Immunometabolic and Cellular Traits in Cardiovascular Disease

LUKAS TOMAS | FACULTY OF MEDICINE | LUND UNIVERSITY

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Research is a process of constant questioning and probably also the hunt for the truth, if it exists. It can be exciting but also overwhelming at times. My past years as an apprentice in research and most likely the years of apprenticeship to come are nicely recapitulated in the journey of Joseph Knecht to become the Magister Ludi in one of my favourite books.

> "Oh, if only it were possible to find understanding," Joseph exclaimed. "If only there were a dogma to believe in. Everything is contradictory, everything tangential; there are no certainties anywhere. Everything can be interpreted one way and then again interpreted in the opposite sense. The whole of world history can be explained as development and progress and can also be seen as nothing but decadence and meaninglessness. Isn't there any truth? Is there no real and valid doctrine?"
>
> The master had never heard him speak so fervently. He walked on in silence for a little, then said: "There is truth, my boy. But the doctrine you desire, absolute, perfect dogma that alone provides wisdom, does not exist. Nor should you long for a perfect doctrine, my friend. Rather, you should long for the perfection of yourself. The deity is within you, not in ideas and books. Truth is lived, not taught. Be prepared for conflicts, Joseph Knecht – I can see that they already have begun."
>
> – Hermann Hesse, The Glass Bead Game

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DOCTORAL DISSERTATION

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Abstract

Atherosclerosis and its sequelae myocardial infarction and stroke are the major global health burden. Previous efforts like the introduction of lipid-lowering therapy have led to a substantial decrease of atherosclerotic death rates. In recent years, however, the mortality due to atherosclerotic cardiovascular disease (ASCVD) has been on the rise again in the Western world. Continuous research into the pathogenesis of atherosclerosis to develop novel diagnostic measures and therapies are warranted to counteract this trend. Atherosclerosis is initiated by the subendothelial retention of apolipoprotein B-containing lipoproteins. The retention and modification of the lipoproteins trigger an inflammatory reaction, in which both, the innate and adaptive immune system take part. The progression of the atherosclerotic plaques is driven by the chronic inflammatory response that fails to transit to the resolution phase. The chronic inflammation in atherosclerosis is the overarching topic of this thesis.

The first part of this thesis evolves around cellular metabolism and specifically the metabolism of immune cells, also known as immunometabolism. In Paper I we have investigated the metabolic profile and expression of metabolic enzymes in human carotid plaques by mass-spectrometry and RNA sequencing. We found that atherosclerotic plaques can be divided into two main groups according to the abundance of metabolites. The group of high-risk plaques – characterized by histological vulnerability, association with pre-operative symptoms and the abundance of inflammatory mediators – showed a metabolic profile that is consistent with elevated glycolysis and amino acid utilization with concomitant reduction in fatty acid oxidation. In Paper II we followed up on these results by investigating the role of myeloid PKM2, which is a glycolytic enzyme and metabolic regulator, in experimental atherosclerosis. We transferred bone-marrow from mice with a myeloid-specific knockout of PKM2 and control bone-marrow into *LDLr*$^{-/-}$ and evaluated atherosclerotic burden after 13 weeks on high-fat diet. Despite a decreased frequency of aortic macrophages in the mice lacking myeloid PKM2, we could not detect an amelioration of advanced atherosclerosis development. In summary, high-risk human carotid plaques provide evidence for an altered metabolism in atherosclerotic plaques, which might be due to the reprogrammed metabolism in activated leukocytes. Nonetheless, myeloid expression of the metabolic regulator PKM2 does not seem to play an important role in advanced atherosclerosis.

The second part investigated the role of various T cell subsets in experimental atherosclerosis and human ASCVD. Paper III re-examined the effect of CD4$^+$ T cell on experimental atherosclerosis by investigating MHC II$^{-/-}$ ApoE$^{-/-}$ mice. We could confirm previous results on the overall athero-protective effect of CD4$^+$ T cells in experimental atherosclerosis. In Paper IV we investigated the association of invariant natural killer T (iNKT) cells with the incidence of first-time coronary events in over 400 participants of the Malmö Diet and Cancer Study. Although iNKT cells have been repetitively shown to be pro-atherogenic in mice, our results did not confirm a positive association of iNKT cells with future coronary events and rather support a subset-specific anti-atherogenic effect. The Malmö Diet and Cancer Study was also used in Paper V to investigate the association of another T cell subset, CD4$^+$CD28^{null} T cells, with the incidence of first-time coronary events. Surprisingly we found a negative association CD4$^+$CD28^{null} T cells and incident first-time coronary events in these individuals without pre-existing ASCVD. In contrast, in patients undergoing carotid endarterectomy we found a positive association of CD4$^+$CD28^{null} T cells and incident cardiovascular events. In conclusion, Paper III confirmed previous reports on CD4$^+$ T cells, whereas Paper IV and Paper V warrant additional investigation of the precise roles of these T cell subsets.

Key words
Atherosclerosis, Vascular inflammation, Immunometabolism, Human plaque, PKM2, Glycolysis, Macrophage, Lymphocytes, CD4$^+$ T cell, Natural killer T cell, CD4$^+$CD28^{null} T cell

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For my family who taught me life
and that 6×6 is 36

Contents

We need less research, better research, and research done for the right reasons. Abandoning using the number of publications as a measure of ability would be a start.

Doug Altman
The scandal of poor medical research
BMJ 1994[1]

Preface

Hundreds of years ago, atherosclerotic cardiovascular disease was a rare cause of death. Nowadays, almost everyone shares an experience with clinical manifestations of atherosclerosis at one point in their life, either by suffering a stroke or myocardial infarction themselves, or by knowing someone who did. Today, atherosclerosis is recognized as a chronic inflammatory disease triggered by our Western lifestyle and high blood lipids. The role of the immune system in this disease has been the focus of my work over the past 5 years, cumulating in this PhD thesis.

Herein, I want to demonstrate the importance of scientific efforts leading to a better understanding of the disease and explain my own contribution to this progress. In a holistic approach, I attempt to set out the current pathological concept of atherosclerosis and how we arrived there, with a particular focus on the immune system (*Atherosclerosis*). Given the publication of more than 2 000 PubMed indexed articles only in 2018, this can only happen in a condensed form focussing on topics relevant for the thesis. The next chapter (*The Killer of the 21st Century*) will try to prove that a continuous or even intensified investment in understanding atherosclerosis is warranted. I will showcase why the research in this thesis is of importance and not just an academic exercise. After critically discussing the main overarching methods (*Methodological Considerations*) for this thesis and their limitations, an inherent feature of science, I will proceed to an extended description and discussion of my results in the two main topics in this thesis – the *Immunometabolic* and *Cellular Traits* in Cardiovascular Disease.

I have tried to stick to plain language as much as possible, particularly in the background chapters. Thereby I hope to make the general topics of atherosclerosis and research in atherosclerosis more accessible to anyone and that you will enjoy reading the results of my exciting work over the past years.

Acknowledgments

It has been some time since I started my journey to become a scientist – a challenging but fascinating one. During these years many people have considerably contributed directly or indirectly to my resilience, development, and the findings presented in this thesis. I want to express my deepest gratitude to everyone who has been there for me and with me. Representative of all the people involved in the progress over the past years I want to acknowledge a few people in particular.

First of all, my main supervisor **Harry Björkbacka**. I admire your calmness and like to think I've ingested some of it in these years. Thank you for your guidance and for fostering my independence. I will always remember our stimulating, sometimes philosophical discussions and my very first and very long journey together with you to my first publication. My co-supervisor **Eva Bengtsson**. If it weren't for you, I probably wouldn't have ended up in research. Thank you for your support and guidance already before I even embarked on my PhD journey and throughout. I really appreciate that your door and your ears were always open to me. My co-supervisor **Isabel Gonçalves**. I really appreciate your honesty and that you did not hold back with your opinion. But most of all, thank you for your support and your caring about my well-being. It was always a joy to be greeted by your smile and being able to laugh together. I also would like to thank **Jan Nilsson** for putting together a research group with great resources, which are a prerequisite for meaningful science.

My former officemate and fellow PhD student **Jenifer**. I'm grateful that I had such a fantastic 'PhD partner-in-crime' with you. Your presence was greatly missed since you chose sunny San Diego over rainy Malmö. Thank you, **Eliana**, for all the good laughs and beers together, as well as the continuous mutual support of overdue PhD students.

Thanks to my officemates **Goran** and **Alex** for generating an almost always funny office atmosphere, at least for me. It was a pleasure to work with you and also enjoy weekends and evenings together.

A particular thanks goes to the good soul of the lab, **Gertrud**. Thank you for knowing almost everything and answering hundreds of questions, for organising all the great lab days and taking care of the bureaucratic hurdles of us PhD students.

I was lucky to have the help of **Linda** and **Wiaam**, who sat countless hours in front of the flow cytometer to generate data I could analyse. Furthermore, I got answers to all kinds of laboratory questions from **Irena** and **Lena**, who have also been a great help in my mouse studies.

I would also like to say thank you to all the other current and former colleagues who provided help, input and always a good laugh either at coffee breaks or in the corridor. Thank you: **Ana**, **Anna**, **Ann-Margreth**, **Anna-Maria**, **Andreas**, **Christina**, **Daniel**, **Fatema**, **Fong**, **Gunilla**, **Ida**, **Karin**, **Lisa**, **Maria G**, **Maria W**, **Mihaela** and **Pontus**.

Particularly, I want to thank my fellow PhD students over the years, for the fun and interesting times together: **Cat**, **Christoffer**, **Helena**, **Ingrid**, **Sara** and **Xenia**.

Special thanks go to all my friends outside the laboratory, who not only once had to listen to my frustration and to support me, but also celebrated my victories with me.

The final thank you is reserved for my family. I'm fortunate to have a family that believes in me, supports me and made all this possible. Thank you for all you have done!

Thesis Papers

1. **Tomas L**, Edsfeldt A, Mollet IG, Perisic Matic L, Prehn C, Adamski J, Paulsson-Berne G, Hedin U, Nilsson J, Bengtsson E, Gonçalves I,* Björkbacka H.* Altered metabolism distinguishes high-risk from stable carotid atherosclerotic plaques. *Eur Heart J* 2018; **39**: 2301–10.

2. **Tomas L**, Rattik S, Andersson L, Ljungcrantz I, Sundius L, To F, Nilsson J, Gonçalves I, Bengtsson E, Björkbacka H. Deficiency of Pyruvate Kinase M2 in myeloid cells does not influence the development of advanced atherosclerosis in $LDLr^{-/-}$ mice. *Manuscript.*

3. Wigren M, Rattik S, Yao Mattisson I, **Tomas L**, Grönberg C, Söderberg I, Alm R, Sundius L, Ljungcrantz I, Björkbacka H, Fredrikson GN, Nilsson J. Lack of Ability to Present Antigens on Major Histocompatibility Complex Class II Molecules Aggravates Atherosclerosis in $ApoE^{-/-}$ Mice. *Circulation* 2019; **139**: 2554–66.

4. **Tomas L**, Bengtsson E, Andersson L, Badn W, Tengryd C, Persson A, Edsfeldt A, Nilsson PM, Schiopu A, Nilsson J, Gonçalves I,* Björkbacka H.* Conflicting associations between $CD4^{+}CD28^{null}$ T cells and cardiovascular risk in a general population and in patients with advanced atherosclerosis. *Manuscript in revision*.

5. **Tomas L**, Badn W, Andersson L, Nilsson J, Schiopu A, Gonçalves I, Bengtsson E, Björkbacka H. Invariant natural killer T cells and incidence of first-time coronary events. *Manuscript.*

*HB and IG share senior authorship.

Other Papers

Marinković G, Grauen Larsen H, Yndigegn T, Szabo IA, Mares RG, Weiland M, **Tomas L**, Gonçalves I, Nilsson J, Jovinge S, Schiopu A. Inhibition of pro-inflammatory myeloid cell responses by short-term S100A9 blockade improves cardiac function after myocardial infarction. *Eur Heart J* 2019; **40**: 2713–23.

Grönberg C, **Tomas L**, Rattik S, Eriksson U, Yao Mattisson I, Larsson E, Andersson L, Sundius L, Kornfeld H, Fredrikson GN, Nilsson J, Björkbacka H. Interleukin-16 Reduces Atherosclerosis Development in Apolipoprotein E Deficient Mice. *Manuscript in revision.*

Abbreviations

AIT	Adaptive intimal thickening
AMPK	AMP-activated protein kinase
apoB-LP	Apolipoprotein B-containing lipoprotein
***ApoE*$^{-/-}$**	Apolipoprotein E knockout
ASCVD	Atherosclerotic CVD
BCR	B cell receptor
CMV	Cytomegalovirus
CoA	Coenzyme A
CPIP	Carotid Plaque Imaging Project
CRP	C-reactive protein
CVD	Cardiovascular disease
DAMP	Damage-associated molecular pattern
FAO	Fatty acid oxidation
FBP	Fructose-1,6-phosphate
GLUT1	Glucose transporter 1
HDL	High-density lipoprotein
HIF	Hypoxia-inducible factor
HLA	Human leukocyte antigen
IFN	Interferon
IgE|IgG|IgM	Immunoglobulin E|G|M
IHD	Ischaemic heart disease
IL	Interleukin
(i)NKT cell	(invariant) Natural killer T cell
LDL	Low-density lipoprotein
***LDLr*$^{-/-}$**	LDL receptor knockout
LysM	Lysozyme M
MDCS	Malmö Diet and Cancer Study
MCP-1	Monocyte chemotactic protein 1
MHC	Major histocompatibility complex
MI	Myocardial infarction
MMP	Matrix-metalloproteinase
NAD(H)	Nicotinamide adenine dinucleotide
NADP(H)	Nicotinamide adenine dinucleotide phosphate

NLRP3	Nucleotide-binding domain, leucine-rich-repeat-containing family, pyrin-domain-containing 3
OXPHOS	Oxidative phosphorylation
PAD	Peripheral artery disease
PAMP	Pathogen-associated molecular pattern
PCSK9	Proprotein convertase subtilisin/kexin type 9
PIT	Pathological intimal thickening
PK	Pyruvate kinase
PPP	Pentose-phosphate pathway
ROS	Reactive oxygen species
TCA cycle	Tricarboxylic acid cycle
TCR	T cell receptor
T_{EMRA}	Terminal effector memory T cells re-expressing CD45RA
TGF	Transforming growth factor
T_H	Helper T cell
TNF	Tumor necrosis factor
T_{REG}	Regulatory T cell
VEGF	Vascular endothelial growth factor
(V)SMC	(Vascular) Smooth muscle cell

Introduction

The heart, consequently, is the beginning of life; the sun of the microcosm, even as the sun in his turn might well be designated the heart of the world; for it is the heart by whose virtue and pulse the blood is moved, perfected, and made nutrient, and is preserved from corruption and coagulation; it is the household divinity which, discharging its function, nourishes, cherishes, quickens the whole body, and is indeed the foundation of life, the source of all action.

Exercitatio Anatomica de Motu Cordis et Sanguinis in Animalibus
William Harvey
Translated by Robert Willis[2]

THE CELL is the fundamental unit of life.[3] The human body is a complex multicellular organism, in which each of the more than 10^{13} cells has a specialized function to enable life as we know it. Sufficient supply of nutrients and oxygen is a prerequisite for each cell to perform its function and survive. Whereas cells in unicellular and simple multicellular organisms are in close or direct contact with their environment and can directly extract the necessary nutrients, complex multicellular organisms need dedicated circulatory systems as a means of transporting nutrients in close proximity to the individual cells and maintain life. The cardiovascular system in humans is arguably one of the most sophisticated systems, in which the heart pumps the blood through the vessels throughout the body, thereby facilitating the transport of nutrients, enabling cellular communication and migration.[4,5]

Diseases affecting the heart or blood vessels, cardiovascular disease (CVD), can lead to a malfunction of this system and thus have the potential to be disastrous. In this context it might not be surprising that CVD can be accounted for one third of all deaths worldwide.[6] CVD is a broad term for several diseases of different aetiology. Examples include cardiac arrythmias, infectious diseases of the heart as well as hypertension or myocardial infarction. The majority of CVD, however, is caused by atherosclerosis. Atherosclerosis, a lipid-induced chronic inflammatory disease of the arteries, and its clinical manifestations ischaemic heart disease, including myocardial infarction, and ischaemic stroke are the topic of the work in this thesis.

Atherosclerosis

Atherosclerosis has been plaguing humans probably since the beginning of humankind. Only in the past centuries has atherosclerosis evolved from a previously rare disease to a worldwide pandemic (see *The Killer of the 21st Century*). One of the oldest medical papyri, the Ebers Papyrus (dated at approximately 1550 B.C.), contains a description that is reflective of anginal chest pain, the syndrome caused by myocardial ischemia due to either atherosclerotic luminal narrowing or an artery-occluding thrombus in the coronaries.[7]

Atherosclerosis is a chronic vascular disease, characterized by lipid deposition and a concomitant inflammatory response within the intima, the normally thin unicellular luminal layer of arteries. The development of atherosclerosis starts very early in life. Fatty streaks and adaptive intimal thickenings (AIT) can already be found in the coronary arteries of unborn children and more advanced atherosclerotic lesions can frequently be detected in individuals below 30 years of age (Figure 1).[8-12] Fatty streaks, clusters of lipid-laden macrophages in the intima, and AIT, intimal accumulation of vascular smooth muscle cells (VSMC) and fibrous tissue, are atherosclerotic precursor lesions.[13,14] These early pre-atherosclerotic lesions develop in arteries throughout the body but mainly at predilection sites of disturbed laminar blood flow in atherosclerosis-prone arteries (Figure 2).[9,13,15]

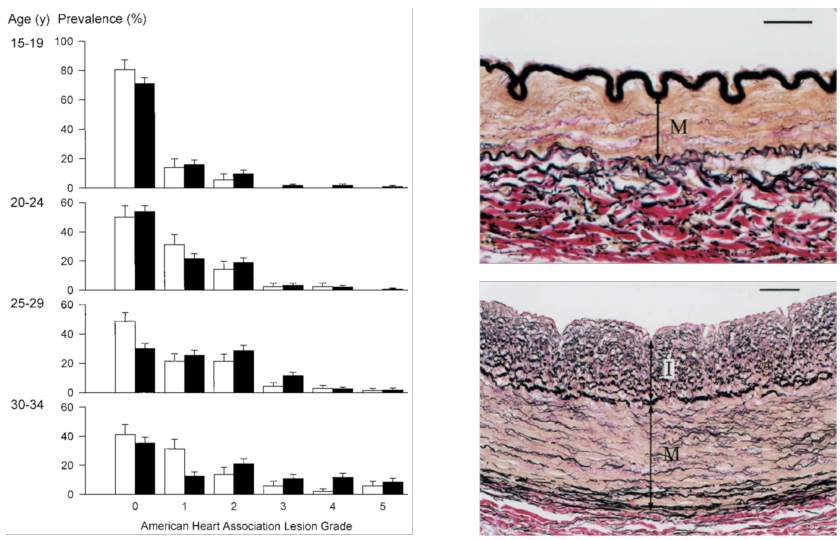


Figure 1. Adaptive intimal thickening in children and young adults. The bar graph illustrates the prevalence of atherosclerotic lesions of various severities in a branch of the left coronary artery, stratified by the age of the examined individuals at autopsy. Lesion grade 0=normal tissue; 1,2=fatty streaks and AIT; 3-5=more advanced lesions. Reproduced with permission from (12), © Wolters Kluwer Health Inc. and American Heart Association. On the right are representative histological pictures of the right coronary artery in a 7-day old female (top), showing the very thin non-diseased intima on top of the media. The bottom picture depicts a branch of the left coronary artery in a 15-year old female with AIT. I=intima, M=media. Scale bars indicate 25µm and 50µm. Reproduced with permission from (9), © Springer Nature.

Fatty streaks and AIT can regress again, but some will latently progress over several decades into advanced atherosclerotic plaques and eventually become clinically apparent.[17,18] Often, an acute thrombosis of an advanced plaque will manifest as a life-threatening myocardial infarction (MI) or stroke and uncover the simmering disease. Alternatively, the atherosclerotic plaque might critically narrow the arterial lumen merely due to its size. Thereby limited blood flow manifests for instance as stable angina or intermittent claudication, depending on the artery affected (Figure 2).

