T2D drug, activates adenosine monophosphate-activated protein kinase (AMPK), which results in activating phosphorylation of early autophagy related genes ULK1 and BECN1. Similarly, statins are also known to activate AMPK and thereby induce autophagy [73].

1.4.2 Molecular machinery of autophagy

Autophagosomal elongation and later maturation are governed by two parallel ubiquitin-like conjugation systems; the ATG5-12-16L1 system and the ATG7-ATG3 system. Ultimately, the complexing of these ATGs and consequential activation, results in the conjugation of phosphatidylethanolamine (PE) to the cytosolic form of the microtubule-associated protein 1 light chain 3 (LC3), converting LC3-I to its membrane-bound counterpart LC3-II [74]. LC3-II indicates autophagosome formation, and has therefore become the mainstream readout in investigation of autophagy. LC3-I is first cleaved by ATG4 then, ATG7 and ATG3 (which are E1- and E2-like enzymes, respectively), which accomplishes PE conjugation to LC3 by their sequential activation [75, 76]. Concurrently, ATG7 and ATG10 together mediate the covalent binding of ATG5 to ATG12. The recently formed ATG5-12 complex subsequently complexes with ATG16L1, and the ATG5-12-16L1 complex dimerises and can act as an E3-like ligase to conjugate PE to LC3 [75-78].

Conveniently, PE-conjugation of LC3 (also referred to as lipidation of LC3) causes a downward shift of LC3-II from LC3-I on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), giving rise to a double-band at approximately 18 and 16 kDa [79]. Therefore, western blotting for LC3-II has emerged as one of the golden standards for measuring autophagic activity. However, once the autophagosome has fused with the lysosome, LC3-II will be recycled into the cytoplasm and reconverted into LC3-I. Effectively, both increases and decreases of LC3-II may be indicative of high autophagic activity. Therefore, blockers of autophagosome-lysosome fusion (e.g. chloroquine and bafilomycin A1) are efficient in inhibiting the LC3-recycling and results in an accumulation of LC3-II during high autophagic activity, allowing for a semi-quantitative determination of autophagic activity using western blot. The LC3-II expression, relative to an endogenous control, with or without the supplementation of a blocker of autophagosome-lysosome fusion is used to measure autophagic flux [79, 80]. LC3-II may form a complex with sequestosome 1 (SQSTM1 or p62), which is attached

to the target selected for degradation. Levels of p62 are, in contrast to LC3-II levels, inversely correlated with autophagic activity [80].

1.4.3 Variations of autophagy

There are several variations of autophagy, including microautophagy, chaperone-mediated autophagy, lipophagy, mitophagy and xenophagy. This section will cover the variations relevant to the research project.

1.4.3.1 Lipophagy

Lipophagy is a form of macroautophagy where the autophagosome specifically degrades lipid droplets in order to mobilise lipids from intracellular storages. Under basal conditions, autophagy sequesters both lipid droplets and other organelles targeted for destruction via the lysosome. When cells are subjected to an acute increase in intracellular lipids, rates of lipophagy are increased to buffer the increased lipid load and thereby maintain cellular homeostasis [68, 81]. However, when lipid levels are chronically increased, levels of both lipophagy and autophagy may be reduced [81]. Several lines of evidence disseminate the importance of autophagy in lipid homeostasis. Case in point, mice deficient of proteins essential for liver autophagy show massive hepatic steatosis [81]. In macrophage foam cells, cholesterol efflux is facilitated by autophagy and is believed to protect from oxidative stress [82].

1.4.3.2 Chaperone-mediated autophagy

Chaperone-mediated autophagy (CMA) is a process by which cargo is directly delivered to the lysosome for degradation. It is distinct from microautophagy, where invaginations of the lysosome facilitate the transfer of cargo into the lysosome, in that only proteins can be targeted and the target needs a chaperone for its delivery to the lysosome.

CMA targets contain a pentapeptide motif that is biochemically similar to Lys-Phe-Glu-Arg-Gln (frequently referred to as the KFERQ-motif) [83], which can be recognised by heat shock protein family A 70 (Hsc70), a constitutive cytosolic chaperone delivering target proteins to the lysosomal surface [84]. Once the substrate-chaperone complex is situated at the lysosomal surface, it interacts with the receptor lysosome-associated membrane protein type 2A (LAMP2A), which multimerises and forms a translocation complex carrying out the transfer of the substrate into the lumen of the lysosome [85-89].

CMA has been assigned functions in cellular quality control, cellular energy metabolism, neuronal survival, kidney growth, antigen presentation and transcription regulation. Failure of CMA in any of these processes may give rise to increased susceptibility to stress, neurodegeneration, kidney disorders and altered immunity [90, 91]. CMA is also a means by which the cell is able to mobilise lipids. Several proteins involved in lipid metabolism are CMA targets, including perilipin-2 (PLIN2) and perilipin-3 (PLIN3) [92]. Most significant for the purpose of this thesis is the targeting of the lipid droplet-associated proteins PLIN2 and PLIN3. Kaushik and Cuervo elegantly showed that PLIN2 and PLIN3 are not only CMA targets but also that their degradation through CMA allows cytosolic lipases (e.g. ATGL) access to the lipid droplet, thereby facilitating lipolysis [93, 94].

1.4.4 Autophagy in atherosclerosis

As previously stated, autophagy has been shown to serve as an important regulator of intracellular lipid homeostasis and macrophage reverse cholesterol transport. Thus, autophagy is a crucial regulator of atherosclerosis initiation – foam cell formation [95, 96]. In VSMCs, autophagy regulates phenotypic switching, and may thereby modulate plaque stability. While basal levels of autophagy may be atheroprotective, based on *in vitro* experiments by Ouimet and colleagues, excessive autophagy activity may contribute to a more unstable atherosclerotic plaque phenotype, by promoting autophagic smooth muscle cell death [56]. In advanced disease, disruption of macrophage autophagy by ablating Atg5 has been shown to enhance apoptosis, efferocytosis and oxidative stress in advanced atherosclerotic lesions [97]. Despite attempts to link deregulated autophagy to disease initiation and progression *in vitro* and *in vivo*, the role of autophagy in human clinical atherosclerosis and CVD remains poorly studied.

2 HYPOTHESES

2.1 GENERAL HYPOTHESES

PLIN2 is a central player in lipid metabolism. A functional single nucleotide polymorphism (SNP) in *PLIN2* has repercussions on plasma lipid profiles, which may modulate foam cell formation and cardiovascular risk. Further, since *PLIN2* is located in the crossroads between autophagy and lipid metabolism, the functional SNP in *PLIN2* may also alter autophagy activity. This may be the means by which this SNP mediates its effects on foam cell formation.
Despite efforts in trying to delineate the involvement of autophagy in atherosclerosis, data on the contribution of autophagy on human atherosclerosis development, progression and plaque stability are largely lacking. This thesis expands on existing data from animal studies and *in vitro* systems, which have implied that autophagy plays a significant role in foam cell formation, cholesterol metabolism and murine atherosclerosis.

2.2 SPECIFIC HYPOTHESES

2.2.1 Ser251Pro polymorphism in PLIN2 influences plasma lipid profiles and intracellular lipid metabolism

We hypothesised that the Ser251Pro polymorphism alters plasma lipid profiles and intracellular lipid metabolism.

2.2.2 Subclinical atherosclerosis and its progression are modulated by PLIN2 through a feed-forward loop between LXR and autophagy

In light of previous data generated by our laboratory, we hypothesised that the Ser251Pro polymorphism modulates macrophage foam cell formation and cardiovascular risk measured as carotid intima-media thickness.

2.2.3 ATG16L1 Expression in Carotid Atherosclerotic Plaques is Associated with Plaque Vulnerability

We hypothesised that ATG16L1 is expressed in atherosclerotic plaques, affects plaque vulnerability and is upregulated during foam cell formation.

2.2.4 Repression of *MAP1LC3A* during atherosclerosis progression plays an important role in regulating vascular smooth muscle cell phenotype

We hypothesised that the autophagy-related *Microtubule Associated Protein 1 Light Chain 3 Alpha/Beta* (*MAP1LC3A/B*) are expressed in advanced atherosclerotic plaques and modulates plaque vulnerability and subsequent symptom development.

3 METHODOLOGY

3.1 SEQUENCE ANALYSIS AND STRUCTURE MODELLING

The U.S. National Center for Biotechnology Information (NCBI; Bethesda, MD, USA) Entrez Protein database was used to download the PLIN2 protein sequence (NP_00112.2/ GI: 34577059) and the ClustalW programme [98] was adopted for sequence comparison across several species. The 3D-JiqSaw software was utilised to predict the secondary protein structure of PLIN2 [99]. Protein images were created using the University of California–San Francisco (UCSF) Chimera package from the Resource for Biocomputing, Visualization, and Informatics [100].

3.2 PRIMARY HUMAN MONOCYTE-DERIVED MACROPHAGES

Human monocytes were isolated either from fresh buffy coats of healthy donors (Blood Transfusion Centre, Karolinska University Hospital, Stockholm, Sweden), as described elsewhere, or from whole blood from healthy donors recruited by genotype [101]. Where the Ser251Pro polymorphism in *PLIN2* has been studied, monocytes were isolated from whole blood from healthy donors using a high-purity protocol outlined below. Briefly, human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats by endotoxin-free Ficoll density gradient centrifugation. Monocytes were then separated from lymphocytes by high-density hyper-osmotic Percoll density gradient centrifugation and separated from platelets and dead cells on a low-density iso-osomotic Percoll density gradient.

The high-purity protocol separates monocytes from PBMCs using a magnetic beads isolation kit from Miltenyi Biotec, which was carried out according to the manufacturer's instructions (Pan Monocyte Isolation Kit, #130-096-537). Monocytes were cultured in RPMI-1640 medium (Invitrogen) supplemented with penicillin-streptomycin, L-glutamine (2 mM) and 5% FBS (Invitrogen). The cells were seeded in 6-well plates at a density of 0.75×10^6 cells/mL and differentiated to macrophages in the presence of human recombinant macrophage colony stimulating factor (M-CSF, 100 ng/mL; PeproTech) over seven days.

3.3 LIPID DROPLET AND FOAM CELL FORMATION IN HUMAN MONOCYTE-DERIVED MACROPHAGES

Lipid droplet (LD) formation was induced in monocyte-derived macrophages by treating them with oleic acid (OA; 360 μM), complexed to fatty acid-free bovine serum albumin (BSA). Total RNA was isolated from mononuclear cells and reversely transcribed according to standard protocols.

In Paper II, where the beneficial effect of the Ser251Pro SNP in *PLIN2* was studied, human primary monocyte-derived macrophages were treated with oxLDL for 24 hours at 25 μg/mL. Untreated monocyte-derived macrophages served as controls. For autophagy flux assessment, the cells were pre-treated for 2 hours with bafilomycin A1 (100 nM, Sigma-Aldrich).

In Paper III, where the role of ATG16L1 in atherosclerosis was studied, human primary macrophages were treated with oxLDL at different time points (2, 6, 24 or 48 hours) and at

different doses (25, 50 or 100 μg/mL). Monocyte-derived macrophages treated with native LDL (indicated as oxLDL dose 0), served as controls. For autophagy flux assessment, cells were pre-treated for 2 hours with bafilomycin A1 (100 nM, Sigma-Aldrich).

3.4 LIPID DROPLET STAINING AND SUBSEQUENT LIPID QUANTIFICATION

In order to quantify intracellular LDs in monocyte-derived macrophages, cells were plated on coverslips, and after treatment fixed with formaldehyde (4%) in phosphate-buffered saline (PBS), and stained with oil red O (OrO) and haematoxylin as described previously [50]. Samples were scanned using the BioPix software (Biopix AB) [102]. Intracellular TG content was quantified by high-performance liquid chromatography as described previously [103].

3.5 PLIN2 CONSTRUCTS AND STABLY TRANSFECTED HEK293 CELLS

To generate human *PLIN2* expression constructs, *PLIN2* cDNA was amplified from the image clone IOH5658 (Invitrogen), using primers containing the NheI and NotI restriction enzyme sites. The fragments were then inserted into pcDNA3.1$^+$ plasmid using the NheI and NotI restriction sites, generating a p*PLIN2* containing plasmid. A fragment containing the IVS and IRES (IIRES) from pIREShyg (Clontech) was amplified and inserted into the p*PLIN2*-IIRES plasmid using NotI and XbaI restriction sites. Finally, EGFP from pEGFP-1 (Clontech) was amplified and inserted into the p*PLIN2*-IIRES plasmid using AgeI and XbaI restriction enzyme, generating a p*PLIN2*-IIRES-EGFP construct.

The QuickChange II site-directed mutagenesis kit (Stratagene) was used to introduce the rs35568725 missense polymorphism (Ser251Pro) in the p*PLIN2*-IIRES-EGFP construct. Mutated *PLIN2* clones (Pro251) were identified and verified by sequencing and inserted directly into the p*PLIN2*-IIRES-EGFP vector using NheI and NotI restriction sites, replacing the wild-type *PLIN2* fragment. Fragments generated by PCR (Pfu Ultra; Stratagene) were all subcloned into pCR2.1 TOPO and positive clones were then identified by restriction analysis and verified by sequencing.

Human embryonic kidney 293 (HEK293) cells were stably transfected with human p*PLIN2*-IIRES-EGFP constructs containing the wild-type sequence (Ser251) or the minor allele sequence (Pro251) lipofectamine 2000 (Invitrogen). Stable clones were selected by culturing transfected cells with 600 μg/mL Geneticin (Invitrogen) and then maintained in 50 μg/mL Geneticin. Clones were selected by assessing PLIN2 protein expression (Western blot). HEK293 cells were cultured in DMEM (Invitrogen) supplemented with 10% iron-supplemented calf serum (JRH Biosciences), 100 U/mL penicillin G sodium, and 100 mg/mL streptomycin sulfate (Invitrogen). Where indicated, the cell culture medium was supplemented with 360 μM OA (Nu-Check Prep) complexed to fatty acid-free BSA.

All manipulations of autophagy and/or LXR activity followed an OA treatment, which was used to stabilise *PLIN2*.

3.6 IMMUNOCYTOCHEMISTRY AND CONFOCAL MICROSCOPY

PLIN2 immunocytochemistry was performed on monocyte-derived macrophages using a polyclonal antibody (Fitzgerald Industries) and Alexa488 goat anti-guinea pig secondary an-

tibody (Invitrogen), as described previously [104]. In stably transfected HEK293 cells, immunofluorescent detection of LDs and LD-associated proteins were performed, as described previously [105]. Briefly, cells were treated in 4-chamber culture slides, and then fixed in formaldehyde 4% for 30 mins. To detect the LDs, the cells were stained with BODIPY493/503 (Invitrogen) for 10 mins. To assess the localisation of PLIN2 and PLIN3, immunocytochemistry against PLIN2 was carried out and/or PLIN3 using a mouse monoclonal antibody against PLIN3 (Progen Biotechnik) at 1:500. The secondary antibodies were AlexaFluor 594 goat antiguinea pig and AlexaFluor 488 donkey anti-mouse, respectively (Invitrogen).
Images were obtained using a Leica SP5 confocal laser microscope using an ×63 oil, 1.4 NA objective lens (Leica Microsystems). Each image consisted of a Z stack of 10 to 20 optical slices taken at 0.2 μm intervals. The 2D images of flattened Z stacks consisted of 10 representative cells per condition in four separate experiments. The number, diameter, and area of the LDs were obtained using the spot detection module of the Imaris 7.2 software (Bitplane AG). The threshold of LD detection was automatically adjusted according to the absolute intensity of green pixels with an estimated LD diameter of 0.5 μm (average obtained by manual measurement on 10 cells) but including only spots larger than 0.2 μm. For colocalisation studies, undeconvoluted datasets were analysed using the Imaris software (Bitplane). The statistics modules in the Imaris colocalisation package determined quantitative data of colocalisation events. Intensities were given as the sum of all colocalised voxels. For quantitative analysis of colocalisation, Pearson's correlation coefficients were calculated.

3.7 TRANSMISSION ELECTRON MICROSCOPY

Cells were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at room temperature for 30 mins and then overnight at 4°C. Samples were postfixed in 2% osmium tetroxide in 0.1 M imidazole buffer (pH 7.4), at 4°C for 2 hours, dehydrated in ethanol followed by acetone, and embedded in LX-112 (Ladd). Ultrathin sections (40-50 nm) were cut with a Leica EM UC 6. Sections were contrasted with uranyl acetate followed by lead citrate and examined in a Tecnai 12 Spirit Bio TWIN transmission electron microscope (Fei Co.) at 100 kV. Digital images were acquired with a Veleta camera (Olympus). Images and LD size were analysed using ImageJ.

3.8 ISOLATION OF LIPID DROPLETS AND SILVER STAINING OF LIPID DROPLET-ASSOCIATED PROTEINS

LDs were isolated from cultured cells as described previously [106]. OA-treated HEK293 cells were scraped into cold PBS, pelleted, and resuspended in Hepes buffer. Samples were incubated on ice for 10 mins and homogenised with a 25-gauge needle. Cell homogenates were overlaid with Hepes buffer and centrifuged at 26,000 g for 30 mins. To remove contaminating cytosolic proteins, floating LDs were adjusted to 25% sucrose and transferred on top of a 60% sucrose cushion. The samples were overlaid with Hepes buffer and centrifuged again at 26,000 g for 30 mins. To further concentrate the samples, LDs were collected from the top and spun at maximum speed for 20 mins. The amount of protein in each sample was determined with the Bradford Protein Assay (Bio-Rad, Hercules). An equivalent amount of LD

protein from each cell line was separated on 11% polyacrylamide gels and visualised by silver staining.

3.9 LIPOLYSIS

Lipolysis experiments were performed as described previously [107]. Briefly, cells were plated in 24-well dishes and loaded with 360 μM OA complexed to fatty acid-free BSA for 24 hours to promote LD formation. ^{3}H-OA (0.4 μCi/well) was added as a tracer. Under these loading conditions, OA is stored intracellularily as TGs in LDs, and OA released upon lipolytic stimulation originates from these storages [108]. Cells were washed in PBS containing 4% BSA, followed by incubation with an efflux medium during the lipolysis experiment. Efflux medium contained 2.5 μM of the acyl-CoA synthethase inhibitor Triacsin C (Sigma-Aldrich), to prevent re-esterification of OA, and 1% fatty acid-free BSA, as acceptor. The efflux of ^{3}H-OA into medium was determined by scintillation counting.

3.10 THE STOCKHOLM COHORT AND OXFORD BIOBANK

The Stockholm cohort consists of 620 healthy 50-year-old males who are permanent residents of Stockholm and of northern European descent. This study was designed to carry out detailed studies on biochemical and molecular genetic mechanisms of atherosclerosis. Men identical in age were selected in order to minimise confounding factors. The participants have been extensively characterised with respect to anthropometric, metabolic and inflammatory parameters. The study population has previously been described [109]. The Oxford Biobank consists of an age-stratified random sample of men and women (aged 30 to 50 years) from Oxfordshire, UK. All participants are of white European origin. Data collection has been described in detail previously [110]. DNA was isolated and stored, and written informed consents were obtained to allow subsequent genotyping for SNPs of potential importance. Physical, demographic and laboratory data, as well as DNA were available for 1,493 individuals. Both studies were approved by local ethics committees.

3.11 GENOTYPING

The rs35568725 (Ser251Pro) SNP was genotyped by TaqMan technology (Life Technologies) using tailored primers and probes. In all cohorts adopted, the genotype frequency adhered to Hardy-Weinberg equilibrium.

3.12 ISOLATION AND MODIFICATION OF HUMAN LIPOPOTEINS

Lipoproteins used for foam cell formation as well as for cholesterol efflux assays were isolated from human plasma obtained from the blood bank at Karolinska University Hospital through sequential ultracentrifugation. Briefly, plasma was ultracentrifuged for >22 hours at 40,000 rpm at 4°C. The uppermost phase containing chylomicrons and VLDL was discarded and the intermediate phase containing LDL and high-density lipoprotein (HDL) was collected. The density of the LDL/HDL phase was adjusted to 1.063 g/mL with potassium bromide (Sigma-Aldrich) and ultracentrifuged as described. The upper phase containing LDL was collected and desalted using a PD-10 column (GE Healthcare). LDL was oxidised overnight

at 37°C using 20 μM copper sulphate [$CuSO_4$] (Merck). The reaction was stopped using 1 mM ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich). The lower phase containing HDL was desalted, sterile-filtered and diluted to a concentration of 2 mg/mL. The resulting diluted HDL was used as an acceptor in cholesterol efflux assays. For cholesterol efflux assays, copper-oxLDL was labelled with 2 μCi/ml ^{3}H-cholesterol (Perkin Elmer) at 4°C overnight, followed by removal of excess ^{3}H-cholesterol using a PD-10 column.