Although clinical manifestations of atherosclerosis were already described in ancient Egypt and even Leonardo da Vinci illustrated coronary atherosclerosis, the late 19th century marks the beginning of an intensified scientific interest in the disease pathomechanisms.[7,19]

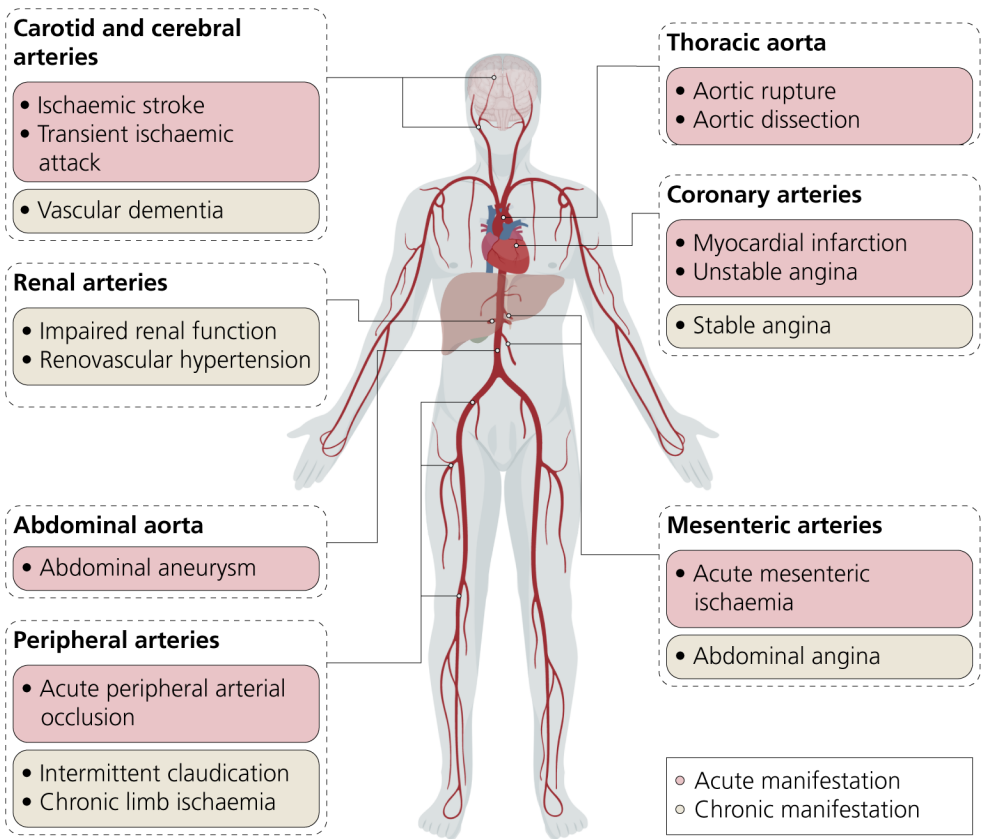


Figure 2. Clinical manifestations of atherosclerosis. Atherosclerosis can affect all vessels but typically presents itself in specific vascular beds, which results in the vessel-specific manifestations. Acute manifestations of atherosclerosis are most often caused by thrombotic occlusion of the vessels, whereas chronic manifestations of the disease are typically caused by a narrowing of the artery because of the growing plaque. Adapted from (16).

Past Hypotheses

Rudolf Virchow is often cited as the founder of modern pathology. While his main interest focused on thrombosis and infarction, already *Virchow* acknowledged the presence of inflammatory cells in the intima of atherosclerotic arteries. He proposed a chronic inflammatory aetiology of atherosclerosis, with cholesterol deposition as a secondary phenomenon. Another authority at that time, *Carl von Rokitansky*, dismissed the idea of a chronic inflammation and saw atherosclerosis rather as a result of repetitive thrombotic events with the remnants being incorporated in the arterial wall. The different views lead to a quite keen and public controversy between the two.[7,20] Despite their controversies, both notions are nowadays part of our current paradigm of atherosclerotic pathophysiology.

In the following years, some pathologists moved from a descriptive to a more experimental approach. Following the findings of increased atherosclerosis development in rabbits fed certain diets by *Ignatowski* and later *Stuckey*, *Nikolai Anitschkow* singled out cholesterol feeding as the responsible factor for increased atherosclerosis.[7] Intriguingly, *Anitschkow* did not only provide the first evidence of the causality of cholesterol-rich lipoproteins in atherosclerosis – he also described several features of the atherosclerotic process, which hold true up until today, including the presence of various leukocytes in atherosclerotic plaques.[21] *Antischkow*'s experiments mark the beginning of the lipid hypothesis of atherosclerosis, which despite intermittent setbacks and scepticism has cemented itself in science since. The identification of the low-density lipoprotein (LDL) receptor by Nobel Laureates *Joseph L. Goldstein* and *Michael S. Brown* was a scientific milestone in this process.[22,23]

Despite the repeated observations of leukocyte infiltration in atherosclerotic lesions, the interest in the inflammatory aetiology of atherosclerosis was basically absent in the decades following their description.[24] Main interest, besides the lipid hypothesis, was devoted to the role of VSMC proliferation. Various hypotheses, like the monoclonal hypothesis, suggesting atherosclerosis as a result of a monoclonal VSMC expansion similar to leiomyomas, or the clonal senescence hypothesis, advocating enhanced age-dependent VSMC proliferation, were proposed.[25-27] In 1976, an initially VSMC focused theory, the response-to-injury hypothesis, was put forward by *Ross* and *Glomset*.[28] They suggested a focal injury of the endothelial cells and the denudation of this cell layer as the initiating event, followed by platelet aggregation and VSMC invasion and proliferation. The potential injury was envisioned to be either mechanical or non-mechanical, e.g. haemodynamic forces and hyperlipidaemia. This paradigm, with modifications, would persist in the scientific society for decades.

Around this time, interest in the inflammatory contribution to atherosclerosis grew and the response-to-injury hypothesis was later refined to incorporate the growing evidence of a participation of monocytes and macrophages in the pathogenesis.[29] Another important hallmark from the perspective of inflammatory atherosclerotic research were the first observations of an involvement of the adaptive immune response

in atherosclerotic lesions. These came from the laboratory of *Göran K. Hansson*, who showed the presence of HLA-DR (human leukocyte antigen) as well as of activated T cells in human atherosclerotic plaques.[30,31] Later, in 1999 *Ross* published his famous article "Atherosclerosis – an inflammatory disease",[32] and since then the inflammatory feature of atherosclerosis has gained much momentum.

Atherosclerosis research has seen several 'rivalling' hypotheses in its history, but as *Daniel Steinberg* puts it for the 'rivalry' between LDL cholesterol and inflammation "There has been an unfortunate tendency to consider atherosclerosis as being either a lipid disorder or an inflammatory disorder. This is a false dichotomy. (…) they are two facets of a single pathogenesis".[33] Indeed, in the current concept and discussions of atherosclerotic pathogenesis we can recognise several features of previously proposed hypotheses.

Current Paradigm

The most widely accepted current paradigm of atherosclerosis development is based on the 'response-to-retention' hypothesis by *Williams* and *Tabas*. Based on a large body of evidence they proposed that intimal or subendothelial retention of cholesterol-rich apolipoprotein B-containing lipoproteins (apoB-LP), most prominently LDL, is the *sine qua non* condition for atherosclerosis initiation.[34] Numerous following studies examining the role of intimal retention of apoB-LP have produced convincing evidence consistent with the response to retention hypothesis.[35] Emerging evidence suggests that any apoB-LP, including lipoprotein(a) and triglyceride-rich apoB-LPs, can be the culprit in atherosclerosis initiation and progression.[36-38]

Initiation of atherosclerosis

Atherosclerosis develops focally in large and medium-sized arteries at the sites of arterial branches or curvatures with non-laminar blood flow.[9,15,39,40] The altered biomechanical forces due to the disturbed flow at these sites lead to characteristic changes of the arterial wall, which are fundamental to atherosclerosis development.

The intima shows an increased amount and altered composition of extracellular matrix components as well as abundant synthetic VSMCs, which produce the fibrous tissue. The histological appearance of the thickened intima is the previously mentioned AIT. The extracellular matrix in these precursor lesions comprises specific proteoglycans, such as versican and biglycan, which are known to be pro-retentive or 'sticky' for lipoproteins (Figure 3).[18,41-48]

The disturbed flow also impacts the endothelial cell layer and VSMCs at these sites. Endothelial cells that are exposed to disturbed blood flow are primed for inflammatory responses via NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), express the leukocyte adhesion molecule VCAM-1 (vascular cell adhesion molecule 1) and become more permeable for inflammatory cells.[49-52] This is a phenotype that might

be expected at sites of injuries or wounds, where even worse blood flow disturbances are present. Although endothelial cells are more permeable for migrating cells, disturbed blood flow per se does not seem to enhance apoB-LP permeability.[46,47,53,54] Vascular smooth muscle cells are usually restricted to the media, the muscular layer in the arterial wall, where their contractility participates in blood flow regulation. The pre-atherosclerotic AIT, however, is characterized by high numbers of VSMCs in the intima. These VSMC are considered to derive from the media and be of mono- or oligoclonal origin. Importantly, the intimal VSMC in these early lesions have lost their contractile phenotype. Instead they are characterized by a synthetic organelle repertoire and production of extracellular matrix proteins, contributing to the apoB-LP retention susceptible environment.[55,56]

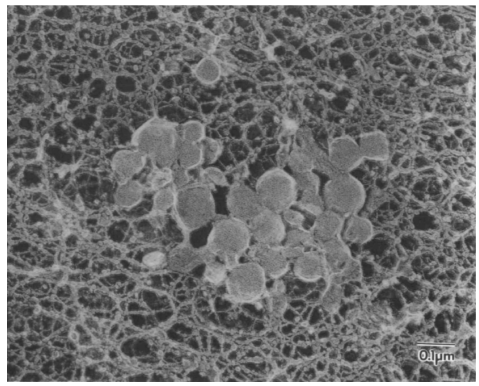


Figure 3. Lipoprotein retention. Clusters of human LDL are visible in the intima of the aortic arch of a rabbit 2 hours after injection. Reproduced with permission from (565), © Wolters Kluwer Health Inc. and American Heart Association

Apolipoprotein B-containing lipoproteins, like other molecules, continuously pass the endothelial layer and, under homeostasis, egress again. Preceding the atherosclerotic lesion development, a small fraction of the apoB-LPs is retained in the 'sticky' environment of AITs. The negatively charged proteoglycans of the AIT bind the positively charged apoB molecule via weak ionic interactions.[57] The initial retention makes the apoB-LPs susceptible to proteases and lipases, which leads to the aggregation and immobilization of the apoB-LPs.[58-60] The probability of LDL retention is dependent on blood LDL levels, explaining the proportionally increased risk for atherosclerotic cardiovascular events with increasing levels of LDL.[54,61] Intriguingly, the tendency of LDL particles to aggregate and thus the susceptibility to atherosclerosis also varies between individuals and associates with future atherosclerotic cardiovascular events.[62]

The deposited lipoproteins and lipids generate an initially restricted inflammatory response carried out by the immune systems' first responders, i.e. cells of the innate immune system. Mainly resident monocyte-derived macrophages and dendritic cells attempt to 'clean up' the subendothelial space by removing the modified and aggregated apoB-LPs.[63-69] Macrophages are able to recognise and ingest the retained lipids via phagocytosis and pinocytosis, as well as by scavenger receptors.[70-76] Notably, VSMCs also have the ability to take up cholesterol and other lipids.[77-79] The ingested lipids are metabolized and transferred to high-density lipoproteins (HDL) for the transport back to the liver, called reverse cholesterol transport.[80] Cholesterol efflux might also be facilitated by the emigration of the phagocytes from the intima.[81,82] If the uptake of cholesterol and other lipids exceeds the efflux, lipid vacuoles become visible in macrophages, dendritic cells and VSMCs, leading to the typical appearance of foam

cells. In extreme cases overwhelming ingestion of apoB-LPs might even result in apoptosis because of the toxicity of free intracellular cholesterol (Figure 4).[83]

The trapped apoB-LPs contain several molecules, including cholesterol and saturated fatty acids that convey danger signals to macrophages and other immune cells.[80,84] In addition, reactive oxygen species (ROS) from activated endothelial cells as well as myeloperoxidase from invading neutrophils can lead to oxidative modifications of phospholipids and the apoB protein.[84-86] These damage-associated molecular patterns (DAMP) activate immune cells, similarly to danger signals from infectious agents, which are called pathogen-associated molecular patterns (PAMP).[84] Damage-associated molecular patterns in the progressing lesion lead to further recruitment and activation of immune cells as well as triggering pro-inflammatory programs in endothelial cells and VSMCs. Inflammatory activation leads to the secretion of a plethora of pro-inflammatory cytokines, including interleukin (IL)-1β and IL-6 as well as chemokines, like monocyte chemotactic protein (MCP)-1, to attract additional monocytes.[87] Artery wall derived IL-6 can trigger an acute-phase reaction in the liver, leading to the secretion of C-reactive protein (CRP; see *Biomarkers*).[88] Importantly, the immune cells do not only secrete pro-inflammatory mediators but also anti-inflammatory cytokines, like IL-10 and inflammation resolving lipid mediators.[87] Depending on the balance of pro-inflammatory and pro-resolving mediators, the inflammation can cease, which leads to a halt of the progression or even regression of the atherosclerotic lesion.[89] Indeed, particularly early lesions, like AITs, can still regress, and it is thought that lowering the circulating apoB-LPs is a prerequisite for regression.[90] Intriguingly, however, regression of early pre-atherosclerotic lesions like fatty streaks seems to occur in young individuals, whose blood lipids were not lowered pharmaceutically.[14,18]

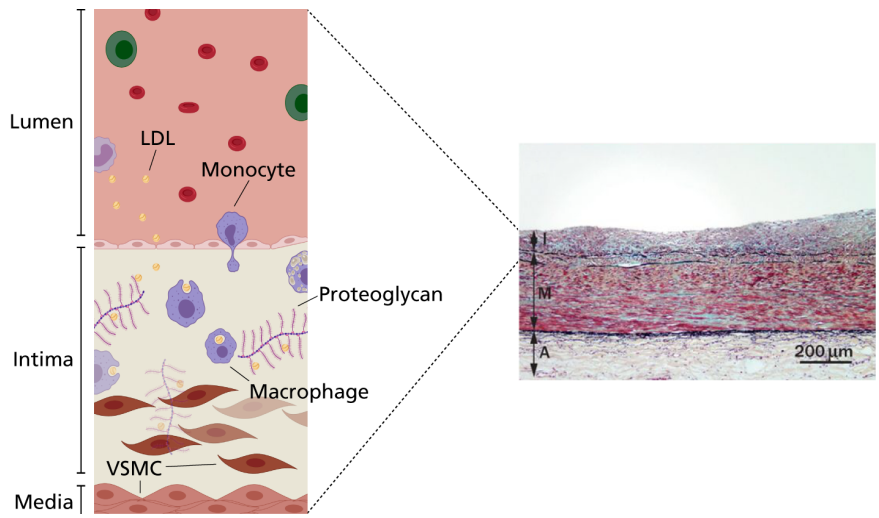


Figure 4. Initiation of atherosclerosis. Schematic representation (left) and histological appearance (right) of the adaptive intimal thickening. Histological image reproduced with permission from (566), © Springer Nature.

Progression of atherosclerosis

If the influx and retention of apoB-LPs continues, the initially beneficial immune response becomes detrimental. In the nascent atherosclerotic lesion, the retention of apoB-LPs is amplified, e.g. by macrophages secreting lipoprotein lipase and an increase in LDL permeability.[57,91] Thus, a vicious amplification loop of lesion development is started.[57,92] The sustained hyperlipidaemia leads to reduced emigration of macrophages and dendritic cells, which prime invading T cells by presentation of neo-antigens derived from the oxidative modification of retained apoB-LPs.[81,82,93-96] The misguided chronic inflammation has now progressed to the involvement of the adaptive immune system, which normally is restrained from reacting to endogenous antigens. In addition to hindering the migratory capacity of antigen-presenting cells, sustained influx of lipids exceeds the capacity of lipid uptake by macrophages and VSMCs.[60] Intracellular accumulation of free cholesterol as well as oxidatively modified lipids can result in the apoptosis of VSMCs and macrophages.[77,97] This leads to the appearance of extracellular lipid pools derived from continuous lipoprotein influx and plasma membranes of dead cells,[59,77] a characteristic of a more advanced atherosclerotic lesion, the pathological intimal thickening (PIT; Figure 5).[18] In this setting, the accumulating cholesterol can precipitate inside macrophages, forming cholesterol crystals, which in turn drive the activation of the NLRP3 (nucleotide-binding domain, leucine-rich-repeat-containing family, pyrin-domain-containing 3) inflammasome, boosting the secretion of IL-1β and IL-18, and potential cell death via pyroptosis.[80,84,88,89] The increased cell death and pro-inflammatory milieu, with a concomitantly defective removal of cell debris (defective efferocytosis) precedes the formation of a necrotic core, which contains a myriad of DAMPs and thrombogenic material.[89,98,99]

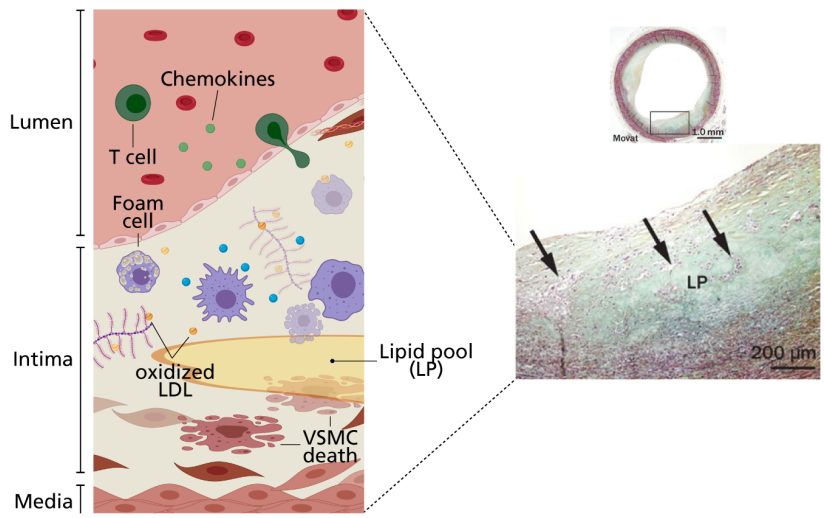


Figure 5. Progression of atherosclerosis. Schematic representation (left) and histological appearance (right) of the pathological intimal thickening. Arrows in the histological image correspond to foamy macrophages. Histological image reproduced with permission from (566), © Springer Nature.

In an attempt to stabilize the advancing lesion, synthetic VSMCs produce extracellular matrix proteins, like collagen I and III, and form a fibrous cap on top of the necrotic core.[18,56] This shields the thrombogenic material from exposure to the blood stream and the thrombocytes therein. The generation of a fibrous cap on top of the necrotic core prohibits the formation of a thrombus in the now advanced fibroatheroma.[18] Inflammation and plaque growth-induced nutrient and oxygen scarcity lead to the expression and activation of several factors including hypoxia-inducible factor (HIF)-1α, which in turn results in the secretion of vascular endothelial growth factor.[100,101] Triggered by the growth signals, new vessels grow into the plaque from the surrounding tissue, the adventitia (Figure 6).[18]

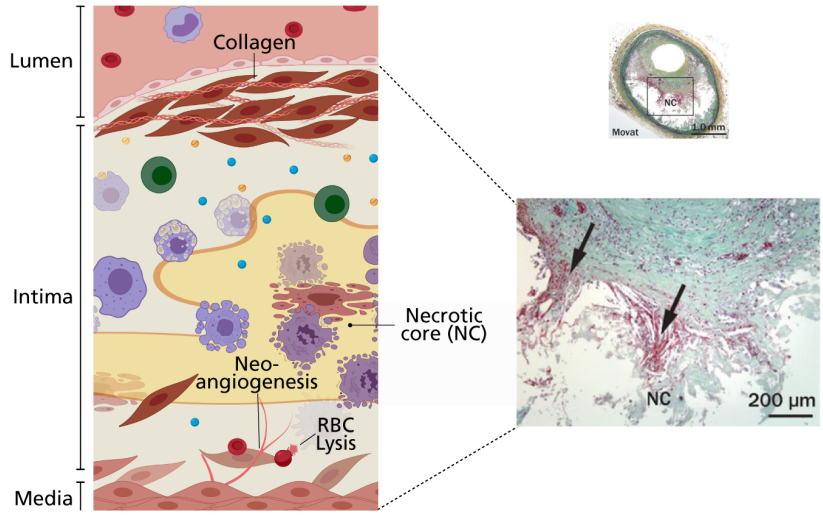


Figure 6. Development of an advanced atherosclerotic plaque. Schematic representation (left) and histological appearance (right) of the late fibroatheroma. Arrows in the histological image correspond to intraplaque haemorrhage. RBC = red blood cell Histological image reproduced with permission from (566), © Springer Nature.

The vessels growing into the plaque are known to be leaky, probably as a result of the highly inflammatory milieu.[102] The increased permeability leads to increased immune cell recruitment as well as to extravasation of red blood cells. The cell membranes of the red blood cells are rich in cholesterol, which is deposited within the plaque after cell lysis, thereby further amplifying the chronic inflammatory reaction.[100,102] Continuous accumulation of dead cells and debris might lead to regions of calcifications within the plaque. The understanding of the triggers and the consequences of calcification is limited. It has been suggested that small and spotty calcifications impair plaque stability, whereas sheets of calcium deposition associate with a higher stability.[103]

The raging inflammatory response produces extensive collateral tissue damage and compromises the integrity of the advanced atherosclerotic plaque.[18,89,104-106] Enzymes

such as matrix-metalloproteinases (MMP) and ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) degrade the fibrotic material, and cellular death destabilizes the lesion. These processes cumulate in the appearance of a thin-cap fibroatheroma, the histological equivalent of the 'vulnerable' or rupture-prone plaque (Figure 7).[18]

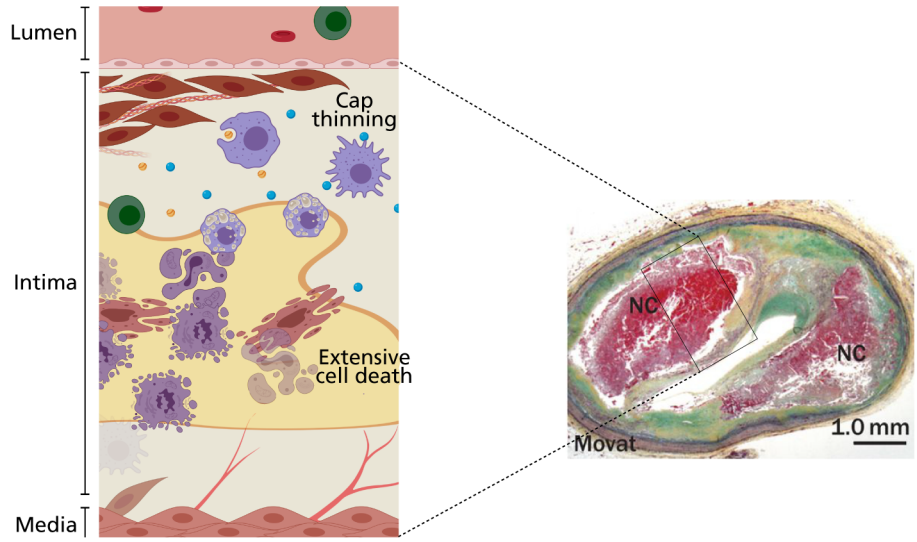


Figure 7. Progression to a high-risk lesion. Schematic representation (left) and histological appearance (right) of the thin-cap fibroatheroma. Histological image reproduced with permission from (566), © Springer Nature.

Complications of atherosclerosis

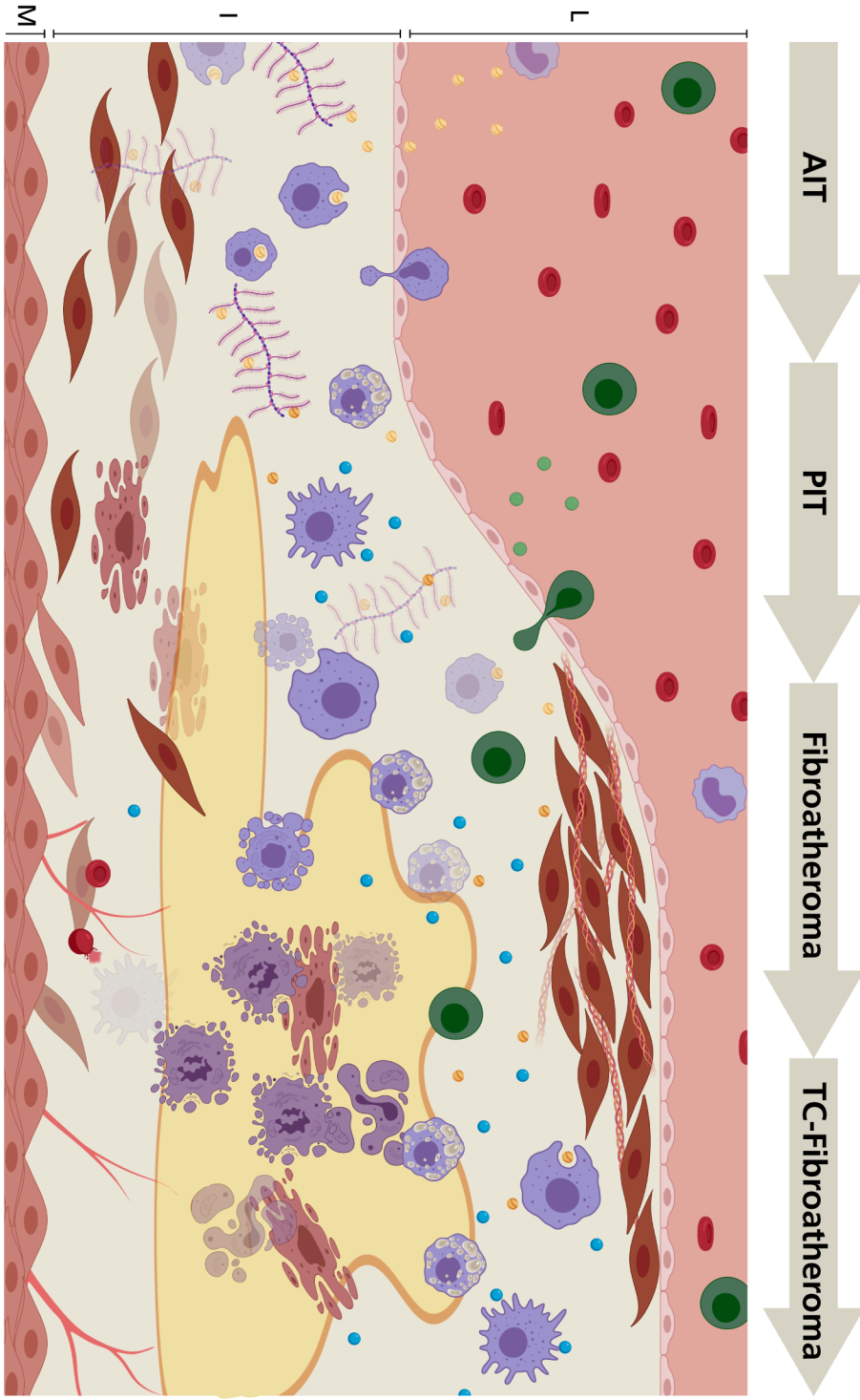
The progression of pre-atherosclerotic AITs to rupture-prone advanced plaques usually takes decades. Importantly, atherosclerotic plaques can stabilize or probably even regress at various stages during the development from a pre-atherosclerotic AIT to the thin-cap fibroatheroma.[90,99] At the same time, however, the disease development most often takes place unnoticed and without causing symptoms. Despite the growth of the atherosclerotic plaque in the luminal layer of the arterial wall, arterial outward remodelling initially prevents the protrusion of the plaque into the vessel lumen.[107,108] Only at advanced stages and after decades of growth, can atherosclerotic plaques grow large enough to compromise the calibre of the vessel lumen and cause chronic low-grade tissue ischemia, resulting, for instance, in stable angina.

The life-threatening complications of atherosclerosis, ischaemic stroke and MI, occur very suddenly and acutely, despite the otherwise silent and chronic course of the disease. The cause of these sudden events is almost always a thrombotic episode, leading to the occlusion of the vessel and substantial ischaemic injury in the tissue the vessel is feeding. In the majority of cases (approximately 70 % of the MI cases and approximately 90% of the ischaemic stroke cases),[18,109] the rupture of an

advanced atherosclerotic plaques exposes highly thrombogenic material, spurring the formation of a thrombus. Plaque rupture occurs most frequently at the shoulders of the fibrous cap. Here, abundant immune cells, including T cells, mast cells and macrophages secrete cytokines and enzymes, such as myeloperoxidase and MMPs, that weaken the fibrous layer by inducing VSMC death and collagen degradation, which eventually leads to rupture.[99,110-112] The remaining thrombotic cases are mainly caused by atherosclerotic plaque erosion.[99,109] Erosion may occur in plaques at earlier developmental stages, PITs and early fibroatheromas, and is characterized by endothelial denudation.[18,99] The mechanisms of erosion are even less well understood than the pathophysiology of plaque rupture. Inflammatory signalling via toll-like receptor 2 and neutrophils are implicated in the events leading up to endothelial cell death and denudation.[113] Importantly, however, there is compelling evidence that most thrombotic events, whether caused by plaque rupture or erosion, do not lead to substantial tissue ischemia and remain silent. The small thrombi formed during these events are incorporated into the atherosclerotic lesion and contribute to the volume increase of the growing atheroma. These healed ruptures often contain extensive calcifications.[18,99]

Clinically important is the different magnitude of potential arterial occlusion caused by either plaque rupture or erosion. Early evidence suggests that plaque erosions cause incomplete thrombosis by a platelet-dominated clot, whereas rupture induces fibrin-rich clots and total vessel occlusion.[18,113]

➤ **Figure 8. Atherosclerosis development.** Schematic representation of the development of an atherosclerotic plaque from the precursor lesion AIT to the rupture-prone thin-cap fibroatheroma. TC = thin-cap, M = media, I = intima, L = lumen.


M
I
L
AIT
PIT
Fibroatheroma
TC-Fibroatheroma

The Immune System in Atherosclerosis

The immune system is designed to defend us from pathogens and provide protection against detrimental effects of infections. In their roles as guardians of tissue integrity, immune cells are also key effectors in wound healing, tissue repair and removal of debris and dead cells. By design, the immune system should be a protection against atherosclerosis, by simply removing trapped and modified apoB-LPs. In progressing atherosclerotic plaques, the protection provided by the immune system immunity goes awry, leading to a non-resolving chronic inflammation that drives lesion progression. Atherosclerotic plaques in mice[115-118] and humans[116,119] harbour a diverse repertoire of immune cells, including monocytes, macrophages, neutrophils, B cells and T cells. The first leukocytes tackling the retained apoB-LPs are cells of the innate immune system.

Innate Immune Cells

Resident macrophages and dendritic cells are the first cells to encounter the retained and modified apoB-LPs. As described above, these phagocytes take up the lipids and cholesterol and clear it from the artery wall. When macrophages take up more cholesterol than they excrete, excess cholesterol is stored as cholesteryl ester in intracellular lipid droplets, which generates the typical appearance of foam cells. The cholesterol uptake in this setting leads to a suppression of inflammatory cytokines like IL-1β and an increase in certain metabolic enzymes, including enzymes of the electron transport chain for oxidative phosphorylation (see also *Part II – Immunometabolic Traits*).[117,120,121] Foam cells have recently been associated with the expression of the surface marker TREM2 (triggering receptor expressed on myeloid cells 2).[117,118,122] Intriguingly, $TREM2^{+}$ macrophages have been shown to play a crucial role in homeostasis of systemic metabolism.[123]

The protective response triggered by cholesterol uptake seems to be related to the physiological role of macrophages in clearing apoptotic cells (efferocytosis), e.g. in the setting of wound healing, which contain large amounts of cholesterol. In progressing atherosclerotic lesions, macrophages are activated by DAMPs, which represents a critical proatherogenic process.[120] The precise series of events and even the trigger of this activation remain obscure. Oxidative modification of apoB-LPs and thereby generated DAMPs are implicated as culprits in this process. Human trials targeting this mechanism have, however, failed so far.[124] Alternatively, non-programmed cell death (necrosis) because of the intracellular accumulation of non-esterified cholesterol could be an initial trigger. Necrosis releases several molecular structures that can act as DAMPs and the early atherosclerotic lesions, like the PIT, contain large amounts of cell membranes from dead cells.[18,84]

In any case, inflammatory activation of resident phagocytes sets a systemic inflammatory in motion. Secretion of chemotactic molecules and upregulation of endothelial cell adhesion molecules facilitate the recruitment of neutrophils into the

artery wall.[125-127] Neutrophils in turn cooperate in promoting monocyte infiltration, which differentiate into macrophages to launch an effective immune response.[126,128]

The circulating numbers of the infiltrating monocytes, Ly6C^{hi} in mice and CD14^{+} in humans, are associated with atherosclerosis development and aggravation.[129-131] Intriguingly, chronic stress, sleep deprivation, and myocardial infarction lead to an increase in circulating Ly6C^{hi} monocytes and accelerated atherosclerosis in mice.[132-134]

Inflammatory macrophages, also called M1 macrophages, contribute to lesional inflammation by secretion of pro-inflammatory mediators, such as IL-1β, IL-12 and tumor necrosis factor (TNF)-α. Furthermore, the induction of nitric oxide synthase leads to the release of anti-microbial nitric oxide.[120] Reactive oxygen species, generated by NADPH oxidase in macrophages or neutrophil myeloperoxidase usually aid pathogen destruction, but in atherosclerosis they generate oxidatively modified apoB-LPs.[120,135,136] Importantly, these anti-microbial and inflammatory mediators are non-specific and induce substantial collateral damage in the artery wall. In a physiological immune response, the inflammatory phase is followed by a resolution phase, which also includes efferocytosis and repair of tissue damage.[89,120]

Macrophages also play important roles in the resolution phase. Reparatory macrophages, also called M2 macrophages, represent the other extreme in the continuum of macrophage plasticity. Reparatory macrophages express surface receptors, like MerTK (Mer proto-oncogene and tyrosine kinase), which are crucial for efferocytosis.[120] Efferocytosis avoids secondary necrosis of apoptotic cells and thus the release of new DAMPs.[89] In addition, they secrete pro-resolving and reparatory mediators, like IL-10 and transforming growth factor (TGF)-β.[120] The resolution response impedes further leukocytic influx, promotes an anti-inflammatory state and initiates tissue repair, e.g. by stimulating collagen deposition.[89,126,137,138] In progressing atherosclerotic plaques, the resolution is, however, defective and the chronic activation state of innate or myeloid immune cells are crucial in the lesion initiation. Depletion of myeloid cells early in atherogenesis or inhibiting differentiation of monocytes into macrophages dramatically reduces and delays atheroprogression, showing the importance of these cells in lesion initiation.[139-141]

Another important immune cell type is the dendritic cell, which bridges the innate and adaptive immune response. Dendritic cells take up antigenic material, e.g. from pathogens or oxidized apoB-LPs, emigrate to lymph nodes and present it to the specific arm of the immune system, the adaptive immune cells.[142] In hypercholesterolemia, however, this emigration is impaired.[81,82,93] The decreased efflux of dendritic cells during hypercholesterolemia might be a physiological response. Hypercholesterolemia and continuous influx of cholesterol-containing apoB-LPs into the artery wall might be sensed as excessive cellular death, which also releases substantial amounts of cholesterol. Excessive cellular death because of an infectious agent means extreme danger for the entire organism and to avoid spreading the pathogen, dendritic cells might limit their emigration (similar to the infection with mycobacterium tuberculosis). Nonetheless, some dendritic cells will migrate to lymphatic tissue and

activate the adaptive immune system, or alternatively activate infiltrating T cells directly in the artery wall.[96]

Adaptive Immune Cells

The adaptive immune system constitutes B and T cells. Cells of the adaptive immunity stand out as highly specific defenders that also generate an immune memory. The specificity of B and T cells is mediated by their surface receptors, the B cell receptor (BCR) and T cell receptor (TCR). During the differentiation of B and T cells, their receptors are generated by germ-line rearrangement, which permits the recognition of millions of pathogenic molecular structures or antigens. Adaptive immune cells undergo positive and negative selection processes in their development, thus ensuring a high specificity towards foreign non-self molecules, such as pathogens, and preventing the recognition of endogenous self-antigens (autoimmunity).[143]

The recombination of BCRs and TCRs can be utilized experimentally. The absence of proteins essential for the receptor rearrangement leads to a defective development of adaptive immune cells. Mice with genetically induced defects in these proteins lack adaptive immune cells. Intriguingly, when these mice are crossed with atherosclerosis-prone mice, they only show an amelioration of atherosclerosis development if fed a regular low-fat chow diet. In contrast, feeding these mice a Western-type diet, does generally not affect atherosclerosis development.[144-148] These data, although generated in an artificial experimental system challenge the importance of the contribution of the adaptive immune system, at least in the circumstance of a Western-type diet (see also *Methodological Considerations – Mouse Studies*).

Antibody production by B cells is essential for host defence by neutralizing or marking (opsonization) antigens and thereby initiating an inflammatory response against toxins and invading pathogens.[143,149] Furthermore, antibodies possess housekeeping functions, like mediating the clearance of cellular debris – an important factor going awry in progressing atherosclerotic plaques (see *Current Paradigm*).[149,150] In addition to antibody-producing B cells, which are called plasmablasts and plasma cells because of their histological appearance, B cells participate in the immune response by acting as antigen-presenting cells as well as by modulating the inflammatory reaction through the secretion of various cytokines, including regulatory IL-10 (leading to their naming as regulatory B cells).[149,151] B cells can mainly be found in the plaque-surrounding media and adventitia, and only in low numbers in the atherosclerotic plaque.[115,116,118,119] Even so, antibodies can be found at all stages within the atherosclerotic lesion.[152,153] Furthermore, the genetically induced absence of B cells in mice leads to an aggravation of lesion development.[149,154] Nonetheless, the protection from atherosclerosis by B cells is subset dependent and differential effects of B cell subsets and antibody isotypes have been observed.[149,154,155]

B cells can be roughly distinguished into two groups: conventional B2 cells and innate-like B1 cells.[150,154] The B1 cells are denoted as innate-like because they possess a highly restricted repertoire of BCRs and thus antibodies.[150] The secretion of these

antibodies does not require a B cell activation stimulus but occurs at steady-state and can already be found in human cord blood, that is why they are called natural antibodies. Natural antibodies bind with low affinity to a broad spectrum of self- and foreign antigens and are mostly of an IgM isotype.[149,150,154,156] Typical antigens that are recognized by natural antibodies are oxidized apoB-LPs and lipids, which are also present in dying cells or pathogens. The B1 cells are considered anti-atherogenic, which is mainly attributed to their production of natural IgM.[150,151,154,156] Natural antibodies recognize various antigens present in atherosclerotic plaques and facilitate their removal.[149,155,156] Mouse studies have repetitively shown an anti-atherogenic effect of B1 cells and natural antibodies and in humans IgM against apoB-LPs are associated with less severe atherosclerotic CVD (ASCVD).[149,154,155]

Conventional B cells, or B-2 cells, possess a much more diverse repertoire of BCRs and antibodies, and produce all antibody isotypes, but mainly IgG.[143,149] In contrast to B1 cells and natural IgM antibodies, B2 cells and IgG antibodies seem to exert detrimental functions and aggravate inflammation and atherosclerosis development. The selective absence of B2 cells in mice leads to an amelioration of atherosclerosis development and the levels of IgG antibodies recognizing plaque antigens associate with more severe ASCVD in humans, although some studies report conflicting results.[149,154,155] A special subset of B cells, regulatory B cells, play a role in the resolution of inflammation by mediating anti-inflammatory effects, e.g. through the secretion of IL-10. Consequently, regulatory B cells have been shown to ameliorate disease in experimental models.[149]

In order to differentiate into plasma cells and secrete copious amounts of antibody, conventional B cells require several signals. The BCR needs to bind the specific antigen and the B cell needs to be activated by additional signals, including those provided by helper T (T_H) cells.[143,149] In order to provide B cell help, T_H cells themselves need to be activated first. Antigen-presenting cells display short antigenic peptides (epitopes) bound to major histocompatibility complex (MHC) II molecules to naïve T_H cells. The T_H cell, which is specific for the presented epitope, is activated once it encounters the antigen-presenting cell. In contrast to T_H cells, which are characterized by the surface protein CD4, cytotoxic T cells are identified by the expression of CD8 and antigens are presented on MHC I molecules to cytotoxic T cells.[143]

In addition to peptide antigens, the human body harbours special T cells that are specific for lipid antigens presented on the family of MHC I-like CD1 molecules.[157] A special subset of these lipid-specific T cells are the innate-like invariant natural killer T (iNKT) cells, which are the topic of *Part II – Invariant Natural Killer T cells and coronary events*.[158] Importantly, MHC and MHC-like molecules are not only necessary for the activation of T cells in the periphery but are also essential during the maturation of T cells in the thymus. In mice engineered to lack these molecules, thymic positive and negative selection of T cells cannot proceed, the respective T cell subset does not mature and is missing in the organism (see also *Part II – MHC II deficiency in experimental atherosclerosis*).

Besides the recognition of the specific T cell antigen on MHC molecules with the cell's TCR, additional activating signals are necessary to permit the differentiation of naïve T cells into effector T cells. These costimulatory signals come in form of cytokines and surface receptors on antigen-presenting cells and also influence the phenotype of the effector T cell.[143] One of the most important costimulatory receptors on T cells is CD28, which pairs with CD80 or CD86 on antigen-presenting cells and allows for full differentiation (see also *Part II – $CD4^+CD28^{null}$ T cells and cardiovascular events*).