3.13 QUANTIFICATION OF INTRACELLULAR CHOLESTEROL AND LIPIDS

Upon termination of the foam cell formation assays, intracellular cholesterol and triglycerides were measured. Assessment of intracellular cholesterol and triglycerides were carried out at the Division of Clinical Chemistry, Karolinska University Hospital, Huddinge. Briefly, lipids were extracted from the cell monolayers by adding 2 mL hexane/isopropanol (3:2, v/v). Triglyceride (TG), total cholesterol (TC) and unesterified cholesterol (UC) mass was measured by enzymatic assays using commercially available kits (Roche Diagnostics GmbH, Mannheim and Wako Chemicals, Richmond, VA). The difference between TC and UC content was used to estimate cholesteryl esters. Data were corrected for cell protein content, measured according to the Lowry method in cell monolayers digested with 1 M NaOH. 27-hydroxycholesterol (27HC) levels were quantified by isotope dilution mass spectrometry, as previously described [(111)].

3.14 CHOLESTEROL EFFLUX ASSAY

Monocyte-derived macrophages were loaded with ^{3}H-oxLDL in Roswell Park Memorial Institute (RPMI) medium (+1% foetal bovine serum (FBS) and 0.1% Penicillin-Streptomycin (PEST)) for 24 hours. Cells were equilibrated for 2 hours with 0.5 mL RPMI medium with or without the supplementation of 100 nM bafilomycin A1 in order to inhibit autophagy. After equilibration, cells were washed with 3×1 mL PBS (Invitrogen, Stockholm, Sweden) and 0.3 mL RPMI medium containing the indicated acceptors (20 μg/mL apoA-1 (Sigma), 100 μg/mL HDL from human plasma or 1% total human serum) were added. RPMI medium alone was used as a control. Efflux was measured over 24 hours, after which, the medium was collected from each well and cells were lysed with 300 μL of 0.1 M NaOH. The collected medium and cell lysates were centrifuged at 12,000 rpm for 10 mins and cholesterol efflux was determined by scintillation counting. Protein measurements of the cell lysates were conducted according to the micro Bradford assay in accordance with the manufacturer's instructions (BioRad). Cholesterol efflux data were adjusted to the total protein content in lysates and presented as % efflux/μg protein.

3.15 INFLAMMATORY PHENOTYPING OF MACROPHAGE FOAM CELLS

Cytokine production profiles were used to assess the inflammatory response of macrophage foam cells. Cell culture medium samples were taken at 0, 6 and 24 hours after oxLDL stimulation. Multiplexed enzyme-linked immunosorbent assay (ELISA) was carried out on centrifuged medium supernatants using the Meso-Scale Discovery (MSD) Human Proinflammatory Panel 1 kit, simultaneously quantifying 10 cytokines (INF-γ, IL-1β, IL-2, IL-4, IL-6, IL-8,

IL-10, IL-12p70, IL-13 and TNF-α). All diluents, calibrators and samples were prepared and the assays were carried out according to the manufacturers' instructions.

3.16 THE IMPROVE STUDY

The IMPROVE study (acronym for "carotid Intima-Media Thickness and IMT-Progression as predictors of Vascular Events in a high risk European population") is a pan-European prospective, multicentre, longitudinal, observational study, designed to investigate whether cross-sectional carotid intima-media thickness (cIMT) and cIMT progression are useful predictors of cardiovascular events in European individuals at high risk of cardiovascular disease. The study comprises 3,711 participants, aged between 54 and 79 years, 48% of whom are male. Inclusion criteria were: presentation of ≥3 classical cardiovascular risk factors and absence of previous cardiovascular events at enrolment.

All participants underwent state-of-the-art high-resolution carotid ultrasound examinations following an established protocol applied at all recruitment centres. In brief, the mean and maximum cIMT measurements of the common carotid at the first centimeter proximal to the bifurcation, the common carotid (excluding the first centimeter proximal to the bifurcation), the carotid bifurcation and the internal carotid arteries were taken. cIMT is defined as the thickness of the vessel wall, measured from the leading edge of the lumen-intima interface to the leading edge of the media-adventitia interface. Segment-specific cIMT measurements were used to generate composite cIMT measurements; mean cIMT, maximum cIMT and the mean of the maximum cIMT [112, 113]. The entire cohort was genotyped for Ser251Pro single nucleotide polymorphism in PLIN2 using TaqMan probes as previously described [114]. Local research ethics review boards approved the study.

3.17 HUMAN ATHEROSCLEROTIC TISSUES

Human carotid atherosclerotic plaques from the Carotid Plaque Imaging Project (CPIP) were studied. This biobank includes carotid plaques from patients undergoing carotid endarterectomies at Skåne University Hospital, Malmö. Indications for surgery were: plaques associated with ipsilateral symptoms (transient ischaemic attack, stroke or amaurosis fugax) and stenosis higher than 70%, or plaques not associated with symptoms but with stenosis larger than 80%. Each patient gave informed consent to participate in the study. Assessment of cytokines, matrix metalloproteinases (MMPs) and cleaved caspase-3 was performed by ELISA in plaque homogenates, as previously described [115].

mRNA and protein expression levels were studied in 127 human carotid atherosclerotic plaques, including 10 control non-atherosclerotic iliac arteries, from the Biobank of Karolinska Endarterectomies (BiKE) study. Briefly, patients undergoing surgery for symptomatic or asymptomatic, high-grade (>50% NASCET) carotid stenosis at the Department of Vascular Surgery, Karolinska University Hospital, Stockholm, Sweden, were consecutively enrolled in the study and clinical data recorded upon admission. The BiKE study cohort demographics, details of sample collection, processing and microarray analyses have previously been described in detail [116]. The microarray dataset is available from Gene Expression Omnibus (GSE21545). In addition, atherosclerotic plaques from 18 patients (matched for male gender,

age and statin medication) were analysed using LC-MS/MS, as previously described [117].
As indicated, five human mammary arteries free from atherosclerosis were obtained from the Advanced Study of Aortic Pathology (ASAP), which served as controls. This biobank includes tissue biopsies from patients undergoing elective open-heart surgery for aortic valve disease and/or ascending aortic disease, as previously described [118].
Local research ethics review boards approved the studies.

3.18 IMMUNOHISTOCHEMISTRY

After surgical removal, carotid plaques were snap-frozen, and 1 mm fragments from the most stenotic region were obtained for histology. Transversal cryosections from the fragments were stained for plaque histology markers α-SMC-actin, CD68, OrO and Masson trichrome, as described previously [119] and total core area was estimated. When ATG16L1 expression was studied, primary antibody polyclonal rabbit anti-ATG16L1 (PM040, MBL International Corporation) and secondary antibody polyclonal goat anti-rabbit (DakoCytomation, Glostrup, Denmark) were used. Areas of the different stainings in the plaque (% area) were quantified blindly using Biopix iQ 2.1.8 after scanning with ScanScope Console Version 8.2 (LRI imaging AB) and photographed with Aperio image scope v.8.0.

3.19 IMMUNO-ELECTRON MICROSCOPY

After surgical removal, another 1 mm fragment of the carotid plaque (the most stenotic region), consecutive to the one used for histology, was fixed in 3% paraformaldehyde in 0.1 M phosphate buffer, washed and then infiltrated into 2.3 M sucrose and finally frozen in liquid nitrogen. Ultra-thin sectioning was achieved at -95°C and placed on carbon-reinforced formvar-coated, 50 mesh nickel grids. Grids were placed on droplets of 2% BSA (Sigma Fraction V) and 2% fish gelatin (GE Healthcare) in PBS to block non-specific binding. Sections were incubated in a humidified chamber at room temperature overnight with anti-ATG16L1 antibody in PBS containing 0.1% BSA and 0.1% gelatin. The sections were washed in phosphate buffer and bound antibodies were detected with protein A coated with 10 nm gold particles (Biocell). Sections were washed and fixed in 2% glutaraldehyde and contrasted with 0.05% uranyl acetate. They were subsequently embedded in 1% methylcellulose and examined in a Tecnai 10 microscope (FEI) at 100 kV. Digital images were taken with a Veleta camera (Soft Imaging System GmbH).

3.20 MOUSE MODEL OF ATHEROSCLEROTIC PLAQUE VULNERABILITY

mRNA expression analyses were performed in an atherosclerotic carotid plaque rupture model in ApoE-deficient mice involving carotid ligation with a contralateral, unligated control carotid artery of the same mouse [120]. Briefly, the model consists of an partial ligation (Vicryl 5-0 suture) of the common right carotid artery (just below the bifurcation) retained for four weeks. This triggers intimal hyperplasia and non-ruptured carotid atherosclerotic lesions. To provoke rupture of the developed plaque, a conical polyethylene cuff is placed proximal to the ligation site for four days [121] and roughly 50% of the 16-week old male mice display features of ruptured plaques. These features include endothelial cracks or ul-

cers, and/or intraluminal thrombus formation. After sacrifice, injured and control carotid arteries were embedded for sectioning (Cryomount, Histolab AB) and snap-frozen. The Stockholm Regional Board for Experimental Animal Ethics approved all experiments, and institutional guidelines for animal welfare were followed.

3.21 RAT MODEL OF INTIMAL HYPERPLASIA

Carotid artery balloon injury was performed on male Sprague-Dawley rats, as previously described [117, 122] and mRNA expression analysis of *Map1lc3a* was performed. The left carotid artery was dissected under isoflurane inhalation anaesthesia, an arteriotomy was performed in the external carotid artery and the common carotid artery de-endothelialised three times with a 2F Fogarty catheter. Animals were euthanised with isoflurane directly after injury (0 hours) or after 2 hours, 20 hours, 2 days, 5 days, 2 weeks, 6 weeks and 12 weeks after vascular injury. Both the left (injured) and right (uninjured) common carotid arteries were harvested (n=6 or n=7 animals at each time point). Arteries were washed with PBS to remove blood. Eight additional animals were sacrificed and uninjured carotid arteries used as controls (intact). Arteries were divided in a proximal segment used for RNA isolation and a distal segment used for histology. Total RNA was used for microarray analysis with Affymetrix GeneTitan Rat Gene ST v1.1 arrays. Experiments were performed according to the protocols approved by the Stockholm Regional Board for Experimental Animal Ethics, and institutional guidelines for animal welfare were followed.

3.22 IMMUNOFLUORESCENCE

In human and mouse atherosclerotic plaques, double immunofluorescent staining was used to identify which cell type contributes most to plaque expression of autophagy-related proteins. In short, acetone-fixed cryosections were incubated at 4°C overnight with rabbit primary antibodies against the autophagy-related protein of interest, followed by AlexaFluor 594 labelled goat anti-rabbit secondary antibodies for one hour. Subsequently, the sections were incubated overnight with mouse antibodies directed towards cell-specific markers followed by incubation with AlexaFluor 488 labelled goat anti-mouse antibodies, with subsequent staining of the nuclei using 4',6-diamidino-2-phenylindole (DAPI). Images were obtained using a Zeiss LSM700 confocal laser microscope using ×20, 0.8 NA objective lens (for the atherosclerotic lesions). Each image consisted of a Z-stack of 15 to 20 optical slices taken at 0.3 μm intervals.

Fluorescent triple-staining was used to identify which cell types contribute to MAP1LC3A expression in human atherosclerotic plaques. Four per cent paraformaldehyde-fixed cryosections were incubated with a monoclonal rabbit anti-LC3A antibody at 4°C overnight, followed by incubation with an anti-rabbit AlexaFluor 647 secondary antibody (Jackson Immunoresearch) for 90 mins. Subsequently, sections were incubated overnight with antibodies against relevant cell-specific markers, followed by an incubation with anti-mouse AlexaFluor 488 secondary antibodies (Jackson Immunoresearch). Staining of the nuclei was accomplished using Hoechst. Fluorescence microscope images were acquired on a Vslide scanning microscope (MetaSystems) using ×20 objectives and appropriate filter sets.

Whole microscope slides were scanned at ×2.5 and tissues were detected based on the Hoechst signal. After generating a position map, all tissue-covered areas were scanned using ×20 primary objective. Individual field of view images were stitched to generate a large 3-channel fluorescence image of the entire specimen with microscopic resolution. Images obtained with Vslide were analysed using Metaviewer (MetaSystems). The specificity of antibodies was confirmed by incubation with isotype-matched control IgG.

3.23 *IN VITRO* MODEL OF HUMAN VASCULAR SMOOTH MUSCLE CELL TRANS-DIFFERENTIATION

Primary human carotid artery VSMCs from Cell applications were cultured in SMC medium (SmGM-2, Lonza), supplemented with PEST, L-glutamine (2 mM) and 5% FBS. Human carotid VSMCs (40,000/well) were plated one day before transfection on 6-well plates and transfected at 40% confluence for 48 hours. They were then treated for either 48 or 72 hours with copper-oxidised oxLDL or with a calcification medium (CaPi), as previously described [123] (2.7 mM $CaCl_2$, 2.5 mM inorganic phosphate in M199 medium). Transfections were performed using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Scrambled- (Lot#: ASO107MI), human *MAP1LC3A*- (ref: s39157) and *MAP1LC3B*-small interfering RNAs (ref: s224886) were purchased from Ambion. All experiments were carried out in cell passages 3-6 and in biological as well as technical triplicates. To assess autophagy flux, cells were pre-treated for 2 hours with bafilomycin A1 (100 nM, Sigma-Aldrich), as indicated.

3.24 GENE EXPRESSION ANALYSES AND WESTERN BLOTTING

Total RNA and whole cell lysates were isolated from all the *ex vivo*/*in vitro* systems described above. In short RNA was reversely transcribed using Superscript III (Life Technologies). Polymerase chain reaction (PCR) amplification was carried out in 96-well plates in a 7,900 HT real-time PCR system (Applied Biosystems), using TaqMan® Universal PCR Master Mix (Applied Biosystems) and indicated TaqMan® Gene Expression Assays (*Table 1*). Results were normalised to the housekeeping gene *RPLPO*. The relative amount of target gene mRNA was calculated by $2^{-\Delta\Delta Ct}$ method and presented as a fold change.

A full transcriptome was obtained from the foam cell formation assay, where individuals were recruited by their genotype in the *PLIN2* locus, using the Affymetrix Clariom D platform. Data were RMA-normalised, annotated to the Genome Reference Consortium human genome build hg19 and differential expression was assessed using non-parametric statistics. Co-expression analyses were carried out using Spearman rank correlation coefficients. All downstream analyses were carried out using Bioconductor in R.

Protein expression was analysed by immunoblotting on whole cell lysates. Briefly, cell lysates were migrated on either a 10% or 14% SDS polyacrylamide gel. Proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes, after which immunoblotting was carried out with indicated antibodies (*Table 1*).

3.25 STATISTICS

As a general rule, non-parametric statistics have been applied to the data unless an assumption of normality of data has been fulfilled. In practice, this means that *in vitro* data has been analysed using Mann-Whitney tests and correlations displayed are Spearman rank correlation coefficients.

For genetic associations, the Plink software was utilised, and standard parametric linear models were generated assuming a recessive model.

$P<0.05$ was considered significant, and multiple testing adjustments were applied as appropriate.

Key Resources				
Product	**Company**	**Identifier**	**Application**	**Paper/s**
Anti-PLIN2 antibody	Fitzgerald Industries	AP002	IF, WB	I
PLIN2 assay	Life Technologies	Hs00605340_m1	TaqMan qPCR	I-II
RPLP0 assay	Life Technologies	Hs99999902_m1	TaqMan qPCR	I-IV
Anti-β-actin antibody	Sigma-Aldrich	A1978	WB	I-IV
Anti-PLIN2 antibody	Origene	Cat# TA307596	WB	II
Anti-p62 antibody	Santa Cruz Biotechnology	Cat# sc-28359	WB	II
ABCA1 assay	Life Technologies	Hs01059137_m1	TaqMan qPCR	II
CYP27A1 assay	Life Technologies	Hs0107992_g1	TaqMan qPCR	II
SREBP1c assay	Life Technologies	Hs00231674_m1	TaqMan qPCR	II
Anti-LC3 antibody	Novus Biologicals	Cat# NB100-2220	IF, WB	II-IV
Anti-ATG16L1 antibody	MBL International Corporation	PM040	IHC, IF, WB	III
Anti-CD68 antibody	DAKO	Clone KP1	IF	III
Anti-α-SMC-actin antibody	DAKO	Clone 1A4	IF	III
Anti-CD31 antibody	DAKO	Clone JC70A	IF	III
Anti-F4/80 antibody	Abcam	Clone BM8	IF	III
Anti-cleaved caspase-3 antibody	Cell Signalling	9961	IF	III
Anti-caspase-3 antibody	Cell Signalling	9962	WB	III
AlexaFluor 488 secondary antibody	Life Technologies	A11029	IF	III
AlexaFluor 594 secondary antibody	Life Technologies	A11012	IF	III
Anti-ATG16L1 antibody	MBL International Corporation	M150-5	IF	III.
Anti-MAP1LC3A antibody	Abcam	Ab62720	IHC, IF, WB	IV
MAP1LC3A assay	Life Technologies	Hs01076567_g1	TaqMan qPCR	IV
MAP1LC3B assay	Life Technologies	Hs00797944_s1	TaqMan qPCR	IV
MYOCD assay	Life Technologies	Hs00538071_m1	TaqMan qPCR	IV
ACTA2 assay	Life Technologies	Hs00426835_g1	TaqMan qPCR	IV
BMP2 assay	Life Technologies	Hs00154192_m1	TaqMan qPCR	IV
GABARAPL2 assay	Life Technologies	Hs00371854_m1	TaqMan qPCR	IV
GABARAP assay	Life Technologies	Hs00925899_g1	TaqMan qPCR	IV
GABARAPL1 assay	Life Technologies	Hs00740588_mH	TaqMan qPCR	IV

Table 1. List of key resources, producers, their identifiers and application. The "Paper/s" column indicates the papers in which the specific products were used. IF=Immunofluorescence; IHC=Immunohistochemistry; WB=Western blotting.

4 RESULTS

4.1 PAPER I

Selection of a PLIN2 protein variant for functional studies of the C-terminal 4-helix bundle

Since little is known about the physiological functions of the C-terminal 4-helix bundle domain of *PLIN2*, we sought to identify protein variants of *PLIN2* that could be used as tools to delineate the function of this region. The NCBI database for single nucleotide polymorphisms was adopted to find amino acid variants in the region spanning amino acid 220–437 of *PLIN2*. Fourteen protein missense variants in this particular region of *PLIN2* have been reported to the SNP database. The Ser251Pro (rs35568725) was the only variant that had a reported minor allele frequency >1% and heterozygosity >5%, which allows for recruitment by genotype through the reasonable high likelihood of finding carriers of the SNP. A Ser251Pro variant is also particularly interesting due to the substitution of serine to proline, which has a significant impact on the chemical properties of the peptide likely to ultimately influence the secondary structure of the protein. The serine residue at position 251 in PLIN2 is conserved across species. The PLIN2 secondary structure was created using 3D-JigSaw for modelling and the UCSF Chimera package for visualisation. The substitution of serine to proline at 251 was predicted to disrupt the α-helix between Thr225 and Phe262 located in a 4-α-helix bundle. The proline residue ablates the potential of making hydrogen bonds with the previous turn of the helices, resulting in conformational changes to the secondary structure. As helical distortions imposed by proline residues in a peptide chain may impact the biological function of the protein, we hypothesised that the Ser251Pro polymorphism would bring about an altered intracellular lipid metabolism.

The Pro251 protein variant of PLIN2 alters lipid accumulation, lipolysis and lipid droplet composition

Individuals carrying either variant of PLIN2 were recruited by genotype and primary monocyte-derived macrophages were prepared from whole blood. Monocyte-derived macrophages were treated with OA and lipid accumulation was assessed. Cells carrying the minor Pro251 displayed approximately a 1.75-fold increase in intracellular lipids as measured by OrO ($p=0.026$). This was replicated in an *in vitro* system of stably transfected HEK293 cells, where intracellular lipids increased more than 1.5-fold in cells carrying Pro251 ($p=0.04$). Also, TG content as assessed by high-performance liquid chromatography was increased 2-fold in cells carrying Pro251 ($p=0.002$). Significantly, no changes to PLIN2 protein or *PLIN2* mRNA were detected.