The activation process influences the functional phenotype of T_H cells, leading to the differentiation of different subsets.[143] Four of the most studied subsets are T_H1, with interferon (IFN)-γ as signature cytokine; T_H2, which secretes IL-4, IL-5, and IL-13; T_H17, secreting mainly IL-17; and follicular helper T cells, which provide help to B cells in lymphoid follicles. Helper T cells act mainly through the secretion of cytokines, most prominently their signature cytokines, but can also interact directly with MHC II expressing cells.[143,159,160]

Interferon-γ from T_H1 cells amplifies the recruitment of macrophages and T cells into the progressing plaque and increases the activation of macrophages.[159,161,162] In the advanced plaque, IFN-γ exerts detrimental effects by promoting VSMC death and inhibiting collagen production as well as augmenting the secretion of MMPs, which ultimately leads to a rupture-prone plaque.[161,162] Macrophage-derived IL-18 potentiates the secretion of IFN-γ by T_H1 cells and in turn T_H1 cells can directly interact with macrophages to further activate them.[143,163,164] Given these roles of T_H1 cells, it is not surprising that there is ample evidence of a proatherogenic role of T_H1 cells in experimental atherosclerosis.[159] In humans, T_H1 cells are associated with increased subclinical atherosclerotic disease but surprisingly do not associate with future cardiovascular events,[165,166] although further studies assessing this association are pending.

The roles of T_H2 and T_H17 cells in atherosclerosis are more controversial. Whereas genetic deletion of IL-5 and IL-13, two of the T_H2 signature cytokines, aggravates disease, partly by reducing protective natural antibody titres,[167,168] mice lacking IL-4 have a decreased atherosclerotic burden.[169,170] Part of the proatherogenic role of IL-4 might be its importance in providing help to B cells to switch their antibody isotype to IgE, which is associated with more severe atherosclerotic disease in humans and in mice.[143,149] Complicating the interpretation of T_H2 cells' roles even more is the fact that circulating T_H2 cells have been associated with a lower incidence of cardiovascular events in humans.[166]

Interleukin-17 and T_H17 cells have been associated with increased plaque collagen and stability in mouse and human atherosclerotic plaques,[171,172] but the results for human lesions are conflicted by other studies.[173] Deficiency in IL-17 signalling in mice has been shown to be atheroprotective,[174-178] whereas other reports have found the opposite.[171,179]

The nidus for the conflicting results might lie in the plasticity of T_H cells. Previously it has been thought that once a T_H cell becomes a differentiated subset, e.g. T_H17, it is

lineage committed. It is now recognized that T_H cells can change their phenotype depending on environmental and other cues, similar to innate immune cells.[180,181] A detrimental feature of the T_H cell plasticity is the loss of regulatory T (T_{REG}) cells.[182,183] Regulatory T cells play an important role in the resolution of inflammation, in concert with reparatory macrophages and other cells. Through the secretion of various cytokines, like IL-10, IL-13 and TGF-β as well as direct cell-contact, they dampen the inflammation and promote efferocytosis, thus stabilizing the atherosclerotic lesion.[184,185] It is thus not surprising that T_{REG} cells are diminished in progressing atherosclerosis and associate with less severe ASCVD in humans and mice.[186-194] Despite the predominance of cytotoxic $CD8^+$ T cells in advanced human atherosclerotic plaques, the roles of these cells in atherosclerosis are even less well understood.[119] As for other immune cells, they might have protective and pathogenic functions depending on the environmental instructions.[195]

Unresolved Questions

Although our understanding of the pathomechanisms in atherosclerosis has seen significant advances since the earliest dispute of *Virchow* and *von Rokitansky*, considerable gaps in our knowledge persist. Two exemplary features of these voids can be found in the early stages of disease initiation and the very late stage respectively.

The precise mechanism(s) firing up the detrimental chronic inflammation that cause disease initiation as well as the 'defect' in the resolution of this response are poorly understood. In particular, why can local factors and enzymes in the arterial wall lead to the enhanced generation of DAMPs from endogenous substances, or even the native LDL particle be antigenic? Why is this happening to apoB-LPs, despite the abundant influx of other molecules and particles, including HDL? Why do the recruited phagocytes not just clear the intima of the retained endogenous lipids and lipoproteins, as they would do with cellular debris in wound healing, without starting a vicious cycle of self-accelerated inflammation?

Our understanding of the pathophysiological events leading to acute complications of atherosclerosis is also very limited. The culprit lesion for most plaque ruptures, and thus most MIs and ischaemic strokes, is the thin-cap fibroatheroma. The PROSPECT trial found that less than 5 % of the, with intravascular ultrasonography, detected rupture-prone plaques caused an MI within the following 3.5 years.[114] At the same time, however, some of the almost 600 detected thin-cap fibroatheroma might have ruptured but remained clinically silent. How can we detect a plaque, or a patient bearing a plaque(s) that will cause an event (and not just silently rupture and heal)? Moreover, what would be the treatment leading to stabilisation of this/these plaque(s)?

The Killer of the 21st Century

In 1971, Abdel R. Omran coined the epidemiological transition theory to connect the demographic changes over the past centuries to epidemiology.[196] An important notion of the epidemiological transition is the mainly sanitation-driven decline of infectious disease mortality, while chronic diseases, or "*degenerative and man-made diseases*" as Omran called them, took over the lead as prime mortality factor in industrialized countries (Figure 9). Thus, mainly lifestyle modification, i.e. hygiene, together with medical advances (antibiotics and vaccines) lead to a massive decline in total mortality, and a switch to CVD as the main cause for mortality in industrialized countries. [196,197]

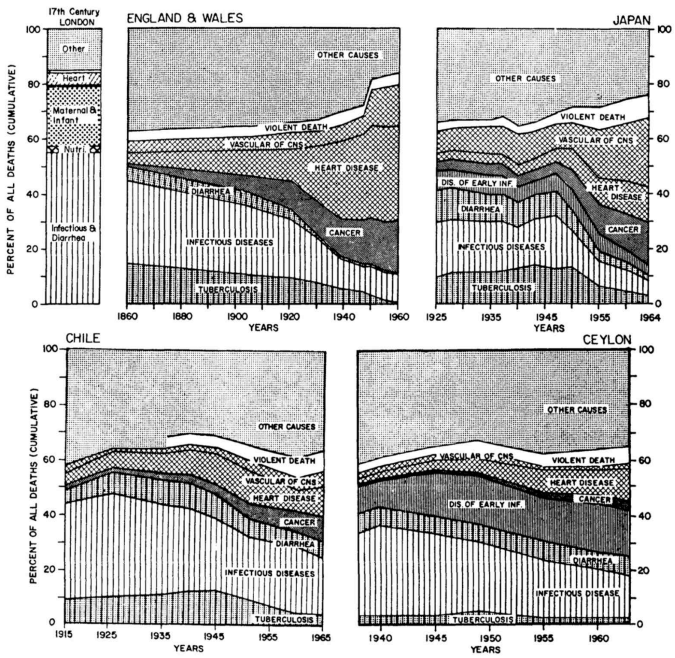


Figure 9. Epidemiological Transition. Changing mortality patterns in England and Wales from a mainly maternal complication and infectious disease burden in the 17th to the 19th century, to a CVD dominated burden from the early 20th century. Japan shows an accentuated transition starting in the mid 20th century, whereas Chile and Sri Lanka (formerly: Ceylon) had not yet experienced such a transition in the mid 19th century. Reproduced with permission from (196), © Milbank Memorial Fund. Published by Blackwell Publishing.

Nowadays, CVD still remains the number one cause of mortality in the Western world and has evolved to be the leading cause of death globally (Figure 10). In 2017, 17.8 million people died of CVD, accounting for 31.8 % of the deaths worldwide. Importantly, the majority of CVD deaths are caused by atherosclerosis (ASCVD), such as ischaemic heart disease (IHD), ischaemic stroke and peripheral artery disease (PAD), adding to a total of 11.7 million global deaths.[6] Naturally, and in line with the epidemiological transition, a significant proportion of the increased mortality due to ASCVD can be accounted for by population ageing.[199] The prevalence of IHD in individuals older than 50 years in countries with a high income per capita, according to the World Bank, adds up to 22639 per 100000 men and 13080 per 100000 women.[200]

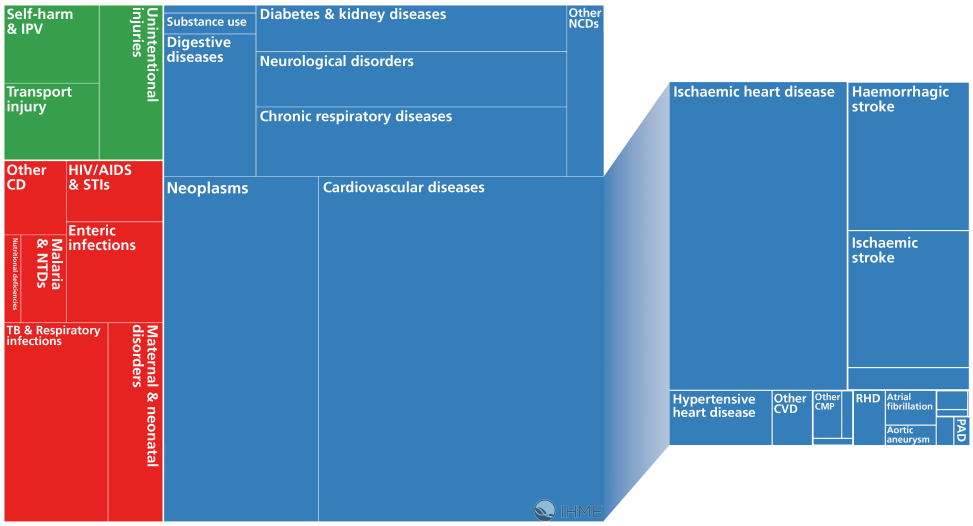


Figure 10. Global Mortality. Treemap showing the worldwide causes of death in 2017. The size of each rectangle corresponds to the accountable fraction of the respective disease on all-cause mortality (left) and cardiovascular CVD mortality (right) among individuals of both sex and all ages. The treemap on the right shows the various underlying pathologies for cardiovascular deaths, with IHD as the main pathology accounting for 50 % of the deaths due to CVD and consequently for 16 % of all-cause mortality. The colours indicate their overarching aetiology as non-communicable (blue) and communicable (red) diseases as well as injuries (green). IPV = inter-personal violence, CD = communicable disease, STI = sexually transmitted infection, NTD = neglected tropical disease, TB = tuberculosis, NCD = non-communicable disease, CMP = cardiomyopathy, RHD = rheumatic heart disease. Adapted from (198)

Population ageing is only responsible for a fraction of the increased cardiovascular mortality, though. Population growth and changes in the risk factor patterns also play a significant role in the increased deaths, whereas improved medical care has ameliorated this development.[201,202]

Mortality numbers do not show the full impact of ASCVD on the population's health. With 2 159 years of life lost (per 100 000 individuals), IHD is the single most common cause for premature death worldwide.[6] In addition to outranking all other diseases in premature mortality rates, IHD also leads the charts for total health loss, described by disability adjusted life years (DALY), a combined measure of premature mortality (years of life lost) and morbidity (years lived with disability).[200,203] The fact that, with IHD, an atherosclerotic disease tops the rankings for total and premature mortality, despite an impressive 28 % reduction of the age-standardized loss in life years rate over the past 27 years, emphasizes the importance of further research into the disease mechanisms as well as into its prevention and treatment.[200]

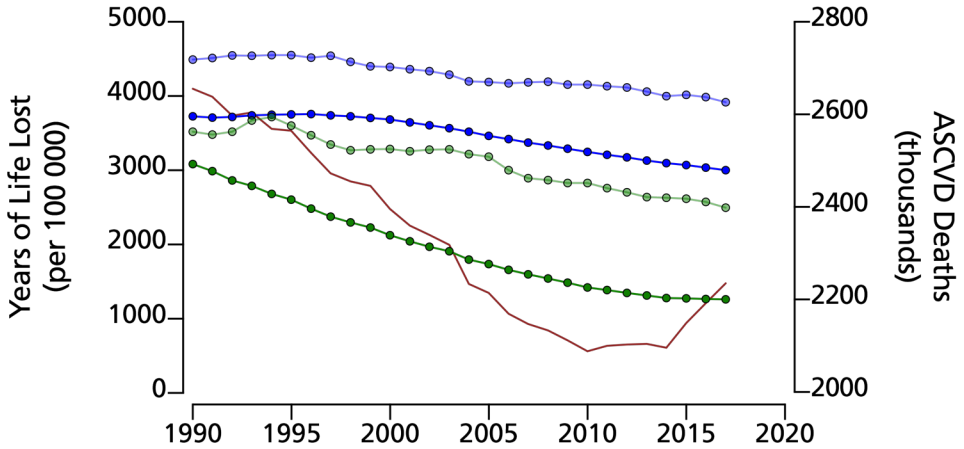


Figure 11. Premature death according to World Bank income levels. Age-standardized years of life lost (per 100 000 individuals) due to ischaemic heart disease, ischaemic stroke and peripheral artery disease in ●high-income, ●upper-middle, ●lower-middle, and ●low-income countries. Red line indicates total number of deaths in high-income countries for all ages. Data from (200).

Medical advances have contributed to a decreasing rate of ASCVD-induced premature death in the past decades, particularly in high-income countries. In recent years, however, we have seen an alarming stagnation of the decrease in the age-standardized premature death rate in wealthy nations, and a concomitant increase in absolute mortality due to ASCVD of 11.9 % since 2014 (Figure 11). These developments occur in countries like Austria, Sweden, or Canada, where effective prevention strategies, including smoking legislation, and state-of-the art healthcare based on previous medical advances, foremost the introduction of statins, have been established. This clearly shows the importance of continuous knowledge gain in ASCVD.[204-207]

Risk Factors

Numerous different risk factors for atherosclerosis and ASCVD have been suggested and identified in observational studies and randomised clinical trials.[208] Risk factors are traditionally divided in modifiable and non-modifiable. Non-modifiable risk factors for ASCVD include age and male sex, as well as the individual's genetic repertoire. In contrast, modifiable risk factors appear to be responsible for up to 90 % of the ASCVD risk, although many of them have a genetic component.[209] In line with this, recent results indicate that the contribution of genetic risk to atherosclerotic cardiovascular events might be of similar amplitude as lifestyle-related modifiable risk factors. A healthy lifestyle, consisting of non-smoking, regular physical activity, non-obesity and

a healthy diet has a similarly low risk for coronary events as a favourable genetic profile.[210]

Modifiable risk factors have been the targets of preventive and therapeutic efforts in ASCVD, explaining the steady decline in atherosclerotic premature death rate in wealthy nations over the past 20 years.[211] A prominent example of the lowered burden of traditional risk factors is the decline in tobacco usage in many high-income countries.[200,212,213] Other traditional risk factors, such as the causal LDL cholesterol and high systolic blood pressure have also been improved by medical care.[201,214]

In contrast, unfavourable dietary patterns, physical inactivity and obesity have dramatically increased in wealthy nations recently and might partly explain the stagnating premature death rates.[201,215-219] Consequently, some epidemiologists speak of the age of obesity and inactivity as the next phase in the epidemiological transition theory.[220] It is interestingly to note that the previous epidemiological transition from infectious diseases to mainly CVD was primarily lifestyle-driven (sanitation) and today's burden could likely also be resolved with lifestyle modifications.

In addition to the increasing prevalence of obesity, other comorbidities associated with an increased risk for atherosclerotic cardiovascular events, including diabetes mellitus and autoimmune diseases like rheumatoid arthritis, have added to the burden of ASCVD.[221-223]

Importantly, risk factor patterns are used to guide preventive and treatment efforts in individuals with subclinical atherosclerotic disease. The Framingham Heart Study pioneered this work by identifying risk factors for atherosclerotic cardiovascular events and developing the Framingham risk score.[224,225] In Europe, the European Society of Cardiology has developed the systemic coronary risk evaluation (SCORE), which takes the traditional risk factors (age, sex, total cholesterol, systolic blood pressure, smoking) in a country-specific manner into account.[226] The resulting risk estimate is used to stratify individuals according to their 10-year risk for a first-time fatal atherosclerotic cardiovascular event and guide the respective preventive measures.[204] An inherent problem of risk scores, like SCORE or the Framingham risk score, is their population-specificity, which makes them not universally applicable for risk estimation.[227,228] This means that the risk is calculated on the basis of the average individual in the respective country, e.g. the average genetic risk, which might differ substantially from the genetic risk that each individual possesses. More importantly, a significant number of individuals develop atherosclerotic cardiovascular events despite the absence of risk factors.[229-232] Thus, scientific efforts in order to discover sensitive and universally applicable biomarkers, which identify the individual at high risk, are needed.

Biomarker

> *The challenge is to develop nonivasive screening methods to detect coronary atherosclerosis in its earliest stages.*
>
> Michael S. Brown and Joseph L. Goldstein
> Heart Attacks: Gone with the Century?
> *Science 1996*[233]

A biomarker (biological marker) is a biological feature or characteristic, e.g. a measured blood analyte, cell type or imaging target, that associates with a biological process or disease.[234] A biomarker can be indicative for the presence of a disease, e.g. as a diagnostic biomarker or also associate with the risk of a disease or disease outcome, which would be called a predictive biomarker. Hence, a risk factor is also a prognostic biomarker.[235] Novel biomarkers could aid the identification of high-risk patients with subclinical disease and the detection of high-risk plaques as well as guide a tailored treatment strategy. The emergence of B-type natriuretic peptide (BNP) and cardiac troponins as diagnostic cornerstones in heart failure and myocardial infarction illustrate biomarker success stories in CVD.[236,237] A biomarker able to assess whether significant or high-risk atherosclerosis is present in a patient, beyond the pure population-wide risk estimation, could have a significant impact on the global atherosclerotic burden.

European guidelines, however, do not (yet) promote the application of biomarkers beyond the measurement of traditional lipid biomarkers, such as LDL and total cholesterol.[204] Although a vast repertoire of potential biomarkers have been suggested in observational studies, none could dramatically improve the risk stratification, compared to the established risk scores.[238-240] Interestingly, even B-type natriuretic peptide and troponin have been suggested as biomarkers for atherosclerotic vascular disease, although they are elevated in response to myocardial wall stress and myocardial ischemia, respectively.[241] Thus, these two biomarkers indicate processes at the end of the ASCVD continuum, long after the optimal detection timepoint for an early initiation of anti-atherosclerotic treatment.

Currently, high-sensitive CRP (hsCRP) represents the most promising candidate as an additional atherosclerosis biomarker and is part of the American Reynolds risk score.[242,243] The acute phase protein CRP mirrors the low-grade chronic inflammation in atherosclerotic plaques and has repetitively proved to be a good predictor of atherosclerotic cardiovascular event risk.[244,245] High-sensitive CRP measurements could in the future be of particular use in stratifying patients whether to receive anti-inflammatory therapies to prevent atherosclerotic cardiovascular events (see *Treatments*).[246,247] Nonetheless, this biomarker is neither causal nor atherosclerosis specific, and influenced by any inflammatory process in the body.[244,245]

In summary, a universally applicable, sensitive biomarker could improve the population-based risk estimation by risk scores, and identify the individual patient bearing high-risk atherosclerotic plaques.
The study presented in ***Paper V*** represents an effort of evaluating a potential diagnostic/prognostic biomarker.

Biomarkers or risk factors are not necessarily causally linked to the associated disease. Prominent examples for ASCVD are HDL and hsCRP. Both are non-causal biomarkers, i.e. their levels in blood do not ameliorate or aggravate atherosclerosis and its sequelae.[248-257] In stark contrast, LDL is a causal biomarker for atherosclerosis. Consequently, LDL lowering has evolved to a main pillar of anti-atherosclerotic therapy since its discovery as a biomarker of atherosclerotic disease.[61] Low-density lipoprotein is a stereotype of how biomarker studies can yield novel insights into disease mechanisms, in addition to their value as risk estimation and diagnostic tools. Thereby, biomarkers might be able to unveil unrecognized pathological mechanisms and identify novel treatment targets.
The studies in ***Paper I*** and ***Paper IV*** represent biomarker efforts to identify novel mechanisms and targets for diagnosis and therapy.

Treatment

A healthy lifestyle with regular exercise, healthy diet and no tobacco use is among the most effective therapies of atherosclerosis, besides being probably the most cost-effective. Measures to achieve a healthy lifestyle are thus a cornerstone of atherosclerotic cardiovascular event prevention. An additional therapeutic layer is the management of comorbidities, mainly hypertension and diabetes.[204]

Causal pharmaceutical therapy targeting the atherosclerotic development and progression relies on lipid-lowering and anti-inflammatory therapy, although only lipid-lowering drugs are clinically used so far. Although platelets are implicated in atherosclerosis development, anti-thrombotic therapy is currently solely used to prevent recurrent thrombus formation and ischaemic events, but not in patients without overt ASCVD.[204]

Lipid-lowering therapy. The WHO Cooperative Clofibrate trial and the Coronary Primary Prevention Trial (CPPT) studies were the first large randomised placebo-controlled trials that finally put the seal of causality on the connection of heightened LDL cholesterol and atherosclerotic cardiovascular events around 1980.[258-260] Nonetheless, scepticism surrounding the case for LDL cholesterol lowering remained. It took until the Scandinavian Simvastatin Survival Study (4S) and the following statin trials which provided evidence that statins decrease mortality by lowering LDL levels. Only after that did lipid-lowering become a more widely accepted clinical reality.[261]

Since then, statins have become the first-line therapy in the prevention and treatment of ASCVD.[204] Statins lower LDL cholesterol levels by inhibition of the endogenous cholesterol synthesis through competitive inhibition of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and concomitant upregulation of the LDL receptor in the liver.[262,263] The reduction of atherosclerotic cardiovascular event risk as well as the development of atherosclerotic plaque volume have been shown to be proportional to the magnitude of LDL cholesterol lowering by statins. Furthermore, the reduction in relative risk is similar in individuals at high or at low risk for atherosclerotic cardiovascular events.[61] Thus, even low risk individuals have a considerable risk reduction by statin treatment.[264,265]

Although statins have dramatically improved medical prevention, a significant portion of patients continue to suffer life-threatening atherosclerotic cardiovascular events despite the high-intensity statin therapy.[266] This is commonly referred to as the residual risk, or specifically as the residual cholesterol risk. Thus, additional lipid-lowering drugs are used and developed. With the discovery of gain-of-function mutations in PCSK9 (proprotein convertase subtilisin/kexin type 9) as additional cause of autosomal dominant familial hypercholesterolemia and the association of loss-of-function sequence variations with lowered LDL cholesterol levels and risk of IHD, PCSK9 has become a new treatment target.[267-269] The recent trials of alirocumab and evolocumab have shown that treatment with a monoclonal antibody against PCSK9 does indeed lower LDL cholesterol and leads to an additional 15 % reduction in the relative risk for atherosclerotic cardiovascular events in statin treated patients.[270,271]

The main effect of statins is the lowering of circulating LDL levels, but anti-inflammatory activities are also ascribed to this class of drugs. It has been shown that the inflammatory biomarker hsCRP is lowered following statin treatment.[245] Nonetheless, it is unclear whether the anti-inflammatory effects are independent of or caused by the lipid-lowering.[272] Consequently, specific anti-inflammatory therapy might lead to a further reduction in the atherosclerotic cardiovascular event risk by targeting the residual inflammatory risk.

Anti-inflammatory therapy. Inflammation is an integral part of atherosclerosis pathogenesis (see *Atherosclerosis – The Immune System in Atherosclerosis*). There is broad evidence from animal models that by blocking essential constituents of the innate or adaptive immune system, atherosclerosis development and progression is hampered.[159,273] Several observational studies have consistently shown the increased atherosclerotic cardiovascular event risk in individuals with elevated hsCRP, and a Mendelian randomisation study provided evidence for a causal role of the IL-6 inflammatory axis in ASCVD.[274,275] Nonetheless, studies providing definite evidence for a causal role of and the potential to therapeutically interfere with the inflammatory process in human atherosclerosis had so far been missing. There had been a few unsuccessful phase II/III trials evaluating different approaches to dampen

inflammation, including antibodies against oxidized LDL, inhibitors of soluble or lipoprotein-associated phospholipase A2, or methotrexate.[273]

In 2016, the Canakinumab Antiinflammatory Thrombosis Outcome Study (CANTOS) provided the long sought first proof of concept, namely that the risk of atherosclerotic cardiovascular events can be reduced by dampening the inflammatory response. In CANTOS, a monoclonal antibody targeting IL-1β (canakinumab) was given to patients with a history of MI and a residual inflammatory risk, defined as hsCRP ≥ 2 mg/L. Patients receiving canakinumab had a 15 % reduction in the relative risk of experiencing an atherosclerotic cardiovascular event and concomitant dramatic reductions in hsCRP and IL-6. Importantly, blood lipids, including LDL cholesterol, were unaffected by the anti-IL-1β treatment, stressing the specific anti-inflammatory effect of canakinumab.[276]

The dosing regimen of canakinumab was similar to the one applied for its approved clinical use in rheumatic disorders, like systematic juvenile idiopathic arthritis and cryopyrin-associated* periodic syndrome.[277,278] Consequently, a therapy that is in clinical use to treat severe inflammatory disorders that come with fever, severe fatigue, arthritis and even chronic meningitis, lead to only a modest risk reduction of 15 % in a disease with low-grade inflammation. In this context, it might not be surprising that CANTOS also documented an increased rate of fatal infections in the canakinumab treated patients. Another intriguing finding in CANTOS was the lack of an increased response with elevated doses of canakinumab. Whereas the 150 mg dose lead to a statistically significant lower relative risk of atherosclerotic cardiovascular events, the 300 mg did not show statistical significance. The lower dose (150 mg) even showed a higher risk reduction for MI than the 300 mg dose (24 % vs. 16 % reduction in the relative risk, respectively).[276]

Clearly, CANTOS was an important proof of concept study that taught us several important lessons, e.g. that targeting the NLRP3–IL-1β–IL-6 axis does ameliorate the atherosclerotic risk. But CANTOS also showed us that there are still big unknowns about targeting the residual inflammatory risk in ASCVD. There is now a need for the identification of more efficient, targeted and safer treatments of the residual inflammatory risk in ASCVD.

The studies in ***Paper II*** and ***Paper III*** focus on improving our understanding of the inflammatory pathomechanisms in atherosclerosis, which might eventually lead to novel, targeted anti-inflammatory therapies.

* Of note, cryopyrin is an alternative name for the NRLP3 inflammasome, which also plays an important pathogenic role in atherosclerosis (see *Atherosclerosis – Current Paradigm*).

Methodological Considerations

INFERRING BIOLOGICAL questions from observations and formulating hypotheses is at the heart of the scientific process. Testing the generated hypotheses requires rigorous and increasingly more sophisticated research methods. Appropriate and reliable methods and study designs are the cornerstone of all scientific advances and every scientific breakthrough. Every scientist should strive for using the best available methods. But even the most sophisticated method will possess limitations. We must stay aware of these and avoid over-interpretation of scientific results by neglecting this important factor. Herein, I want to critically review two main research methods used in this thesis, experimental mouse and observational human studies.

Mouse Studies

Animal models are valuable tools to study the complex pathophysiological interplay in disease. There is a continuous effort to reduce the use of animals in research but modelling whole organism physiology *ex vivo* without the use of animals remains a considerable obstacle.[279-281] Several scientific breakthroughs affecting clinical practice have been enabled by preclinical research in animal models, not least the identification of the role of LDL in atherosclerosis.[21,282,283] Nonetheless, an even higher percentage of findings in animal studies has not held true for the human situation and translating the results into clinical practice has failed.[284,285]

The biology of the animals used in studies is not entirely overlapping with human biology and experiments are often done in artificial settings to reduce experimental cost, time and confounding.[286-288] These factors bring about limitations that might lead to wrong conclusions, if the researcher is not or cannot be aware of them, which in part explains the high failure rate in the clinical translation of preclinical findings. An example of an animal model limitation being initially overlooked is the early research conducted by the previously mentioned *Nikolai Anitschkow*. He induced atherosclerotic lesions by feeding cholesterol to rabbits and correctly inferred that cholesterol is the culprit in atherosclerotic disease. When other laboratories and *Anitschkow* himself tried to reproduce these results in other animals, including dogs and rats, they failed. This led to the wrong conclusion by many that cholesterol is not the causal factor for atherosclerosis and an abandoning of the cholesterol hypothesis for years. Eventually,

the different metabolic handling of cholesterol in rabbits and dogs was shown to be accountable for the conflicting results.[21]

Contemporary atherosclerosis research, including ***Paper II*** and ***Paper III*** in this thesis, relies heavily on the mouse as a model organism. Reasons for using the mouse as animal model are multiple. Mouse colonies can easily be maintained in laboratory settings and reproduce very fast. Inbreeding reduces genetic variability dramatically and the generation of transgenic or knock-out animals by genetic manipulation is fairly easy.

There is one major issue with the mouse as a model for atherosclerosis research. Mice are relatively resistant to atherosclerosis development, particularly in the short experimental time scales of weeks we are using to keep up with scientific pace. The solution to this paradox is to engineer the genetics of mice so that they readily develop severe hypercholesterolemia and atherosclerosis. These manipulations are typically performed in a specific mouse strain or genetic identity, called C57Bl/6. This strain is more susceptible to atherosclerosis, which is attributed to the T_H1 skewed immune system and low HDL levels.[289-293]

The most common genetically modified models and the ones used in this thesis are knockouts of apolipoprotein E (*ApoE*$^{-/-}$)[294,295] and of the LDL receptor (*LDLr*$^{-/-}$)[296]. *Apolipoprotein E*$^{-/-}$ mice have a decreased clearance and marked increase of the cholesterol-rich LDL precursor molecules, very-low density lipoproteins and chylomicrons, and develop hypercholesterolemia on a regular chow diet.[294,295] The *LDLr*$^{-/-}$ mouse is characterized by elevated levels of plasma LDL, which more closely resemble the human lipoprotein profile.[296,297] Whereas hypercholesterolemia in *ApoE*$^{-/-}$ mice can be further accentuated by feeding a Western-type diet (21 % fat, 0.15 % cholesterol), *LDLr*$^{-/-}$ mice require a Western-type diet for advanced atherosclerosis development.[294,295,297,298] The lesion development in these mice is quite similar to what can be observed histologically in humans, despite the accelerated time-course of disease progression in the genetically engineered mice. The lesions, however, do not progress to the stage of a rupture-prone thin-cap fibroatheroma, show no calcifications and almost never cause thrombotic events. Similar lesional features can be observed in the human disease equivalent of the *LDLr*$^{-/-}$ mouse, called homozygous familial hypercholesterolemia. In this devastating disease thrombotic events appear to be a rare cause of premature death, which occurs in the first decades of life if the condition is left untreated.[299] Thus, the atherosclerotic disease in *LDLr*$^{-/-}$ and *ApoE*$^{-/-}$ mice models familial hypercholesterolemia. Familial hypercholesterolemia is tightly connected to the multifactorial atherosclerotic disease in the general population, but has some distinctive features, e.g. the accelerated disease progression and relative absence of rupture-prone plaques.

In contrast to the LDL receptor, apoE is a multifunctional protein that affects many biological processes other than lipoprotein metabolism. Apolipoprotein E influences adipose tissue biology, platelet function as well as the proliferation and migration of SMCs.[300] Most importantly, apoE conveys anti-inflammatory properties. Pro-

atherogenic type I inflammatory responses are ameliorated by apoE and expression of apoE in macrophages as well as other immune cells has been shown to reduce atherosclerosis independently of plasma lipid levels,[301-304] although this might not be specific to immune cells.[305] Furthermore, efferocytosis, which is important for the effective resolution of inflammatory responses, is hampered in the absence of apoE.[306] Recently, it has also been reported that apoE is an important inhibitor of the complement cascade which contributes to atherosclerosis development.[307] Thus, the application of *ApoE*$^{-/-}$ mice in studies examining the inflammatory mechanisms in atherosclerosis does not only represent a limitation but should be questioned.

Experimental studies in animal models are not only limiting variability by using genetically virtually identical mice, comparable to the study of only monozygotic human siblings of one family, but also by restricting environmental influences. The human phenotype develops as a result of host genetics in combination with environmental influences, including the genes of the microbiome (bacteria, archaea, viruses, fungi) colonializing the human body. This is particularly true for the phenotype of the human immune system. Studies in human monozygotic and dizygotic twins have revealed that a substantial fraction of the inter-individual variability in the immune system's effector functions are non-heritable factors.[308,309] A prominent factor responsible for 58 % of all measured immune parameters in one of the studies was a virus, namely cytomegalovirus.[308]

Importantly, cohabitation has been shown to be a major factor in non-genetic assimilation of the immunoprofile.[310] The microbiome, presumably, is a major factor driving the convergence of the immune system functions, given that a shared environment leads to a shared profile of colonializing pathogens and infections that even extends to pets in the same household.[311-313]

Despite the importance of environmental influences on the immune system, laboratory mice are kept in an over-sanitized environment to avoid any contact with pathogens.[287,314] Not surprisingly, the immune system varies considerably in laboratory mice compared to their free-living 'wild' counterparts. The immune system of laboratory mice has never faced the bulk of microorganisms and thus partly mirrors the naïve state of human neonates.[315,316] The pathogen-experienced immune system in 'wild' mice also reacts with a less intensive cytokine response upon challenge with PAMPs, and probably also DAMPs.[316] This effect, supposedly due to a more elaborate immune homeostasis in the face of continuous pathogenic challenges, might have substantial effects on the immune response in experimental atherosclerosis. As for humans, cohabitation of laboratory mice with their 'wild' counterpart leads to an adaptation of the naïve immune system in laboratory mice that more closely resembles the immunoprofile of human adults.[315] Even more intriguingly, immune-modulatory therapies that had shown promising results in preclinical studies of laboratory mice, but failed dramatically in human trials, produced a similar (initially unanticipated) adverse immune activation in mice with a 'wild' microbiome.[317] Thus, mouse experiments of the inflammatory reaction in atherosclerosis bear an inherent bias, namely an

immunoprofile that is different from the one of patients suffering from atherosclerotic disease.

Experiments in animal models have improved our understanding of molecular biology and atherosclerosis considerably and contributed to advancements in clinical practice. The two mentioned limitations highlight the circumstance that studies in experimental mice are a first step in expanding our understanding of the atherosclerotic pathomechanisms. It is important to bear in mind that mouse studies might not reflect the human situation and need to be followed up in model systems closer to human reality before a translatability can be assumed.

Human Studies

In contrast to mouse studies, research in humans has one big advantage – it is the species we ultimately want to understand and for which we try to improve clinical management and life conditions. Unlike mouse studies, which utilize inbred mice in tightly controlled environmental settings, research in humans has to deal with the enormous genetic variability and various different environmental influences on each individual. This brings about necessary changes in study designs. One important adaptation that needs to be made is an adjustment of the study size (the number of study participants). In order to produce meaningful or significant results human studies require higher numbers of study participants, in the best cases in the 10 000s, compared to mouse studies, where the numbers are in the best case in the 10s.[318,319] This circumstance obviously impacts the feasibility and affordability of human studies.

The human studies in this thesis (***Paper I***, ***Paper IV***, and ***Paper V***) are observational studies, in contrast to the mouse studies herein, which are experimental. Observational studies investigate a (measured) exposure, such as the metabolites in human plaques or the numbers of circulating iNKT cells and relate it to the presence or development of disease without an experimental intervention. The observational studies in this thesis were aimed to elucidate novel biomarkers for the atherosclerotic disease mechanism (***Paper I*** and ***Paper IV***) as well as to evaluate a potential diagnostic biomarker for ASCVD (***Paper V***).

Like mouse studies, also human observational studies have important limitations that need to be considered when interpreting the study results. As an example, ***Paper I*** in this thesis reports that a specific metabolic profile associates with a high-risk phenotype of human atherosclerotic plaques. This association might be due to true causality, i.e. a certain metabolic profile in plaques causes them to be at high risk of rupture. Contrary, it could also mean that the development of a high-risk plaque induces a change in the metabolic profile, a feature of the data called reverse causality. In addition, the results in ***Paper I*** could reflect a systematic error in the study (bias), confounding (i.e. the metabolic profile associates with one or more unknown factors that are the true

causal agents) or being generated by chance (random error). The knowledge of the precise study design and analytical methods used helps us to ascertain and thereby exclude the possibility of systematic and random errors to a certain degree.[319,320] In addition, statistical significance tests can be used to partly assess the potential of random errors. These tests calculate the probability (the *P* value) that the observed values (in two or more groups) are only extremes of the same.[318,321] Confounding and reverse causality, however, cannot (or only to a limited extent) be resolved in observational studies.

A prominent example, in which most likely confounding led to wrong conclusions is the Heart Protection Study of vitamin supplementation for ASCVD.[322,323] Several observational studies had found that vitamin C was inversely associated with the incidence of atherosclerotic cardiovascular events. A study of almost 20 000 individuals had shown that higher blood concentrations of vitamin C were associated with a lower all-cause mortality as well as mortality due to ASCVD.[324] Nonetheless, the Heart Protection Study, a randomised placebo-controlled trial did not find a beneficial effect of vitamin supplementation, including vitamin C, in more than 20 000 individuals.[322] An analysis in the British Women's Heart and Health Study later on revealed a significant association of vitamin C levels with the individual's socioeconomic status.[323,325] Adjusting the statistical analysis of the association of vitamin C levels and IHD for socioeconomic status led to the disappearance of the inverse association, indicating that the initial observation of a potentially protective effect of vitamin C on ASCVD was confounded by socioeconomic status.[325]

Adjusting statistical regression analyses for (potential) confounders is one way that might minimize the effect of confounding onto the results of human studies. Alternatively, confounding can be accounted for in the study design, e.g. by stratification of individuals or matching cases and controls for certain variables, as was done in ***Paper IV*** and ***Paper V***.[318,319] Matching case-control studies, however, introduces a selection bias, which needs to be controlled for by taking into account the matching in the statistical analysis.[320]

Another inherent limitation of observational studies is the problem of reverse causality, which can be nicely demonstrated on the case of CRP in ASCVD. Emerging in the mid-1990s, a growing body of evidence showed an association of CRP and atherosclerotic cardiovascular events.[245,326,327] Although these results stemmed from observational epidemiology studies, a causal role of CRP in ASCVD was propagated and even inhibitors of CRP were being developed to treat ASCVD.[326-328] Observational studies, though, cannot determine the direction of an association between the exposure (elevated CRP) and the outcome (atherosclerotic cardiovascular events). This limitation, the possibility of reverse causality, has become reality in the case of

CRP. Several Mendelian randomisation[†] studies have since provided strong evidence against a causal role of chronically elevated CRP and instead implicate more severe ASCVD as cause for elevated CRP levels.

In summary, observational studies have one major limitation that must not be overlooked. Observational epidemiology studies do not permit causal inference because of the issue of confounding and reverse causality. Coming back to the association of a specific metabolic profile with high-risk atherosclerotic plaques in ***Paper I***, how can we determine a possible causal role of the metabolism. In order to examine a potential causality of the metabolic profile, we would need to induce changes leading to the described metabolic profile and examine whether a high-risk plaque develops. In fact, this experimental approach was used in ***Paper II***, albeit in mice. But also human research can be experimental in nature, which is then often called human trials. The gold-standard experimental studies for novel drugs are the randomised-controlled trials. In these, the influence of a novel drug (the intervention) on a disease outcome is tested by treating a randomly allocated subset of individuals with the drug and comparing them to a group that received placebo.[319] These studies can report on causality[‡]. Nonetheless, even randomised-controlled trials have limitations.[319,332]

In addition to the specific drawback of not permitting causal inference, observational studies as well as experimental studies have additional limitations which relate to the specific study designs and statistical analysis, e.g. the necessity of including a sufficient number of participants in a study to produce meaningful results.[318,319,332]

Despite the limitations, observational epidemiology studies are extremely valuable tools in medical research. Two famous examples showcase the importance of observational epidemiology studies in atherosclerosis. The Framingham Heart Study and the Seven Countries Study, both of which started in the 1950s, provided crucial evidence for an important role of elevated cholesterol in ASCVD.[224,225,333] This knowledge was capitalized by designing randomised-controlled trials with cholesterol-lowering drugs, which has become the first-line therapy in prevention and treatment of ASCVD (see *The Killer of the 21st Century*). An interesting side-note in the context of being aware of study limitations: Observational studies in the 1980s and 1990s found associations between low cholesterol and cancer risk and even in the initial WHO Cooperative Clofibrate trial there was a slightly, though not statistically significant,

[†] Mendelian randomisation makes use of genetic data in observational studies to create a study design similar to randomised controlled trials. By identifying small mutations in the genes of interest (single nucleotide polymorphisms) that affect the functions or levels of the encoded protein, e.g. CRP, one can compare how for instance a life-time high or low concentration of that protein relates to a disease outcome. Since Mendelian randomisation studies utilize genetic alterations, the measured effects always relate to a life-long change in the protein level or function. Consequently, acute effects of the altered protein that might influence the disease cannot be measured.(329, 330)

[‡] It should be noted, however, that one randomised controlled trial will not suffice to establish causality. Claiming causality traditionally requires multiple lines of evidence and the fulfilment of the nine Bradford-Hill criteria, akin to Koch's postulates for infectious diseases.(331)

increased cancer incidence in the treated individuals.[258,334] Subsequent studies, including randomised-controlled trials and observational studies, could fortunately show that there is no causal connection between low LDL-C and risk of cancer, and instead it might be cancer who causes low cholesterol.[334-336]

Part I – Immunometabolic Traits

General Aim

- To evaluate the contribution of immunometabolic circuits on atherosclerosis development and severity

IMMUNOMETABOLISM IS not a predefined term. In a macroscopic view it is used to describe the interplay of systemic organismal metabolism and inflammation. This is a topic that is becoming increasingly more important, given the rise in obesity with concomitant adipose tissue inflammation and type 2 diabetes (see also *The Killer of the 21st Century*).[337] Alternatively, immunometabolism refers to the cellular metabolic configuration of immune cells during different functional states – the microscopic view.[338] The two are not different entities but rather different aspects of an integrated functional system. The metabolic environment of cells, and thus systemic metabolism, affects the immune cell function. The changes in systemic metabolism due to obesity lead to an altered and dysfunctional immune response.[337,339,340] Conversely, effector functions of immune cells require an adaptation of the cellular metabolism, which leads to a systemic metabolic adaptation to support the immune response.[341,342] Metabolism instructs immunity, and immunity instructs metabolism. In this thesis, the topic of immunometabolism is dealt with in the microscopic, cellular perspective.