PLIN2 influences plasma lipid profiles

The Pro251 PLIN2 protein variant, which was shown to modulate lipid accumulation and lipolysis, was hypothesised to influence plasma lipid and lipoprotein profiles. Since plasma lipid and lipoprotein levels are largely dependent on intracellular lipid handling in hepato-

cytes, in concert with previous data herein, there was a clear rationale for studying the effects of Ser251Pro on plasma lipid profiles in humans. By using a dominant model, we show that the minor Pro251 allele was significantly associated with decreased plasma VLDL-TG concentration in a well-characterised Stockholm cohort (p=0.029). Since the number of homozygotes for the minor Pro251 allele is very low, a joint analysis combining the Stockholm cohort and the Oxford Biobank was performed. In the combined analysis, the minor Pro251 allele was significantly associated with decreased plasma TG concentration, using both a recessive (p=0.042) and a dominant (p=0.012) model. VLDL-TG concentrations were not used since this data was unavailable in the Oxford Biobank.

4.2 PAPER II

PLIN2 modulates atherosclerosis development and plaque growth

Since we previously reported that the Pro251 variant of *PLIN2* is associated with a more profitable plasma lipid profile and *PLIN2* is involved in the initiation of murine atherosclerosis, we investigated whether *PLIN2* modulates the development of human atherosclerosis. A high-risk pan-European population, which had undergone high-resolution ultrasonographic investigation of intima-media thickness (IMT) in carotid arteries, was genotyped for the *PLIN2* Ser251Pro polymorphism. The minor Pro251 allele was associated with decreased carotid IMT at several levels of the carotid artery tree compared to the major Ser251 variant. Most prominently, the Pro251 protein variant was associated with decreased carotid IMT measured as Mean-Max IMT at baseline (β=-0.013, p=0.03) and internal carotid (β=-0.012, p=0.003), as well as mean carotid artery IMT (β=-0.0046, p=0.05) after 30 months of follow-up compared to the major Ser251 allele (*Table 2*).

cIMT at Baseline

CHR	SNP	Major allele	Minor allele	cIMT-phenotype	MAF	BETA	SE	P
9	rs35568725	Ser251	Pro251	Mean Max IMT	0.05	-0.0128	0.006	0.03

cIMT at 30 months Progression

CHR	SNP	Major allele	Minor allele	cIMT-phenotype	MAF	BETA	SE	P
9	rs35568725	Ser251	Pro251	Mean IMT	0.05	-0.0046	0.002	0.05
9	rs35568725	Ser251	Pro251	Mean ICA IMT	0.05	-0.012	0.004	0.003

Table 2. rs35568725 is significantly associated with decreased cIMT both at baseline and at 30 months follow-up in a high-risk pan-European population. CHR=Chromosome; cIMT=carotid Intima-Media Thickness; MAF=Minor Allele Frequency; SE=Standard Error; SNP=Single Nucleotide Polymorphism.

Carotid atherosclerotic plaques originating from patients undergoing carotid endarterectomy were analysed for inflammatory and plaque stability markers. Carriers of the minor Pro251 variant of PLIN2 presented with a reduced plaque core area and plaque CD68 content. These data, in combination with the findings on cIMT, suggest that the 251Pro variant of *PLIN2* indeed possesses properties protective of atherosclerosis development and plaque progression.

PLIN2 modulates cholesterol accumulation, autophagy-dependent choelstero efflux activation, and LXR activation with repercussions on macrophage immunophenotypes

Since PLIN2 has a central role in lipid accumulation, we used the functional Ser251Pro protein variant in PLIN2 as a genetic tool to investigate whether this protein influences foam cell formation. Monocyte-derived macrophages carrying either variant of *PLIN2* were treated

with oxLDL in order to induce foam cell formation. The *PLIN2* protein variant did not alter the expression levels of *PLIN2* itself. However, cells carrying the Pro251 variant displayed reduced levels of cholesteryl esters, increased 27OH-cholesterol (27HC) and augmented autophagy activity. Additionally, Pro251 *PLIN2* increased the cholesterol efflux from oxLDL-loaded monocyte-derived macrophages (*Figure 3*). This was coupled with an increase in *SREBP1c* expression and significant co-expression between *PLIN2* and cholesterol transporters, all of which are LXR target genes. Human primary monocyte-derived macrophage foam cells carrying the rare Pro251 protein variant of *PLIN2* produced increased levels of the anti-inflammatory cytokine IL-10 and autophagy blockade ablated the differences between the two protein variants. Furthermore, Pro251 *PLIN2* mRNA was co-expressed with mRNA of *IL10* and *ARG1*, which are markers of M2-macrophages. Contrasting, Ser251 *PLIN2* mRNA was co-expressed with M1-macrophage markers *IL6* and *CD68*. Data suggest that PLIN2, through LXR and autophagy activation, may modulate the immunophenotype of macrophage foam cells by coercing an M2-phenotype. *PLIN2* is located in the crossroads of lipid metabolism and autophagy and when investigating the influence of autophagy on these parameters, we demonstrated that autophagy modulates the response pertaining to these measurements.

Figure 3

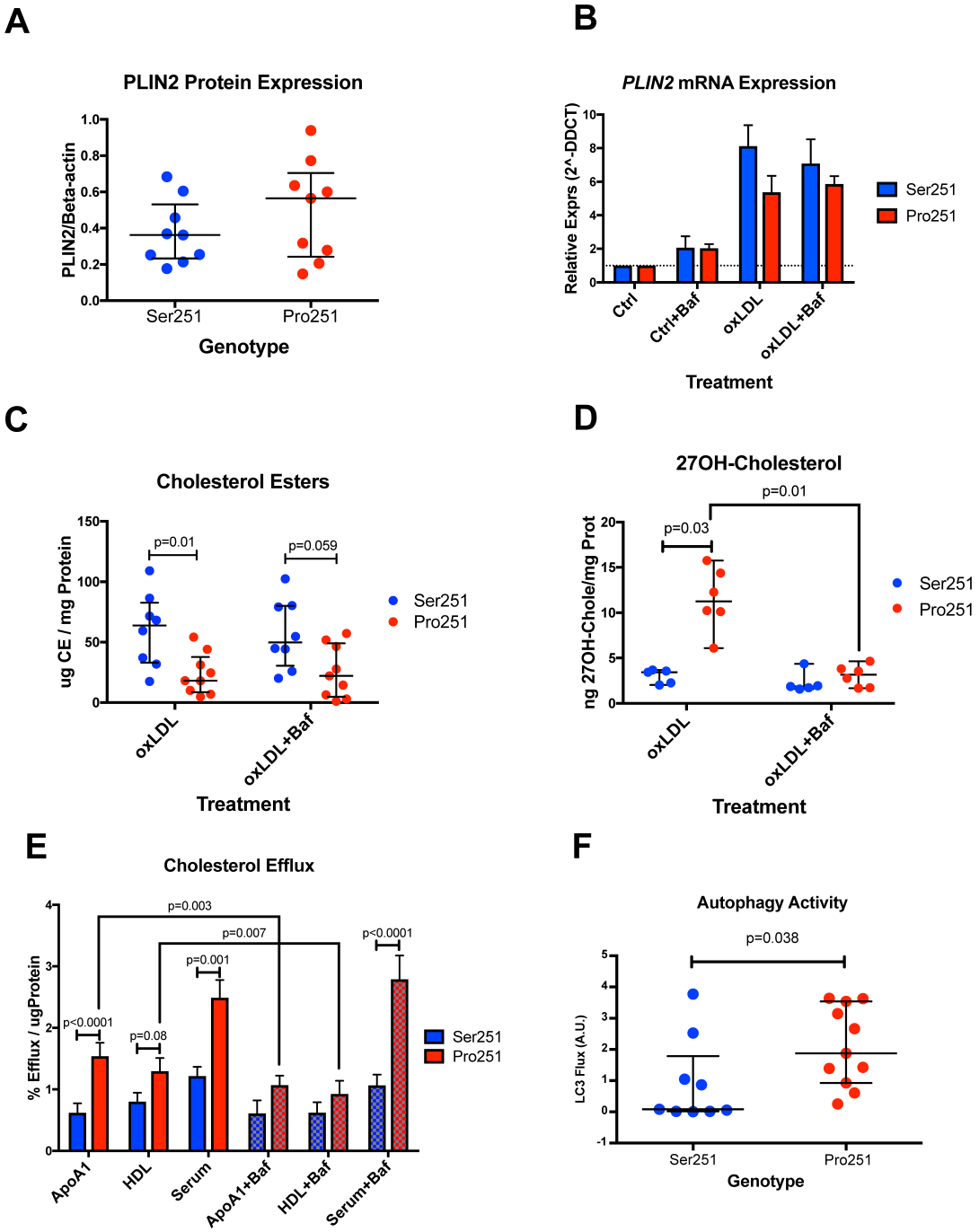

Figure 3. Human primary monocyte-derived macrophages were treated with oxLDL over 24 hours. *PLIN2* expression (**A-B**), cholesterol accumulation and efflux (**C-E**), as well as autophagy activity was assessed (**F**). Cells carrying the Pro251 variant of PLIN2 display with decreased cholesteryl esters, increased 27HC, cholesterol efflux and autophagy activity, while *PLIN2* expression remains constant.

Since the Pro251 variant in *PLIN2* was displayed with higher levels of 27HC, a modulation of LXR target gene expression, and increased cholesterol efflux, we thus sought to investigate whether the two variants of *PLIN2* presented with different levels of LXR activation. Stably transfected HEK293 cells carrying either variant of *PLIN2* were transfected with a luciferase reporter construct carrying an LXR responsive element. The LXR agonist GW3965 induced *SREBP1c* mRNA expression and the response was 2-fold higher in cells carrying Pro251 compared to non-carriers. Conversely, LXR antagonism resulted in negligible mRNA levels of *SREBP1c*. Bafilomycin A1 supplementation blunted the response of GW3965 in cells carrying the Pro251 variant from a 6-fold increase to a 5-fold increase, suggesting that autophagy modulates stimulation of LXR. Firefly luciferase activity was readily induced by treatment with GW3695. Analogous to previous data herein, the response was significantly higher in cells carrying the Pro251 protein variant. Interestingly, early autophagy blockade using 3MA resulted in a dampened response to the LXR agonist. Notably, HEK-cells carrying the Pro-variant of *PLIN2* presented with roughly 3-fold increase in autophagy flux when LXR is activated, an effect ablated by LXR antagonism (*Figure 4*).

Figure 4

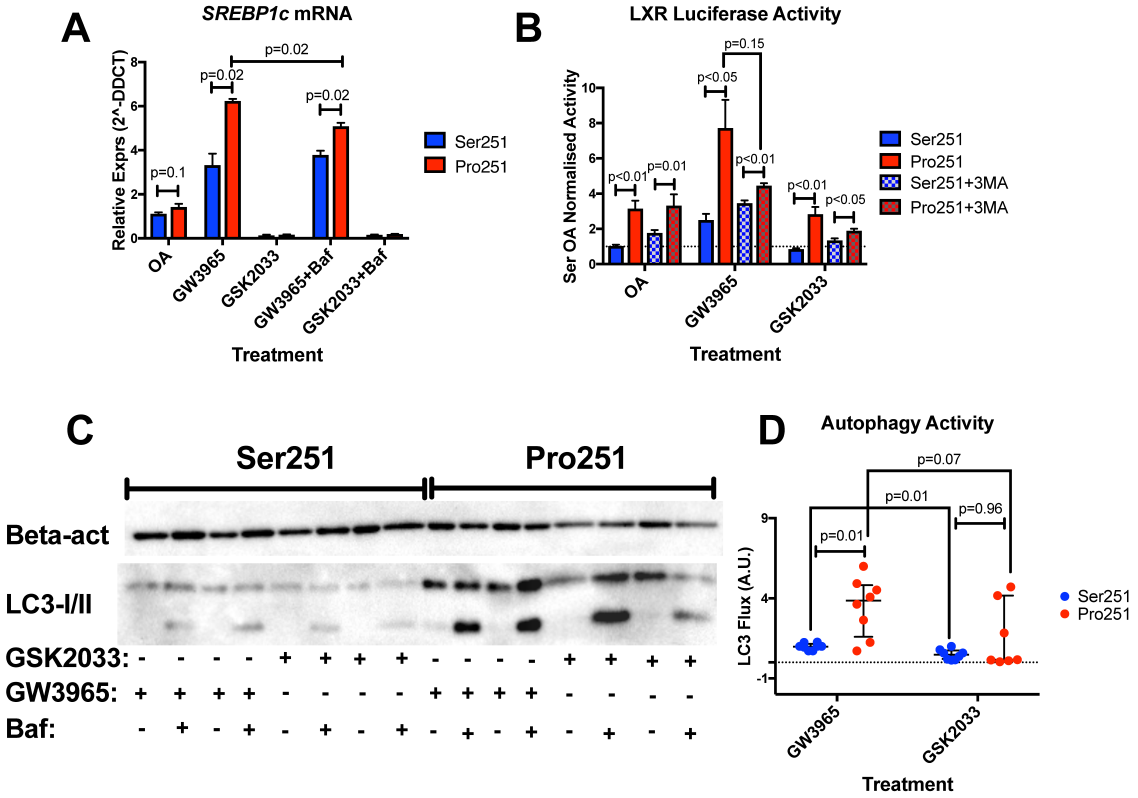

Figure 4. LXR activity as well as *SREBP1c* expression (**A-B**) is increased in HEK293 cells carrying the Pro251 variant of *PLIN2*. As suggested by previous data presented herein, the increased LXR activity was responsible for the observed augmentation in autophagy activity in cells carrying Pro251 (**C-D**).

LXR and autophagy are interconnected in a feed-forward loop through 27HC

LXR stimulation in human primary monocyte-derived macrophages induced the mRNA expression of both *SREBP1c* and *ABCA1*, whereas LXR inhibition significantly reduced their expression. Concurrent with the upregulation of LXR target genes, LXR stimulation resulted in a significant increase in autophagy activity whereas LXR inhibition resulted in blunted autophagy activity. Since we were able to substantially increase 27HC through foam cell formation and autophagy activation, and autophagy inhibition resulted in blunted levels of 27HC, we demonstrate that autophagy-driven LXR activity observed in human primary monocyte-derived macrophages was carried by 27HC (*Figure 5*).

Figure 5

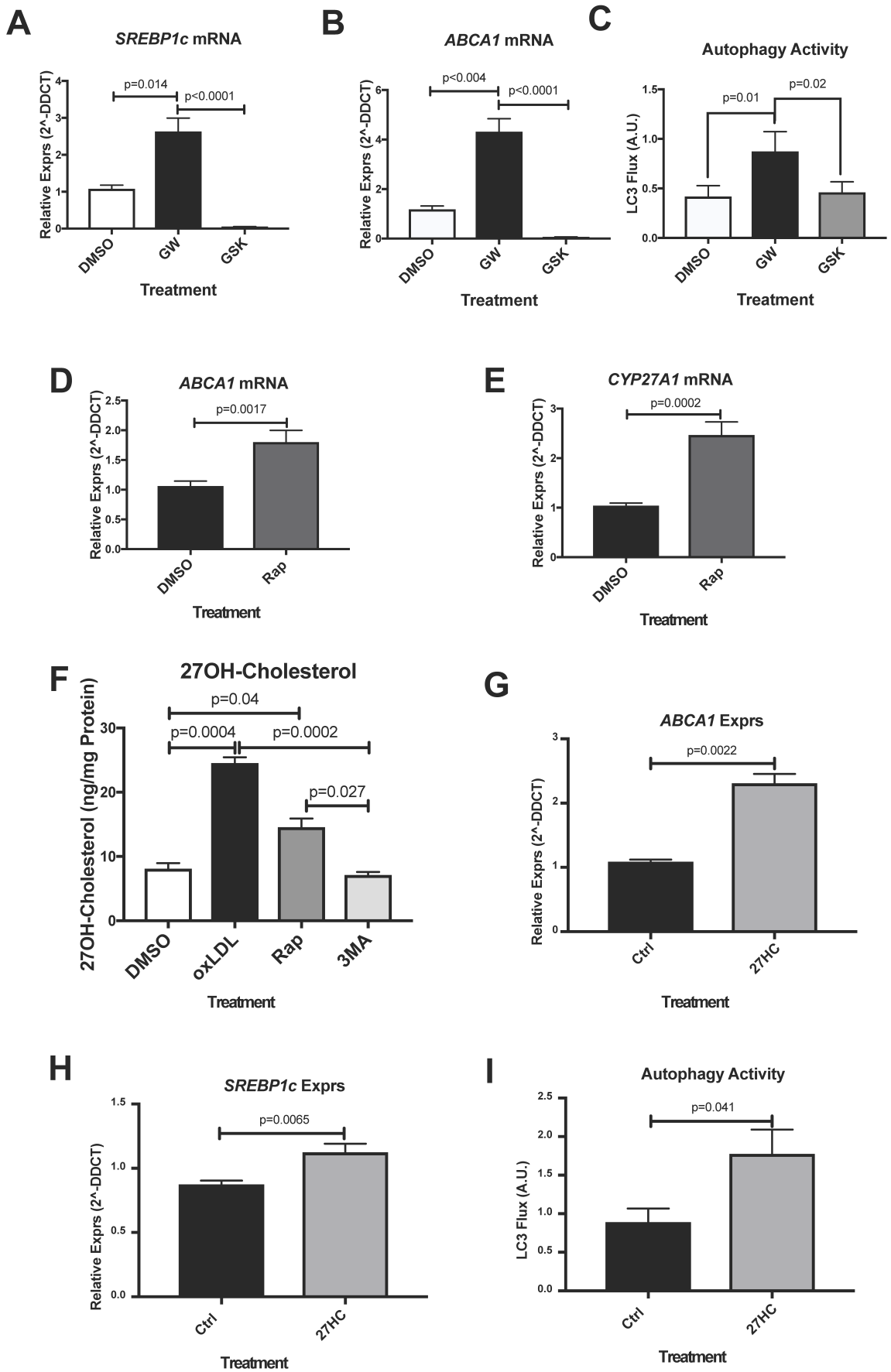

Figure 5. LXR stimulation results in increased expression of LXR target genes and autophagy activity in human primary monocyte-derived macrophages (**A-C**). Conversely, autophagy stimulation results in increased expression of LXR target genes and production of the endogenous LXR ligand 27HC (**D-F**). LXR and autophagy are responsible for their reciprocal activation, which is mediated by the endogenous LXR ligand 27HC (**G-I**).

4.3 PAPER III

ATG16L1 expression patterns in human carotid plaques

Immunohistochemistry micrographs of human carotid plaques were studied and ATG16L1 expression localised to the shoulder region, areas surrounding the necrotic core and ruptured healed regions of the vessel. ATG16L1 expression was homogenous across the entire vessel of mammary control arteries, which were free from atherosclerosis (*Figure 6*). While the amount of ATG16L1 staining was similar between symptomatic and asymptomatic patients, ATG16L1 correlated positively with the amount of lipids retained within the vessel wall (OrO staining, $r=0.341$, $p<0.005$) and the phagocytic marker CD68 ($r=0.455$, $p<0.005$). Furthermore, ATG16L1 expression correlated with pro-inflammatory cytokines IL-6 ($r=0.280$, $p<0.005$) and MCP-1 ($r=0.296$, $p<0.005$), and extracellular matrix degrading proteins.

Immunofluorescent staining and immunogold electron microscopy were adapted in order to determine which cell types are main contributors to ATG16L1 expression in human carotid atherosclerotic plaques. Immunofluorescence staining suggested that CD68+ and CD31+ cells were main contributors of ATG16L1 expression. VSMCs and mast cells contributed to ATG16L1 expression to a lesser extent. Electron microscopy corroborated immunofluorescence data in that macrophages and endothelial cells (CD68- and CD31-positive cells, respectively) were sources of ATG16L1 expression. However, in contrast to immunofluorescence, some ATG16L1 expression was indeed observed in VSMCs containing lipid droplets.