Immunometabolism is a very young field, with the first PubMed entry appearing in 2011. That cellular metabolism has a substantial impact on cellular functions seems trivial, but this fact has been mostly neglected in immunity until recently. The first studies date back to the early 20th century and were profoundly influenced by *Otto Warburg*.[343-346] *Warburg* discovered that cancer cells made heavy use of glycolysis even in the presence of oxygen (aerobic glycolysis), which is until today known as the Warburg effect.[347,348]

Leukocytes, as well as any other cell, regulate their metabolism in response to the cellular demand and the environmental supply (Figure 12). Quiescent immune cells have a limited biosynthetic and energetic demand. While surveying the environment for potential PAMPs and DAMPs and contributing to immune homeostasis, immune cells, like macrophages or naïve T and T_{REG} cells, rely mainly on the efficient oxidative phosphorylation to satisfy their ATP demand.[338] The acetyl-CoA used in

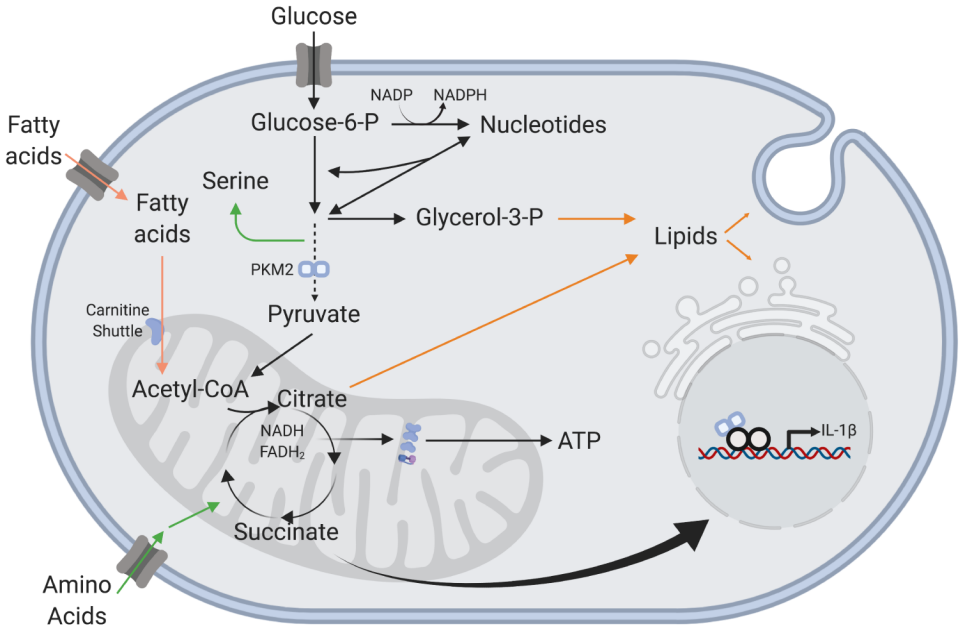


Figure 12. Cellular metabolism. Schematic representation of the major metabolic pathways and their metabolic and non-metabolic implications.

the tricarboxylic acid (TCA) cycle to generate NADH for the oxidative phosphorylation (OXPHOS) in the non-activated cells is mainly derived from fatty acids and glucose.[349] The homeostatic T cell cytokine IL-7 for instance supports this steady-state metabolism of T cells.[350]

When immune cells encounter danger signals and antigens, they rapidly need to adapt their metabolism to facilitate the response against harmful molecules and pathogens. A hallmark of this adaptation is the increase in glycolytic flux irrespective of oxygen levels, the Warburg effect.[338] Upregulated glycolysis facilitates cellular migration towards the centre of inflammation and is directly tied to the necessary intracellular actin remodelling.[351-353] In addition, increased glycolytic flux constitutes a fast, though inefficient, source of ATP production but also generates various biosynthetic precursors.[348,354] Several glycolytic intermediates can be diverted to pathways branching off glycolysis, such as the pentose-phosphate pathway (PPP) or the phosphoserine pathway.[355] The PPP and exogenous glutamine provide the cells with NADPH, which is essential for the microbial killing by macrophages and neutrophils.[338,355] The NADPH is also used for the generation of ROS, via the NADPH oxidase, as well as the cellular protection against ROS by regenerating the antioxidant

glutathione.[338,356,357] Proliferating T cells require *de novo* nucleotide synthesis, which depends on pentose phosphates from the PPP.[355] The increase in cellular mass and the demand for carbon sources is facilitated by the uptake of amino acids, which can feed into the TCA cycle, a process called anaplerosis.[358] Citrate, a TCA cycle intermediate, can be shuttled into the cytosol and together with serine from the phosphoserine pathway and NADPH serves as precursor for lipid synthesis.[338,355] The lipid synthesis supports the increasing demand of membrane lipids for the endoplasmic reticulum and cell membrane in order for macrophages and dendritic cells to produce cytokines and present antigens to lymphocytes.[359,360]

Conversely, reliance on OXPHOS and concomitant FoxP3-induced transcriptional programs allow T_{REG} cells to thrive in an environment of high inflammatory burden with low glucose and high lactate levels. This alternative metabolism of T_{REG} cells enables the regulation of the inflammatory response and initiation of a reparatory response in a metabolically challenging environment, as is the site of an ongoing inflammation.[361] Regulatory T cells secrete IL-10, which in turn suppresses the extensive glycolytic flux in macrophages and skews them towards a reparatory phenotype.[362]

The altered metabolism provides immune cells with the energy and metabolites necessary for their effector functions. Additionally, the metabolic machinery engages in cellular signalling via accumulated metabolites and metabolic enzymes. The TCA cycle intermediate succinate accumulates in inflammatory macrophages and fuels the amplification of the inflammatory response. Increased intracellular succinate levels lead to the secretion of IL-1β via stabilization of HIF-1α.[363] Additionally, succinate drives the generation of ROS, possibly via reverse electron transport, and thereby potentiates IL-1β secretion and inhibits anti-inflammatory cytokine production.[364] Succinate also has extracellular functions and acts as an auto- and paracrine danger signal for macrophages and other immune cells.[365] Inflammatory macrophages also increase their expression of the M2 isoform of pyruvate kinase (PK). Besides its function as a glycolytic regulator PKM2 can, in its dimeric form, translocate into the nucleus and cooperate in the induction of the HIF-1α transcription program, as described in *Myeloid PKM2 in advanced atherosclerosis*.

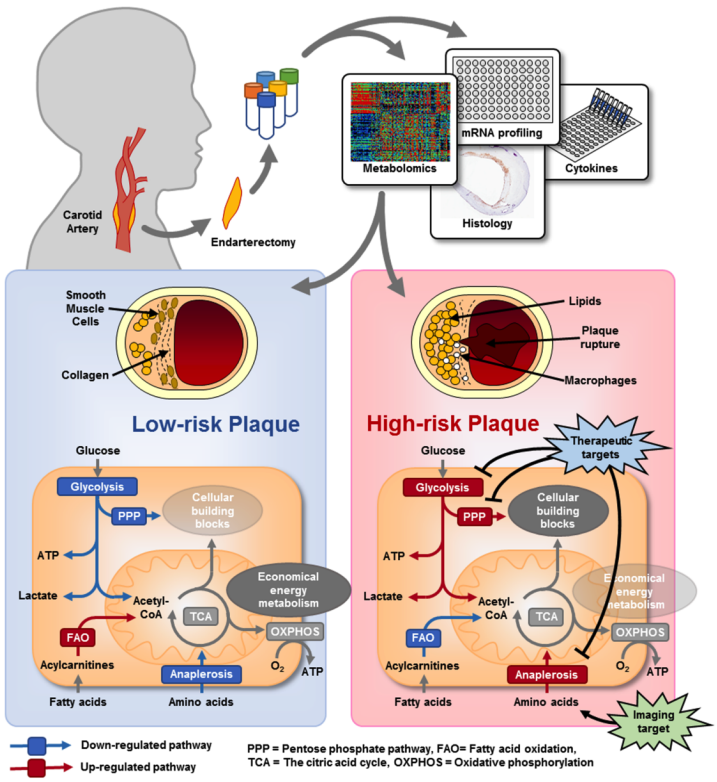
Altered metabolism in high-risk plaques

Specific Aims

- To identify a metabolomic signature in high-risk atherosclerotic plaques
- To improve our understanding of the metabolic basis for advanced atherosclerosis

Key Finding

- Human atherosclerotic plaques that appear to be at high risk of causing a thrombotic event have an altered metabolic phenotype, consistent with an increased glycolysis and amino acid utilization and concomitant reduction in fatty acid oxidation.



Metabolomics in ASCVD

The metabolome, the complete set of metabolites in our body or in a biological sample, has attracted increasing attention in ASCVD recently.[366,367] Traditionally, research in the metabolic pathogenesis of atherosclerosis focused primarily on cholesterol, triglycerides and oxidized phospholipids. The methodological improvements in mass-spectrometric methods and the notion that traditional lipid risk factors only incompletely explain the risk of atherosclerotic cardiovascular events (see also *The Killer of the 21st Century*) have led to the growing interest in other and less abundant metabolites. Numerous studies have investigated the blood plasma metabolome in ASCVD patients in an attempt to elucidate specific metabolic high-risk signatures. Patient blood is readily accessible, thereby making plasma metabolomics feasible. These studies might prove useful in the identification of diagnostic and prognostic biomarkers but are limited in determining pathomechanistic relevance. The circulatory metabolome is more likely to represent exogenous metabolites, relating to diet and medication, than metabolites characteristic of a tissue-specific disease process, like atherosclerosis. Indeed, in ***Paper I*** we show that the local atherosclerotic plaque metabolome does generally not reflect the metabolome in the circulation. To expand our knowledge on the mechanistic role of metabolites in atherosclerosis, we have performed a targeted metabolomics approach on a large number of carotid endarterectomy specimens within the Carotid Plaque Imaging Project (CPIP) biobank in ***Paper I***.

Metabolism of high-risk atherosclerotic plaques

The pathogenetic basis for atherosclerosis is the deposition of lipids through the retention of apoB-LPs in the artery wall. Hence, the metabolomics of carotid endarterectomies were tailored for specifically, but not exclusively, detecting various lipid species. The metabolomic profile could, in an unbiased multivariate analysis, distinguish two groups or clusters of plaques. One cluster, which we denoted the cluster of high-risk plaques, was characterized by plaques with a vulnerable histological appearance, high inflammation and associated with symptoms before the carotid endarterectomy. The opposite was true for the second cluster, which contained stable atherosclerotic plaques. A striking feature in the two clusters of plaques was the acylcarnitine profile. Short- and medium-chain (≤C14) acylcarnitines were decreased in the cluster with high-risk carotid plaques, whereas the C16, C18 and C18:1 acylcarnitine species were higher in these plaques. Fatty acids with 14 or more carbons cannot pass directly through the inner mitochondrial membrane into the mitochondrial matrix for fatty acid oxidation (FAO). Acylcarnitines are a means to transport these long-chain fatty acids (acyls) across the mitochondrial membrane, also called the carnitine shuttle (Figure 13).[368] The accumulation of the long-chain acylcarnitines in the high-risk cluster, thus, hinted towards a relatively decreased

transport of fatty acids and FAO. Intriguingly, potentially fatal inborn errors in two of the enzymes of the carnitine shuttle, carnitine-palmitoyltransferase 2 and carnitine/acylcarnitine translocase, are characterised by the inability to utilize long-chain fatty acids for FAO and cause the specific elevation of C16, C18 and C18:1 acylcarnitine.[369] Furthermore, the type and quantity of short- and medium-chain acylcarnitines mimics the pool of acyl-CoAs, which are the substrate for FAO, providing even more evidence for a relatively downregulated FAO in the high-risk plaques.[368,370,371]

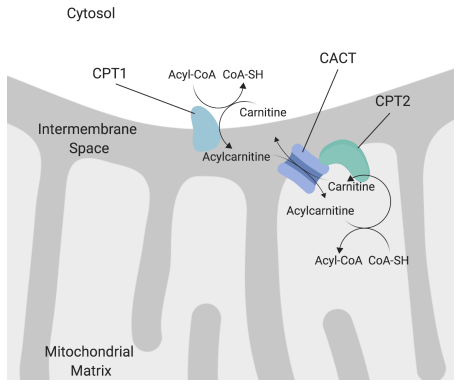


Figure 13. Carnitine shuttle. The transport of long-chain fatty acids into mitochondria requires a sequence of biochemical reactions with acyl-CoA as the starting substrate. The replacement of CoA by carnitine to generate acylcarnitine is catalysed by carnitine-palmitoyltransferase 1 (CPT1) in the outer mitochondrial membrane. The acylcarnitine can now pass the inner mitochondrial membrane by facilitated diffusion through the carnitine-acylcarnitine transporter (CACT). In the final step, the carnitine is replaced by CoA in the mitochondrial matrix, which is catalysed by carnitine-palmitoyltransferase 2 (CPT2)

In conjunction with the additional non-lipid metabolomics and RNA sequencing data, we were able to establish a signature of a metabolic phenotype in high-risk plaques, which was characterized by lower FAO as well as increased glycolysis and amino acid utilization (anaplerosis), compared with stable atherosclerotic plaques. A fascinating and reaffirming finding was the high concordance of the transcriptomic profile in CPIP clusters and in the independent replication biobank (Biobank of Karolinska Endarterectomies). In the replication biobank, the transcriptomic profile of symptomatic plaques, which are associated with a higher risk, mirrored the RNA sequencing data of the high-risk cluster in CPIP, even without a previous stratification according to metabolomics and thus without a potential analytical bias.

The transcriptomic data of both biobanks showed an upregulation of one important regulator of glycolytic capacity, HIF-1α. Together with the glycolytic phenotype and the increased weight of the endarterectomy specimens in the high-risk cluster, this might indicate a higher hypoxic burden. Hypoxia is a long-known important factor in advanced atherosclerotic plaques, that leads, for instance, to neo-angiogenesis through the expression and activation of HIF-1 (see also *Atherosclerosis – Current Paradigm*).[372] Hence, the question arises: did we only measure in the hypoxic burden and is hypoxia the culprit for the different metabolic configuration?

The higher weight of the endarterectomy specimen confers only limited information on the area being insufficiently supplied with oxygen from the lumen or neo-vessels, i.e. the area that extends beyond the critical oxygen diffusion distance of 0.25 – 0.50 mm.[100,373] The weight of the specimen does not necessarily indicate a difference in hypoxic area in the plaques of the two clusters. The lack of information on the macroscopic appearance of the endarterectomy specimens is a limitation of our study

in this aspect. It can be imagined that higher specimen weight is a result of a more elongated plaque but similar cross-section, leading to a comparable distance to oxygen supply as in lighter non-elongated specimens. In line with this idea is the comparable ATP concentration in plaques of both clusters, despite the reported ATP depletion in hypoxic plaques.[374-376] It is also important to note that in neither of the two biobanks did we find a dramatic difference in the expression levels of lactate dehydrogenase A. This enzyme would, however, be absolutely required to regenerate NAD^+ for continuous glycolytic flux in the absence of oxygen.

The increase in glycolytic flux per se is not a hypoxic phenomenon. As outlined before, enhanced glycolysis is characteristic of activated leukocytes and other cells with a prevailing anabolic metabolism, irrespective of oxygen levels. Although activated leukocytes predominantly increase glycolytic flux, in certain instances they concomitantly increase the oxygen consumption, though to a lesser extent.[349] Consequently, leukocyte activation might cause local hypoxic changes due to an increased demand of oxygen,[377] similar to what can be observed in wound healing.[378]

Lastly, how can a factor named hypoxia-inducible factor (HIF) 1 not be a marker of increased hypoxia? Hypoxia-inducible factor 1 is a cellular master regulator, whose expression is increased by and its activity depends on low oxygen tension. In hypoxic environments, cells ensure their survival by activating HIF-1, which leads to the expression of glycolytic enzymes and VEGF (vascular endothelial growth factor). Higher glycolytic activity ensures continuous ATP delivery during hypoxia and VEGF promotes vessel growth, which will eventually improve the oxygen supply. Nonetheless, nuclear HIF-1α, indicative of activated HIF-1, can be seen in atherosclerotic plaques at distances of less than 0.25 mm from the lumen (Figure 14) and thus well below the critical oxygen diffusion distance.

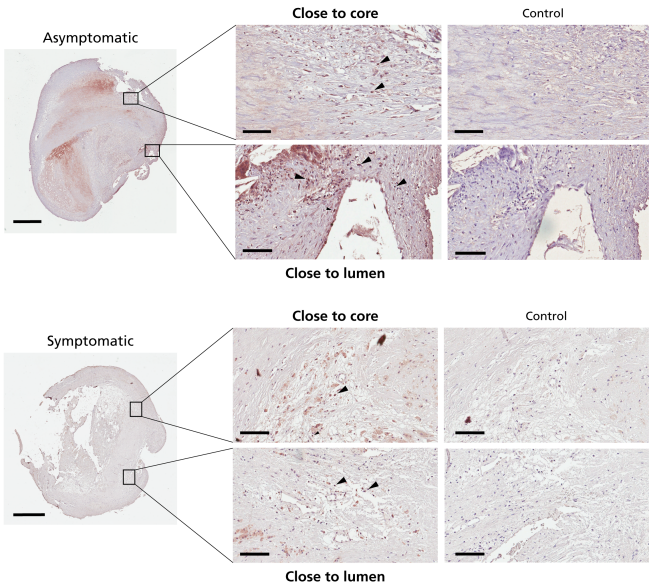


Figure 14. HIF-1α in human atherosclerotic plaques. Carotid atherosclerotic plaques from a asymptomatic and symptomatic patient, stained for HIF-1α. Nuclear staining of HIF-1α is indicative of active HIF-1 and can be seen close to the necrotic core of the atherosclerotic plaques. Additionally, nuclear HIF-1α staining is also visible close to the lumen, well below the critical oxygen diffusion distance. Arrows indicate positive nuclear HIF-1α staining. Scale bars indicate 1mm for the lower magnification and 100µm for the higher magnification.

The reason for active HIF-1 in the likely absence of hypoxia can be explained by its important function as a metabolic regulator. Succinate, which accumulates in activated leukocytes, can activate of HIF-1 even during normoxia. Accumulation of succinate also leads to increased ROS which represents another activating factor of HIF-1. Alternatively, the glycolytic enzyme PKM2, which is specifically expressed in activated leukocytes and proliferating cells, serves as a co-activator of HIF-1 (see *Myeloid PKM2 in advanced atherosclerosis*). Of particular interest for atherosclerosis is the potential of oxidized LDL to activate HIF-1 in the absence of hypoxia. Indeed, and probably not surprisingly, we found elevated levels of oxidized LDL in the high-risk cluster (Figure 15).

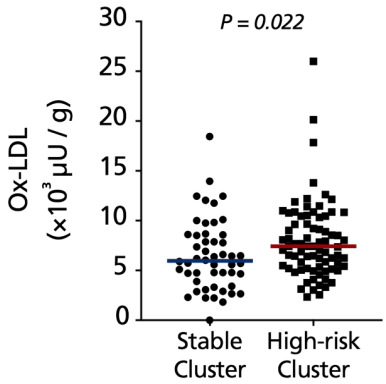


Figure 15. Oxidized LDL in the two plaque clusters. Higher levels of oxidized LDL (Ox-LDL) can be found in homogenates of the high-risk plaques, compared to the plaques with a lower risk of rupture.