Figure 6

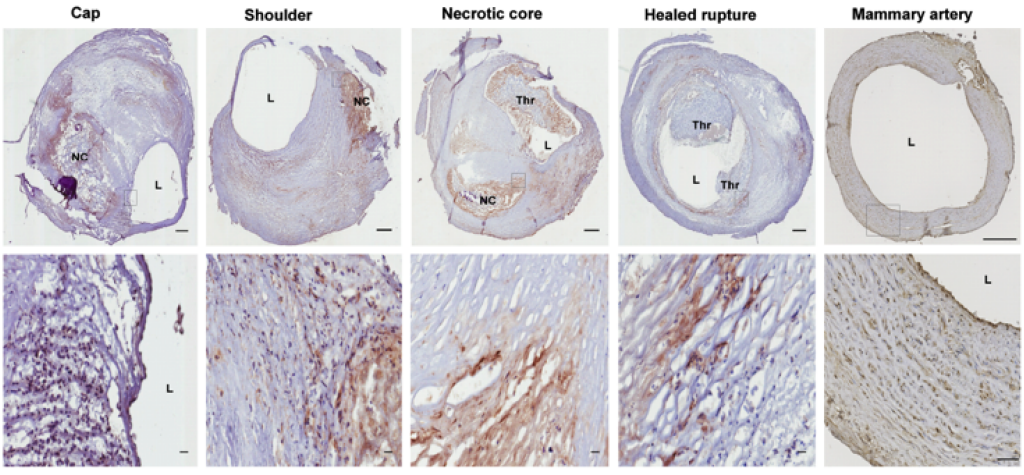

Figure 6. Micrograph of ATG16L1 expression (brown) in frozen sections of human carotid atherosclerotic plaques of different phenotypes and a human mammary artery free of atherosclerosis.

ATG16L1 in plaque vulnerability

Analysis of Atg16l1 expression and localisation was performed in a carotid plaque vulnerability model in atherosclerotic ApoE-deficient mice using immunofluorescence. The model consists of an incomplete ligation of the common right carotid artery (just below the bifurcation). Kept as such for four weeks, triggering intimal hyperplasia and non-ruptured carotid atherosclerotic lesions. Placing a conical polyethylene cuff proximal to the ligation site for four days provoked plaque rupture. Approximately 50% of mice develop atherosclerotic plaques with similar phenotype of a ruptured plaque upon cuff placement. Significant intimal hyperplasia was observed in carotid arteries developing stable fibrous caps upon cuff placement, which coincided with an increase in Atg16l1 expression. Atg16l1 did not colocalise with the macrophage marker F4/80 in stable and vulnerable fibrous caps. Contrasting, Atg16l1 showed a colocalisation with α-SMC-actin in stable fibrous caps only, (*Figure 7*).

Figure 7

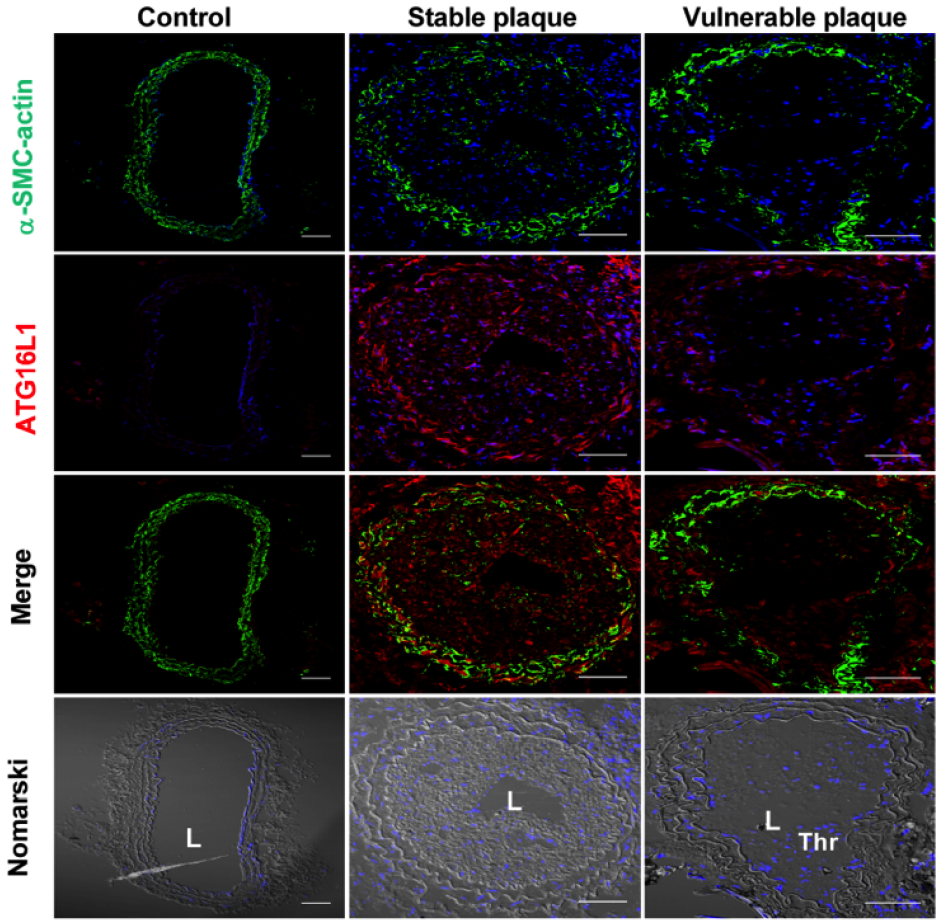

Figure 7. Double immunofluorescence labelling of Atg16l1 (red) and α-SMC-actin (green) demonstrate a colocalisation between Atg16l1 and α-SMC-actin in murine stable fibrous caps. Nuclei are stained with DAPI (blue).

ATG16L1 and apoptosis activation

Since the stability and degradation of ATG16L1 can be regulated by apoptosis through caspase-3 activation, we sought to explore the relationship between ATG16L1 and caspase-3 in human atherosclerosis and foam cell formation. Plaque ATG16L1 expression showed a significant correlation with plaque cleaved caspase-3 content ($r=0.186$, $p=0.026$). Macrophage foam cell formation was induced in primary human monocyte-derived macrophages, and protein expression levels of ATG16L1, caspase-3 and MAP1LC3 were determined using western blotting. ATG16L1 expression increased over time (up to 48 hours of treatment) in macrophage foam cells receiving a low dose of oxLDL (25 μg/mL). This was concurrent with a gradual increase in autophagy activity. However, macrophage foam cells receiving a high dose of oxLDL (100 μg/mL) showed both decreased ATG16L1 levels as well as impaired autophagy. No changes to caspase-3 levels were detected in whole cell protein extracts and cleaved caspase-3 was undetected using western blotting. However, treating primary human monocyte-derived macrophages with a high dose (100 μg/mL) of oxLDL for 48 hours resulted in an increased caspase-3 cleavage, which was dependent on autophagy, as bafilomycin A1 blockade reduced caspase-3 cleavage. ATG16L1 did not colocalise with cleaved caspase-3.

4.4 PAPER IV

MAP1LC3A expression is downregulated in mouse and human atherosclerosis and relates to clinical atherosclerosis manifestation

A time-dependent analysis of *Map1lc3a* and *Map1lc3b* mRNA expression in aortas of atherosclerotic mice was carried out. The atherosclerotic mouse model is known to trigger lipid accumulation to induce atherosclerosis. A sustained *Map1lc3a*, but not *Map1lc3b*, downregulation was observed in atherosclerotic mouse aortas over time, starting from 30 weeks. *Map1lc3a* downregulation coincided with a drastic growth of the lesion area, assessed by Sudan IV staining at 30 to 40 weeks of age. In a carotid ligation and cuff-placement mouse model, re-enacting a plaque rupture significantly reduced *Map1lc3a* mRNA expression.

In the BiKE biobank, *MAP1LC3A* mRNA was significantly downregulated in human carotid plaques compared to healthy iliac control arteries. Symptomatic compared to asymptomatic patients displayed lower *MAP1LC3A* expression. Mass-spectrometry quantification of MAP1LC3A protein levels substantiated the finding that *MAP1LC3A* is reduced in atherosclerosis, particularly symptomatic atherosclerosis. In addition, a delayed time-to-surgery, which has been suggested to reflect plaque phenotype and vulnerability, displayed with a trend towards lower *MAP1LC3A* mRNA expression.

By adopting the CPIP biobank, we determined that MAP1LC3A was abundantly expressed in the shoulder regions and areas surrounding the necrotic core of human carotid atherosclerotic plaques. Further, plaque MAP1LC3A content was lower in patients who had suffered postoperative cardiovascular events either one or two years after endarterectomy compared to patients who did not present with any postoperative cardiovascular events.

Collectively, data support the notion that *MAP1LC3A* is involved in atherosclerosis progression and plaque rupture.

Vascular smooth muscle cells are main contributors of MAP1LC3A expression in human and murine atherosclerosis

Co-expression analyses in BiKE revealed that *MAP1LC3A* was co-expressed with VSMC markers *MYH11*, *SMTN*, *ACTA2* and *TAGLN*, both on the mRNA and protein level. Concurrently, *MAP1LC3A* showed negative or no co-expression with inflammatory markers. Additionally, by using triple-staining of human atherosclerotic plaques from the CPIP cohort, we demonstrate that MAP1LC3A was abundantly expressed in VSMCs expressing α-SMC-actin, cells expressing the phagocytic marker CD68 and VSMCs positive for CD68.
Since VSMCs are important contributors of *MAPLC3A* expression, and intimal hyperplasia in response to vascular injury predominantly involves VSMC migration and proliferation, we sought to investigate the role of *MAP1LC3A* in intimal hyperplasia. In a balloon-injury induced rat carotid intimal hyperplasia model, *Map1lc3a* mRNA expression showed a gradual, transient downregulation from 20 hours up until 5 days post-injury as compared to the expression pre-injury. Moreover, uninjured control carotids displayed a stable, unchanged *Map1lc3a* expression over time. Taken together, data support a role for MAP1LC3A in VSMC biology.