In summary, our results do not support a potentially different level of hypoxia as the causal factor for the different metabolic configuration in high-risk and stable carotid atherosclerotic plaques. Instead, the evidence herein suggests that the metabolic profile and HIF-1α levels are due to cellular, probably immune-cellular, activation. Nonetheless, high-risk plaques might harbour increased but well-defined small local hypoxic zones in areas with sufficient supply, secondary to leukocytic activation. Although differences in hypoxia do not seem to be a constraint in our study, there are some important limitations that need to be taken into consideration in interpreting the findings.

Limitations

The study in ***Paper I*** is a cross-sectional examination at a single time-point. Cellular metabolism is very dynamic. Consequently, we do not know if the metabolic profiles represent a steady-state or a short-term perturbation. Given the replication of the transcriptomic profile in an independent cohort, however, it is more likely to represent a settled profile. More importantly, the synopsis of the metabolomic and transcriptomic data strongly suggest a different metabolism. Nonetheless, the descriptive nature does not allow us to convincingly infer an altered metabolism. Thus, studies in animal models analysing metabolic flux *in vivo* are urgently needed.

These studies could also address the question of which cell type is responsible for the altered metabolic configuration. The observed profile matches the expected metabolism of activated macrophages and T cells and was associated with several implicated cytokines and chemokines, like IL-1β, IL-18, IL-6 and MCP-1. Nonetheless, we could not conclusively address this question with our material. In addition to not being able

to identify the cellular source, we could not normalize the metabolite levels for plaque cellularity. The increased abundance of phosphatidylcholines in the high-risk cluster might hint at an increased cellularity in these plaques. Alternatively, it could indicate increased cell death and deposited plasma membrane components. Phosphatidylcholine is, however, also an important metabolite in activated myeloid cells, enabling the cells to produce cytokines,[379-381] supplying membrane components for phagocytosis[382] and serving as an arachidonic acid source for prostaglandin synthesis.[383,384] The phosphatidylcholine signature could thus stem from an increased activation of myeloid cells and a metabolic adjustment of these cells, in line with the other findings. Furthermore, the metabolite data was normalized for the total metabolite quantity and adjusted for the individual plaque weight. It is, thus, unlikely that a potential difference in cellularity accounts for the different metabolomic profile, particularly since the metabolite data was analysed as an entire metabolic signature (glycolytic, lipid and amino acid metabolites) with increased and decreased metabolites and in conjunction with transcriptomics.

Implications

An altered plaque metabolism has important pathogenetic as well as potential clinical implications. The overutilization of glycolysis might contribute to the generation of an inhospitable environment with nutrient scarcity and acidic pH, compromising cell viability. Indeed, atherosclerotic lesions show an acidic pH, similar to other sites of inflammation.[385,386] The low pH aggravates atherosclerotic lesion development by amplifying apoB-LP retention and modification. Furthermore, an acidic environment can act as a danger signal for immune cells, thereby augmenting the inflammatory response.[386] Another pro-inflammatory mediator, nitric oxide, is important for anti-microbial defence but simultaneously it is a potent inhibitor of the electron transport chain and OXPHOS. Nitric oxide might inhibit OXPHOS in other immune cells in the vicinity of the producing pro-inflammatory cell.[387] This could lead to dysfunction or cell death in surrounding cells, which are committed to non-glycolytic metabolism and dependent on OXPHOS, like T_{REG} cells or reparatory macrophages. This mechanism might play a role in the non-resolving inflammation in atherosclerosis.

Given the suggested pathogenetic importance, the altered metabolism represents a target for clinical interference. In fact, imaging glucose (^{18}F-fluorodeoxyglucose) uptake by positron emission tomography, which is routinely used for cancer imaging, has been shown to correlate with the inflammatory load in human atherosclerotic plaques and atherosclerotic cardiovascular events during long-term follow-up.[388,389] In ***Paper I***, we additionally found a profile of increased utilization of glutamine, also called glutaminolysis. Interestingly, various cancers exhibit glutaminolysis and imaging with positron emission tomography using the glutamine analogue ^{18}F-fluoroglutamine facilitates brain cancer identification in humans, which is hindered with ^{18}F-fluorodeoxyglucose due to the high glucose uptake of the healthy brain.[390] The use of ^{18}F-fluoroglutamine could significantly improve the metabolic imaging of coronary

artery plaques, where ^{18}F-fluorodeoxyglucose is ineffective because of the high basal glucose uptake of myocardial cells.[391] The resemblance of cancer metabolism with the metabolic profile in advanced atherosclerotic plaques could also be exploited therapeutically. Several modulators of metabolic enzymes and transporters are being tested as anti-cancer agents and might be refurbished as anti-atherosclerotic agents. One of the enzymes being targeted in cancer is PKM2, a master regulator of glycolysis. Positron emission tomography with a PKM2 specific tracer was able to identify experimental brain cancer in mice, and a small-molecule modulator of the enzyme suppressed tumorigenesis in mice.[392,393] Most importantly, PKM2 has been shown to be important in the rewired metabolism of inflammatory macrophages.[394] Thus, we have sought to validate the potential of targeting PKM2 in ASCVD, as described in the next section *Myeloid PKM2 in advanced atherosclerosis*.

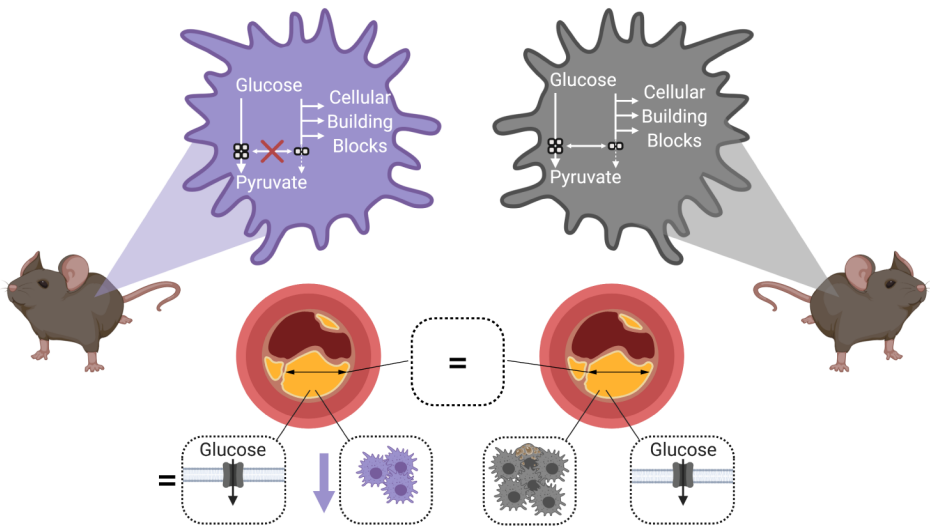
Myeloid PKM2 in advanced atherosclerosis

Specific Aims

- To evaluate the role of myeloid PKM2 in experimental atherosclerosis
- To elucidate the potential contribution of immunometabolism on experimental atherosclerosis
- To test the potential of PKM2 as an anti-atherosclerotic drug target

Key Finding

- Myeloid PKM2 does not considerably affect the development of advanced atherosclerosis, despite a decreased plaque macrophage burden in mice lacking myeloid PKM2.



PKM2 as a target in ASCVD

The knowledge of an altered metabolism in human atherosclerotic plaques opens up novel possibilities to scrutinize for potential anti-atherosclerotic therapies. Although we could not determine the cellular source of the altered metabolic profile in ***Paper I***, it was reminiscent of the reprogrammed metabolism in activated leukocytes and associated with a high inflammatory burden.

A major hallmark of the metabolic profile in high-risk plaques was the glycolytic overutilization – a feature that has already previously been exploited with ^{18}F-fluorodeoxyglucose imaging – in conjunction with elevated levels and presumably increased activation of HIF-1α. A crucial regulator of glycolysis and the metabolic reprogramming in immune cells is the glycolytic enzyme PK and in particular its M2 isoform.[395,396] In addition, PKM2 has been shown to stabilize HIF-1α in lipopolysaccharide (a membrane component of gram-negative bacteria)-stimulated macrophages and act as a co-activator for HIF-1α.[394,397] These features made PKM2 an interesting target in experimental atherosclerosis in light of the results in human high-risk plaques. Intriguingly, a cytokine profile mirroring the one we found in human high-risk plaques, particularly elevated IL-1β and IL-6 but equal levels of TNF-α, has been found in macrophages from patients with IHD and shown to be dependent on dimeric PKM2 (see below), introducing PKM2 as an even more attractive potential target.[398]

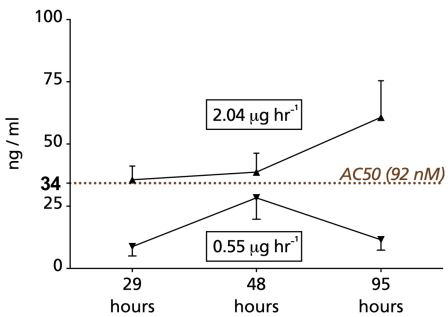


Figure 16. Bioavailability of TEPP-46. Mice (n=3 per group) had osmotic pumps implanted subcutaneously with two different delivery rates of TEPP-46. Subsequently TEPP-46 was measured in plasma at three different time points with gas-chromatography mass spectrometry. Whereas the lower rate (0.55 μg hr^{-1}) was insufficient to reach the half-maximum activating concentration (AC50) of PKM2, the higher rate reached above-AC50 levels as early as 1 day after implantation. This configuration would allow TEPP-46 delivery via osmotic pumps over several weeks. Symbols represent median and range.

Another advantage of scrutinizing PKM2 is the availability of experimental drugs targeting the enzyme. Pyruvate kinase M2 is highly expressed in most cancers and has thus become a high-value pharmaceutical target.[399,400] One of these drugs, the PKM2 activator TEPP-46 (ML-265), which has been developed in an academic setting, has previously been shown to reduce growth of several experimental cancers, including lung cancer.[393] We have verified that TEPP-46 can be administered systemically to mice over extended periods of time and reach sufficient doses (Figure 16). Consequently, TEPP-46 constitutes an interesting molecule to test the importance of systemic PKM2 in experimental atherosclerosis.

In addition to experimental drugs, the interest in PKM2 has also led to the generation of genetically modified mouse models, such as the *PKM2$^{fl/fl}$* mouse,[401] which has been used here in ***Paper II***.

Physiological role of PKM2

Pyruvate kinase catalyses the final step in glycolysis, the conversion of phosphoenolpyruvate to pyruvate with concomitant phosphorylation of ADP to form the high-energy substrate ATP. In mammals, four different isoforms of PK can be found, which are encoded by two different genes. The *PKLR* gene encodes the PKR isoform of erythrocytes and the PKL isoform, which is mostly confined to the liver. The M1 and M2 isoforms are the result of the mutually exclusive splicing of the *PKM* gene.[396,402]

The differentially incorporated exons 9 and 10, for PKM1 and PKM2 respectively, span the coding region for the binding pocket of fructose-1,6-phosphate (FBP) and the contact domain, which is important for forming enzyme oligomers of PKM.[403] The catalytic function of PK depends on the tetrameric configuration of identical subunits, whereas PKM dimers have a low and monomers no catalytic activity.[404]

Pyruvate kinase M1 is expressed in differentiated tissues with a high constant catabolic demand, such as the brain, heart and skeletal muscle. In contrast, PKM2 is the dominant isoform during embryonic development as well as in proliferating and anabolic cells, such as stem but also cancer cells.[396,402] As outlined before, activated immune cells are predominantly anabolic and their metabolic phenotype mirrors the one of cancer cells. Indeed, effector T cells and inflammatory macrophages express mainly PKM2, whereas PKM1 can be found in reparatory macrophages.[394,398,405-408] The expression of PKM isoforms is regulated by splicing factors that favour PKM2, by either suppressing the inclusion of exon 9 or promoting the incorporation of exon 10.[409-413] An absence of these factors leads to the preferential expression of PKM1, which might indicate that PKM1 is the default isoform, unless various signals provide cues for the necessity of PKM2.[399] Two important regulators controlling the splicing factors are the mammalian target of rapamycin (mTOR), which is an important nutrient sensor and metabolic master regulator, and c-Myc.[409,412,414] Interestingly, we found increased levels of c-Myc in the high-risk atherosclerotic plaques that showed an altered metabolic profile (see also *Altered metabolism in high-risk plaques*).

The reasons for the preferential expression of PKM2 in anabolic cells like activated leukocytes are multifaceted but seem to be based on the ability of PKM2 to oscillate between the catalytically active tetramer and less active dimer (Figure 17).[415] This feature is in stark contrast to PKM1, which is a constitutive tetramer and locked in the high-activity conformation. [399] The conformational change of PKM2 into a dimer and the possibility to regulate this process confers a physiological advantage over PKM1.

Pyruvate kinase M2 is subject to extensive allosteric regulation as well as posttranslational modifications. These regulate the catalytic activity through modulating the affinity for phosphoenolpyruvate, but particularly by leading to the disassociation of the PKM2 tetramer into a dimer, and vice versa.[399] The major allosteric activator of PKM2 is the upstream metabolite FBP, whose binding pocket in PKM2 is encoded in the PKM2-specific exon 10.[403,416-418] Another upstream metabolite that acts as allosteric activator is SAICAR (succinylaminoimidazolecarboxamideribose-

5′phosphate), an intermediate of the *de novo* purine synthesis, which requires glycolytic precursors from the PPP.[419] These and other upstream metabolites activate PKM2 mainly by promoting tetramer formation and stabilization.[399] In contrast, the amino acid alanine and the anabolic thyroid hormone triiodothyronine act allosterically to promote the dimeric form and inhibit PKM2 activity.[415,418,420-422] Interestingly, triiodothyronine simultaneously also promotes PKM2 transcription.[423]

Regulation of PKM2 structure and activity is also achieved by phosphotyrosine growth signalling and through numerous posttranslational modifications, including phosphorylation and acetylation.[424-426] Importantly, PKM2 is a target of oxidative modifications and appears to be an important sensor and modulator of cellular redox homeostasis. Reactive oxygen species can lead to an inhibition of PKM2 by directly oxidizing the protein or via succinylation of PKM2, which plays an important role in protecting the cell from oxidative stress and avoiding cell death.[427,428]

The extensive regulation of the reversible inhibition and activation of PKM2 through its quaternary structure serves the integration of growth factors, environmental signals and status of nutrient supply.[402,418] Importantly, the disassociation into the dimeric form has significant functional consequences (Figure 17).

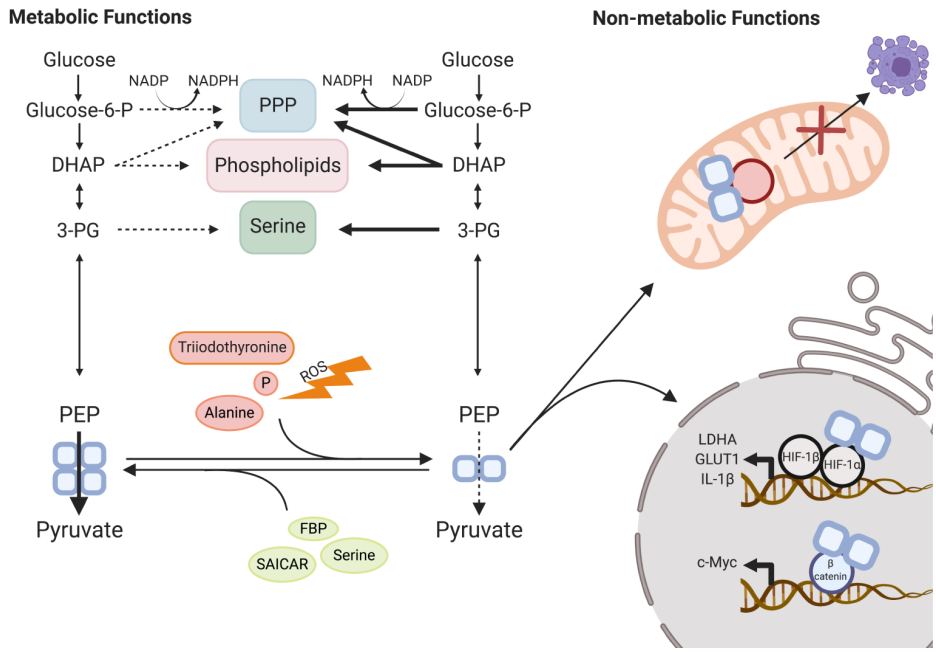


Figure 17. Regulation and functions of PKM2. Pyruvate kinase M2 is extensively regulated which dictates its quaternary structure. The tetramer is catalytically active, leading to a high turnover rate of phosphoenolpyruvate (PEP) to pyruvate. In contrast, the dimer of PKM2 is much less catalytically active, which facilitates the accumulation of upstream metabolites like glucose-6-phosphate, dihydroxyacetone phosphate (DHAP) and 3-phosphoglycerate, which in turn can feed biosynthetic pathways. Furthermore, dimeric PKM2 can translocate to the nucleus and mitochondria, where it executes non-metabolic functions.

The low activity of dimeric PKM2 creates a bottleneck for glycolytic flux into pyruvate. The subsequent accumulation of upstream glycolytic intermediates, such as glucose-6-phosphate, dihydroxyacetone phosphate and 3-phosphoglycerate promotes their diversion into anabolic pathways, like the PPP, phospholipid-synthesis and the phosphoserine pathway.[393,394,402,424,429-431] The increased flux in these anabolic pathways provides activated immune cells, like effector T cells and inflammatory macrophages, with the necessary building blocks (nucleotides, lipids, amino acids, NADPH) to exert their effector functions and proliferate, as outlined previously.

Pyruvate kinase M2 has important additional roles beyond the regulation of glycolytic flux. One of these is the modulation of chromatin binding of MDM2 (mouse double minute 2), thereby promoting antioxidant responses via glutathione in conditions of increased oxidative stress.[432]

Furthermore, as a dimeric protein PKM2 can translocate into the nucleus and participate in transcriptional regulation.[395] Of particular importance for atherosclerosis is the role of nuclear PKM2 in serving as a co-activator and stabilizer for HIF-1α. Increased activity of HIF-1 leads to an upregulated transcription of glycolytic enzymes like lactate dehydrogenase A or the glucose transporter GLUT1 as well as of the inflammatory cytokine IL-1β.[394,397,408,433] An additional function of PKM2 that results in the increased expression of the genes for GLUT1 and lactate dehydrogenase A is its role as a co-activator of the transcription factor β-catenin. This factor, in turn, induces c-Myc, which leads to GLUT1 and lactate dehydrogenase A expression.[434] Thus, the inhibition of PKM2 leads to an increased expression of glycolytic genes, as well as to a feed-forward loop of increased PKM2 expression through the interaction of dimeric PKM2 with HIF-1α and β-catenin.[397,434]

Nuclear PKM2 has also been proposed to act as a protein kinase and phosphorylate various transcription factors.[395,396] The capability of PKM2 to phosphorylate proteins has, however, recently been questioned.[435] In addition to nuclear translocation, PKM2 can also be found in mitochondria.[436,437] In conditions of oxidative stress, PKM2 can translocate into mitochondria and prevent apoptosis by interacting with and regulating the expression of important pro-apoptotic proteins.[436,438]

In summary, PKM2 can be seen as a master regulator of reprogrammed cellular metabolism, considering the important direct and indirect metabolic functions of PKM2 and its high degree of regulation. Blocking the functions of PKM2 leads to an arrest of cellular proliferation, increased susceptibility to oxidative stress but also diminished activation of the NLRP3 inflammasome and IL-1β secretion in macrophages.[393,394,401,408,424,427,431,439] Given the pro-inflammatory role of PKM2 in macrophages we set out to elucidate its role in experimental atherosclerosis.

Myeloid PKM2 in advanced atherosclerosis

In ***Paper II*** we generated mice bearing a myeloid specific knockout of PKM2, while preserving the transcription of PKM1 in myeloid immune cells. For this, we crossed *PKM2*$^{fl/fl}$ mice with *LysM*^{cre} mice and transplanted their bone-marrow into *LDLr*$^{-/-}$.

After feeding the mice a Western-type diet for 13 weeks, we assessed the atherosclerotic lesion development in mice harbouring bone-marrow with a homozygous (*PKM2*$^{\Delta/\Delta}$) and heterozygous (*PKM2*$^{fl/\Delta}$) knockout of myeloid PKM2 as well as in their wild-type counterpart (*PKM2*$^{fl/fl}$).