MAP1LC3 depletion results in defective autophagy and deregulation of phenotypic switch in human carotid vascular smooth muscle cells

By adopting a VSMC *in vitro* system we investigated whether depletion of *MAP1LC3A* was associated with defective autophagy. We first tested that no compensatory mechanisms were observed between *MAP1LC3A* and *MAP1LC3B*. Silencing *MAP1LC3A* mRNA expression levels by more than 90% did not upregulate *MAP1LC3B* mRNA expression and vice versa. Significantly, simultaneous silencing of *MAP1LC3A* and *MAP1LC3B* did not affect *GABARAP*, *GABARAPL1*, *GABARAPL2*; three autophagy genes known to be functionally redundant to MAP1LC3. However, *MAP1LC3A* and *MAP1LC3B* depletion abolished MAP1LC3 protein levels. Importantly, after bafilomycin supplementation, silencing of *MAP1LC3A* and *MAP1LC3B* led to an increase in p62, an autophagy marker known to accumulate when autophagy is defective.
In order to re-enact the heterogeneity of the VSMC phenotypes present in an advanced atherosclerotic plaque and coerce a switch of VSMCs from a contractile to a synthetic phenotype, we adopted a human carotid VSMC trans-differentiation model *in vitro*. Human VSMCs were treated with either oxLDL or CaPi. Concurrent silencing of *MAP1LC3A* allowed us to determine the role of *MAP1LC3A* in VSMC phenotypic switching. Consistent with previous findings, coercing a phenotypic switch induced autophagy flux, as seen by MAP1LC3 lipidation after bafilomycin supplementation. Silencing of *MAP1LC3A* mRNA resulted in a transient compensatory upregulation of *ACTA2* and *MYOCD* mRNA expression at 48 hours compared to scramble in untreated controls. Forcing VSMCs towards a synthetic phenotype substantiated this effect, at least in part. Bone Morphogenetic Protein 2 (*BMP2*), a marker for

VSMC calcification [124], was analysed in calcified VSMCs and *MAP1LC3A* depletion reduced its mRNA expression 0.5-fold.
Data demonstrate that *MAP1LC3A* impacts VSMCs' phenotypic switch, which likely owes to the destabilising properties of low *MAP1LC3A* expression in human and murine atherosclerotic lesions.

5 DISCUSSION

5.1 MODULATION OF PLAQUE VULNERABILITY BY AUTOPHAGY

As previously stated large necrotic cores, inflammation and thin fibrous caps are features of a prone-to-rupture or unstable atherosclerotic plaque [7, 8]. Cholesterol efflux halts atherosclerosis by decelerating macrophage foam cell formation, a feature modulated by autophagy [82, 95, 96]. If foam cell formation is interrupted, so is the build-up of the necrotic core of the atherosclerotic plaque; this results in a plaque, which is less likely to rupture. *Paper II* clearly demonstrates that modulation of autophagy has repercussions on early macrophage foam cell formation, the inflammatory state of foam cells and cholesterol efflux, which has ramifications on both human subclinical atherosclerosis as well as plaque progression.

Atherosclerosis is an inflammatory disease of intermediate-size; large arteries and more inflamed atherosclerotic plaques are at higher risk of rupturing [7, 8]. *Paper III* reveals that ATG16L1 expression within the atherosclerotic lesion correlates with inflammatory markers. Concurrently, ATG16L1 expression is increased in monocyte-derived macrophages treated with extended and high-dose exposures of oxLDL, which is paralleled with an augmentation of autophagy activity.

The fibrous cap of an atherosclerotic lesion consists primarily of VSMCs; the thickness of the fibrous cap and thus also VSMC function, regulates plaque stability [37-39]. *Paper IV* assigns *MAP1LC3A* a role in VSMC trans-differentiation, where low *MAP1LC3A* expression is a feature of phenotypic switch. Further, low *MAP1LC3A* was observed in advanced symptomatic, human atherosclerosis.

Collectively, *Papers II-IV* clearly demonstrate that modulation of autophagy has multifaceted repercussions on subclinical atherosclerosis, plaque stability and overt CVD, where the mechanism by which autophagy exerts its effects spans cholesterol metabolism, inflammation and regulation of VSMC phenotypes.

5.2 AUTOPHAGY AS A THERAPEUTIC LEVERAGE IN ATHEROSCLEROSIS

The means by which autophagy is induced, and the temporal relationship between foam cell formation, initiation of atherosclerosis and autophagy induction, is in all likelihood absolutely essential. Several lines of data support the notion that autophagy induction may be protective of atherogenesis [95-97]. There is limited data available on autophagy in the multi-faceted disease that is atherosclerosis, particularly human atherosclerosis, providing a rationale for further studying its role in disease progression and overt CVD. Due to the pleotropic roles of autophagy in cell biology, it is not surprising that the means by which autophagy exerts its beneficial effects spans over regulating cholesterol metabolism [82, 95, 96], VSMC trans-differentiation [43, 44] and inflammation. Autophagy also has significant repercussions on disease development in animals [97, 125]. Significantly, repercussions on disease development have only been adequately studied in murine models of atherosclerosis. Data herein extends the existing animal data in that human material has been used throughout the four studies. We demonstrate that a protein variant in *PLIN2* modulates not only cardiovascular risk, but also plasma lipid levels, which is a risk factor for CVD. The mechanism by which this protein

variant acts is likely through initiating a feed-forward loop between LXR and autophagy. Significantly, LXR-directed therapeutics have consistently failed in early drug development stages due to off-target effects. Data form *Paper II* suggest that targeting autophagy allows us to simultaneously affect LXR activity, which has known roles in promoting cholesterol efflux and halting atherosclerosis development. Although autophagy has documented roles in cholesterol efflux, this is a significant advancement in unravelling the intricate relationships between autophagy and key players in atherosclerosis development.

ATG16L1 has been implicated in the pathogenesis of Crohn's disease, where it regulates inflammatory responses. Indeed, a functional SNP in ATG16L1 shed light on the role autophagy plays in regulating inflammation in inflammatory bowel disease [126, 127]. In *Paper III* we hypothesised that ATG16L1 also contributes to atherosclerosis development, since inflammation is such a significant element in both development and progression of human atherosclerotic disease. Although *Paper III* is primarily descriptive in nature, we demonstrate that ATG16L1 is expressed in inflammatory and stability-regulating regions of the atherosclerotic plaque, and that inflammatory cells are main contributors of ATG16L1 expression. ATG16L1 expression patterns are also deregulated in vulnerable carotid plaques as shown by a murine model of plaque rupture. Further, both autophagy flux and ATG16L1 expression increased by long and high-dose exposure of monocyte-derived macrophages to oxLDL. The fact that ATG16L1 increases with autophagy activity, and that ATG16L1 is correlated with a more inflamed plaque phenotype, further strengthens the notion that timely and tightly regulated activation of autophagy is essential when evaluating whether the response is atheroprotection or actually deleterious.

In *Paper IV* we assign autophagy-related protein *MAP1LC3A* a role in regulating VSMC trans-differentiation, where VSMCs are main contributors to *MAP1LC3A* expression. In atherosclerotic plaques, *MAP1LC3A* expression is reduced compared to normal vessels. Furthermore, *MAP1LC3A* is downregulated in symptomatic atherosclerosis, whilst gene expression profiles of VSMC signature genes and phenotypic regulators as well as autophagy activity are deregulated when *MAP1LC3A* is repressed. VSMCs switching into a synthetic phenotype orchestrate the modulation of plaque stability, where *MAP1LC3A* may exert a protective effect in preserving VSMC function [128, 129], which in turn owes to the destabilising properties of low MAP1LC3A expression in human atherosclerotic lesions.

Collectively, data from *Papers II-IV* clearly demonstrate the pleotropic effects of autophagy in the pathogenesis of atherosclerosis, which undoubtedly hinders the effective targeting of autophagy in atherosclerosis treatment. Autophagy stimulation may be beneficial in early macrophage foam cell formation, but when reaching a tipping point it may very well be detrimental in more advanced, clinical atherosclerosis. Once one develops advanced lesions, maintaining autophagy activity in VSMCs specifically, may be beneficial in stabilising the atherosclerotic plaque and thus preventing overt CVD. Therefore, *where*, *when* and *how* to target autophagy remain pivotal questions before therapeutics can be introduced.

5.3 GENETIC APPROACHES TO UNRAVELLING THE BIOLOGY BEHIND COMPLEX DISEASES

Above all, *Paper II* has elucidated an intricate biological relationship between autophagy and cholesterol metabolism, where LXR and autophagy are responsible for their reciprocal activation through 27HC. However, *Paper II* also illustrates how one can leverage on genetic associations to conduct translational research finding novel disease pathways.

The genetic era, with its immense efforts in doing whole-genome scans to identify genetic risk factors for common traits must be viewed as rather disappointing. Risk stratification using genetics does not substantially improve risk stratification using classical risk factors. What has been provided is an enormous amount of information, implying genes that may be involved in the pathological pathway of CVD [(3)]. By fully leveraging the advancement of laboratory techniques, including the development of CRISPR-Cas9 as well as the recruit-by-genotype approach described herein, it is now time to harvest the seeds that large GWAS have planted.

5.4 LIMITATIONS AND METHODOLOGICAL CONSIDERATIONS

At first glance, the results from *Papers I* and *II* may seem contradictory. In *Paper I* we describe how monocyte-derived macrophages carrying the Pro251 variant of *PLIN2* increase their lipid content, whereas in *Paper II* we show that cholesteryl esters are significantly reduced. In the first study, neutral lipid content was measured using confocal microscopy and OrO or BODIPY 493/503 staining. Neutral lipids include TGs, non-esterified fatty acids as well as cholesteryl esters. In contrast to *Paper I*, we measured cholesteryl esters in *Paper II* only – thus these results are not necessarily contradictory. Further, OA treatment is quite distinct from oxLDL challenge, where one contains one specific non-esterified fatty acid whereas the other contains both TGs and cholesterol. This clear distinction in experimental set-ups may influence the data obtained.

One of the major limitations of *Paper III* is that the study is primarily observational. Numerous correlations between ATG16L1 and inflammatory markers were observed in human atherosclerotic plaques. Correlation is far distinct from causation, and by including mechanistic studies similar to the ones in *Paper IV*, we could have further delineated the role of ATG16L1 on the inflammatory state of, for example, macrophages and how autophagy is involved in modulating inflammation in macrophage foam cells.

Limitations pertaining to *Paper IV* include the rather inexplicitly defined temporal window and combination of provocations leading to the heterogeneity of VSMC phenotypes present in an advanced atherosclerotic plaque. Moreover, the general decrease in MAP1LC3A and MAP1LC3B expression in the *in vitro* system raises the question whether cell viability is compromised over time; why viability, proliferation and migration assays should be adopted to further characterise the VSMC phenotype.

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