To our surprise, we only found a small, non-significant, decrease in the plaque size in regions of the aortic root with the highest plaque burden in *PKM2*$^{\Delta/\Delta}$ mice, compared with *PKM2*$^{fl/fl}$ mice. Almost as intriguing as the similar lesion size was the amount of plaque development because all genotypes showed unusually big atherosclerotic plaques. The unexpectedly fast development of cross-sectional aortic root plaques in the range of 1 mm^2 might be related to the use of purchased *LDLr*$^{-/-}$ bone-marrow recipient mice. The purchased mice are very likely to have a different microbial colonization than the mice bred in our own animal facility, which arguably affects their immune system and thereby atherosclerotic development (see also *Methodological Considerations – Mouse Studies*).[315,316,440,441] Importantly, it has previously been shown that the atherosclerotic plaque development in mice displays an exponential growth profile over time.[442] Given the enormous plaque size, it is conceivable that the measurement of plaque burden in ***Paper II*** occurred in the plateau-phase at the end of the development, marking a potential amelioration of plaque development in *PKM2*$^{\Delta/\Delta}$ mice.

A further complication arising with the big atherosclerotic burden in the examined mice is the obvious occlusion of coronary arteries in them. In 38 – 56 % of the mice in all three groups, complete or almost complete occlusion of at least one coronary vessel at its orifice in the aortic root could be detected (Figure 18). The occlusion of a coronary artery would most likely give rise to a MI, which would confound the experiment. Myocardial infarction causes an inflammatory reaction and, as mentioned before, leads to an increased mobilization of Ly6C^{hi} monocytes into the blood and accelerated atherosclerosis.[134] Additional histological (fibrotic scar, ischaemic/infarcted area) and serological (troponins) analyses might yield insight into this issue.

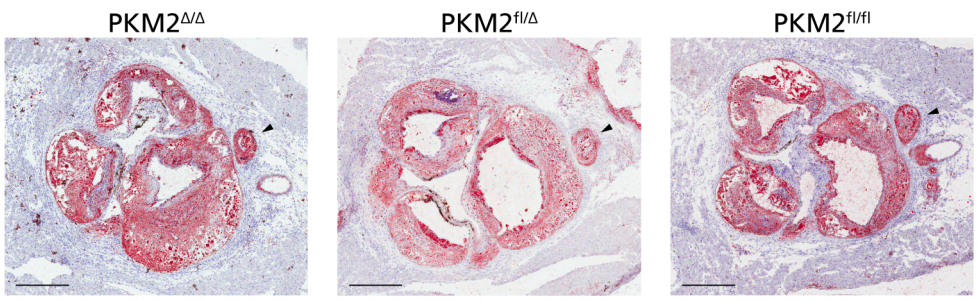


Figure 18. Aortic root sections. Occluded coronary arteries (arrows) are clearly visible in all three genotypes. Scale bars indicate 400µm.

Other potential reasons for the absence of any, expected, difference in the development of atherosclerosis relate to the issue of local plaque chimerism and the role of PKM2 in proliferation and HIF-1α co-activation, as outlined in the manuscript. Furthermore, the complexity of metabolic regulation in immune cells does not permit a black and white scheme of pro- and anti-inflammatory metabolic pathways and enzymes.[443] The metabolic requirements of efferocytosis, which is an essential anti-atherogenic process, are an example of and another possible explanation for the results in *PKM2*$^{\Delta/\Delta}$ mice, as discussed in the manuscript. Another aspect of this complexity is the role of PKM2 in cellular redox homeostasis.

The bottleneck created by dimeric PKM2 at the final step in glycolysis and subsequent increased flux into the PPP creates important reducing potential in the form of NADPH. Thereby generated NADPH can be utilized by the cell to regenerate oxidized glutathione and avoid oxidative stress and cellular death. Additionally, non-metabolic functions of PKM2 include the protection from cellular death during oxidative stress by interacting with mitochondrial apoptosis-regulating proteins. Consequently, the knockout of PKM2 might have abolished this protection from oxidative stress in macrophages. Although other pathways than the PPP exist for the generation of NADPH, several lines of evidence show the importance of dimeric PKM2 in the antioxidant response and avoidance of cellular death thereby.[427,428,432,444,445]

Importantly, oxidative stress is an important pathogenic mechanism in atherosclerosis, e.g. by leading to increased cellular death and subsequent generation of necrotic cores due to the defective efferocytosis (see *Atherosclerosis – Current Paradigm*).[446,447] This could imply that the knockout of PKM2 in myeloid cells, albeit skewing macrophages towards a less inflammatory phenotype, led to defective antioxidant response, counterbalancing the expected anti-inflammatory response of the absence of PKM2.

Limitations

Paper II represents an early report of the attempt to mechanistically expand our results of ***Paper I*** by elucidating the role of the glycolytic enzyme and regulator PKM2 on experimental atherosclerosis. Considering that ***Paper I*** is still work in progress, several additional analyses are yet to be performed. A crucial experiment will be the verification of the PKM2 knockout on a protein level in lysozyme M (LysM)-expressing myeloid cells. Furthermore, more in-depth histological and immunohistochemical analysis should provide us with a better understanding of potential changes in plaque stability (e.g. collagen and VSMC analysis) and assays for oxidative stress and cellular death might yield novel insights into alternative explanations.

Summary and Outlook

In *Part I – Immunometabolic Traits* I have presented work identifying an altered metabolic profile in human atherosclerotic plaques that are at high risk of causing a stroke, a life-threatening complication of atherosclerosis (***Paper I***). This metabolic profile was associated with an increased inflammatory burden and might thus represent the reprogrammed metabolism of activated leukocytes within the plaques. Consequently, ***Paper II*** represents an attempt to further investigate the relevance of the reprogrammed leukocytic metabolism for atherosclerosis in mice and identify potential drug-able immunometabolic targets. For this, we have elucidated the role of a major regulator of the altered metabolism of activated leukocytes, PKM2, specifically in myeloid cells. Although we could not find an effect on advanced experimental atherosclerosis when PKM2 was absent in myeloid cells, ***Paper II*** represents work in progress and additional experiments are necessary before reaching a, at this moment, premature conclusion.

Immunometabolism research in atherosclerosis is in its infancy and has just recently emerged from the likewise very young field of general immunometabolism. In fact, ***Paper I*** was the first comprehensive report of an altered metabolism in human atherosclerotic plaques. Immunometabolism does not only bear significant potential in promoting our understanding of the pathomechanisms in atherosclerosis, it might also reveal diagnostic targets, e.g. with the means of functional imaging, or more importantly pathways and molecules that can be therapeutically exploited. The promising results from the CANTOS trial (see *Atherosclerosis – Treatment*) have showcased the potential benefit of anti-inflammatory treatments in ASCVD and the field of immunometabolism has the potential to put future drug targets on centre stage.

The veteran anti-diabetic drug metformin, for instance, can be repurposed as an immunometabolic drug. Metformin inhibits complex I of the electron transport chain (OXPHOS) and thereby activates a master regulator of cellular (energy) metabolism, AMPK (AMP-activated protein kinase).[448-450] Activation of AMPK limits anabolic cellular metabolism, which is important for activated leukocytes and proliferating cells, like cancer cells.[451] Metformin has been shown to limit IL-1β secretion in macrophages and boost their IL-10 production, skewing them towards a reparatory phenotype.[452] The anti-inflammatory action of metformin can also be seen in humans, independent of its function on blood sugar levels.[453] These effects are mainly attributed to the metformin-mediated inhibition of complex I and subsequent limitation of ROS generation by reverse electron transport.[452,453] Given the metabolic similarities of activated leukocytes and cancer cells, it is not surprising that metformin is being used in clinical trials as candidate anti-cancer drug and has been suggested as anti-inflammatory drug in non-diabetic CVD.[400,454,455] A particular value of a drug like metformin might be found in a personalized medicine approach, e.g. for using

metformin specifically in diabetic individuals with ASCVD and a residual inflammatory risk.

Another, particularly for ASCVD interesting target, is the lipid pathway. Interfering with for instance FAO, e.g. by manipulating the carnitine shuttle, might yield a beneficial pleiotropic drug, aiming primarily at achieving an anti-inflammatory action but simultaneously also an increased lipid utilization. Indeed, it has been shown that carnitine-palmitoyltransferase 1, which mediates long-chain FAO, is crucial for T_{REG} cells and reparatory macrophages.[456,457] The overexpression of carnitine-palmitoyltransferase 1 in macrophages mitigates their inflammatory phenotype and reduces lipid accumulation.[458] Recently, however, the crucial role of long-chain FAO for anti-inflammatory and inflammation resolving processes has become blurry, and it has been suggested that carnitine-palmitoyltransferase 1 and the drug used to study the enzyme, etomoxir, have effects other than affecting long-chain FAO.[443,459] Furthermore, it has become apparent that long-chain FAO, mediated by the carnitine shuttle and carnitine-palmitoyltransferase 1 is also important in activating the NLRP3 inflammasome in inflammatory macrophages.[460]

It is clearly going to be exciting to elucidate the potential of immunometabolic findings on clinical management and possibly watch additional immunometabolic drug targets unfold. Nonetheless, the exact immunometabolic processes require further dissection (an effort that is also part of this thesis in ***Paper II***) before they can be translated into clinical management and potentially reduce the global burden of ASCVD.

Part II – Cellular Traits

General Aim:

- To evaluate the contribution of T cell subsets to the development of atherosclerotic cardiovascular disease and their eligibility as prognostic biomarkers.

ATHEROSCLEROTIC PLAQUES constitute a heterogenous mix of different cell types. Endothelial cells, smooth muscle cells but particularly immune cells participate in the growth of the nascent lesion. The continuous influx of leukocytes from the circulation into the progressing atheroma is a critical pro-atherogenic feature. [159] Higher numbers of circulating leukocytes can be found in patients with ASCVD and the levels of blood leukocytes are a predictor of future atherosclerotic cardiovascular events, in particular coronary events. [461-463] T cells are among the most abundant immune cell populations in advanced human plaques and certain T cell subsets have been shown to associate with future atherosclerotic cardiovascular events. [116,119,464,465] However, the necessity of advanced methodology to study human T cells and the daily growing repertoire of T cell subsets have left us with an incomplete understanding of the relationship between the various T cell subsets and ASCVD.

The study in ***Paper III*** re-examines the role of $CD4^+$ T_H cells in experimental atherosclerosis in mice. However, the biology and particularly the immune system in experimental mice is substantially different from the one in humans (see also *Methodological Considerations – Mouse Studies*). This makes translational studies of T cell subsets in human-beings indispensable. Human observational studies can aid in the identification of potentially novel pathomechanisms and generate unique hypotheses, as has been shown in *Part I – Altered metabolism in high-risk plaques*. The study in ***Paper IV*** represents an effort to translate the findings in mice regarding the pathogenic function of iNKT cells into humans.

The cellular composition of the human immune system is shaped by environmental factors and the individual's genetic repertoire. [308,309,466] Both factors, the genes and the environment, e.g. through the presence or absence of certain infectious agents, affect the development of disease via an alteration of the immune cell repertoire. [308,466-468] Therefore, certain immune cell subsets might be (risk) biomarkers of ASCVD. This

aspect of the *Cellular Traits in Cardiovascular Disease* is the topic of the last study in this section, ***Paper V***.

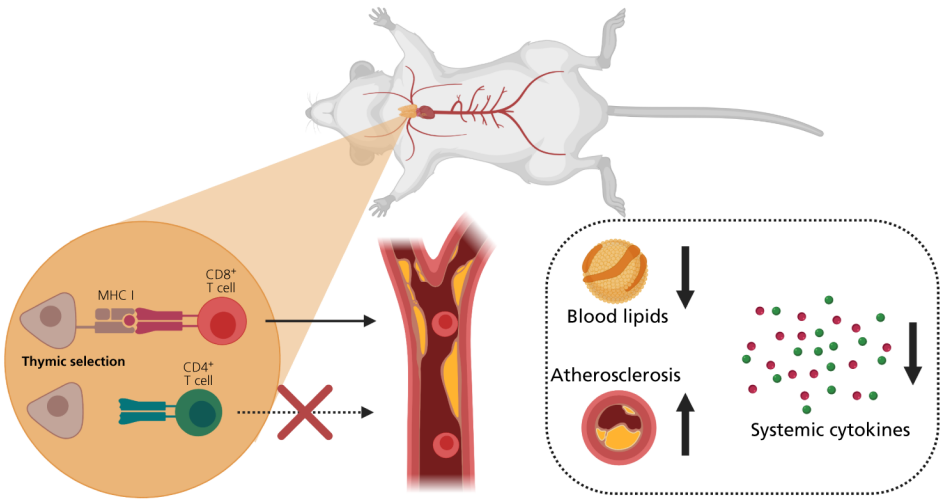
MHC II deficiency in experimental atherosclerosis

Specific Aims

- To investigate the importance of $CD4^+$ T cells in the development of atherosclerosis

Key Finding

- The net effect of $CD4^+$ T cells appears to be protective because MHC II deficient mice, which lack T_{REG} and T_H cells, have a dramatically increased atherosclerotic burden, despite decreased lipid levels and systemic inflammation.



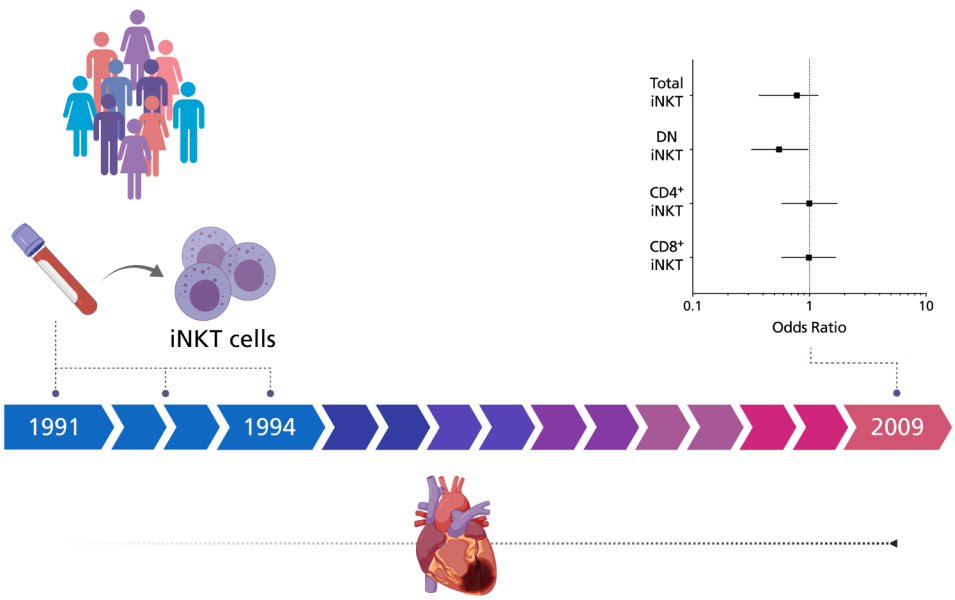
Invariant Natural Killer T cells and coronary events

Specific Aim

- To elucidate the relationship of circulating iNKT cells with first-time incident coronary events.
- To determine whether the pro-atherogenic role of iNKT cells in experimental mice translates into human prospective data on coronary events.

Key Finding

- High numbers of circulating iNKT cells do not associate with an increased incidence of first-time coronary events, but rather with a decreased event risk.



Invariant natural killer T cells

Natural killer T (NKT) cells are innate-like T lymphocytes with a TCR specific for glyco- and phospholipids.[158] These rare lipid-sensing immune cells are thus an intriguing subset in the context of a lipid-induced chronic inflammatory disease, as is atherosclerosis. The TCR repertoire of NKT cells distinguishes two main types of NKT cells, invariant NKT (iNKT) and diverse NKT (dNKT) cells. Invariant natural killer T cells express an invariant TCR α chain (Vα24Jα18 in humans), which pairs almost exclusively with a Vβ11 chain. Conversely, diverse NKT possess a more variable TCR repertoire.[158]

Invariant natural killer T cells seem to be an evolutionary intermediate between the non-specific fast responders of the innate immune system and the surgically precise slow responders of the adaptive immunity.[469] Within hours of activation, iNKT cells exert effector functions constituting of their cytotoxic activity and the secretion of a multitude of cytokines, including IFN-γ, IL-4, IL-10 and IL-17, hence the term 'innate-like'.[158,470,471] The secretion of cytokines and the expression of various surface molecules are at the core of iNKT cell function in orchestrating the immune response by interacting with several innate and adaptive immune cells.[158,469,472] This property of iNKT cells makes them critical players in anti-microbial and anti-tumour immunity as well as in shaping inflammatory and autoimmune conditions.[473-475] In an attempt to exploit the critical role of iNKT cells in bridging the innate and adaptive immune system, clinical trials are testing iNKT activation in novel vaccine adjuvant formulations and anti-cancer immunotherapy.[157,476-478]

Activation of iNKT cells occurs via the presentation of strongly antigenic microbial lipids, like the prototypical antigen α-galactosylceramide, without requiring additional signals. Alternatively, iNKT cells can be activated by the dual signal of an antigenic lipid and inflammatory cytokines from antigen-presenting cells. Microbial lipids, such as glycolipids from pneumococci (Streptococcus pneumoniae), or endogenous self-lipids, which might be specifically synthesized for iNKT activation act in this way. Lastly, and owing to their innate-like origin, iNKT cells can be activated by cytokines in the absence of a TCR signal, as is the case for murine cytomegalovirus infections.[469,479]

Invariant natural killer T cells can be categorized in one of several thymically predetermined subtypes, according to transcription factor expression and their signature cytokines IFN-γ (iNKT1), IL-4 (iNKT2), IL-17 (iNKT17) and IL-10 (iNKT10), analogous to conventional T cells (see *Atherosclerosis – The Immune System in Atherosclerosis*).[480] Although the various subsets have been extensively described in mice, they are yet to be confirmed in humans. Functional heterogeneity in human iNKT subsets have been described with $CD4^+$ iNKT cells as potential iNKT2 equivalent and $CD4^-CD8^-$ double-negative iNKT cells resembling iNKT1.[469]

The frequency of iNKT cells in blood varies considerably among individuals, but is usually around 0.001 – 0.1 % of all peripheral T cells.[475,481] This is well in line with the observed median frequency of 0.02 % in the Malmö Diet and Cancer Study in

Paper IV. Nonetheless, iNKT cells are tissue-resident cells and thus more abundant in peripheral tissues where they also show a differential enrichment of the various functional subtypes. The largest numbers of iNKT cells can be found at two important sites for lipid homeostasis, the adipose tissue and the liver, where lipoproteins are assembled.[475,482] Whereas iNKT1 are abundant in the liver, the adipose tissue harbours almost exclusively iNKT10 and iNKT2 cells, which seem to dampen adipose tissue inflammation and oppose the development of obesity and type 2 diabetes.[482-487] Interestingly, obesity is accompanied by decreasing numbers of iNKT cells in mice and humans.[483,485,488] Similar depletion can be seen in several other autoimmune diseases as well as in patients with ASCVD.[475,489,490]

Invariant natural killer T cells and experimental atherosclerosis

The impact of iNKT cells on atherosclerosis has been investigated in numerous mouse studies and with varying experimental approaches. Deficiency of iNKT cells by genetic manipulation or thymectomy led to a decreased atherosclerotic burden in experimental mice.[491-496] The opposite approach, activation of iNKT cells with the iNKT1-skewing agonist α-galactosylceramide resulted in an increased lesion development.[492,493] In line with these results, the adoptive transfer of iNKT cells, particularly of $CD4^+$ but not double-negative iNKT cells, also aggravates atherosclerosis development.[496-498] In contrast, one group has reported decreased plaque development in mice treated with α-galactosylceramide.[499] A potential explanation for the disparate data might be different environmental microbial cues in the various laboratories (see also *Methodological Considerations – Mouse Studies*). The development and proper function of iNKT cells is particularly dependent on the microbiome early in life.[309,469,500] Intriguingly, a common commensal bacterium in humans, Bacteroides fragilis, has even been reported to produce the prototypical iNKT antigen α-galactosylceramide.[501]

Almost all mouse studies have been performed in either *ApoE*$^{-/-}$ or *LDLr*$^{-/-}$ mice, which poses a major limitation. Both, apoE and the LDL receptor are important in the uptake of lipid antigens and presentation to iNKT cells. In the absence of either of the two, activation of iNKT cells by antigen-presenting cells is significantly hampered or completely abrogated, depending on the type of antigen-presenting cells.[502,503] Furthermore, hyperlipidaemia leads to reduced numbers and an impaired function (anergy) of iNKT cells in *ApoE*$^{-/-}$ as well as in high-fat fed wildtype mice. [483,504-506] Despite these limitations, mouse studies have repetitively found a 1.5 – 2 fold increase in atherosclerosis development. The increased atherosclerosis was attributed to iNKT cell function, but it is unclear if there are differential effects of the various iNKT subsets (iNKT1|iNKT2|iNKT17|iNKT10) since they have not been investigated specifically in atherosclerosis. As outlined previously, human biology differs from the mouse and it is thus important to confirm experimental findings in the mouse in humans. Observational human studies can aid this translation from the mouse to the human.

Atherosclerotic CVD and iNKT cells

Invariant natural killer T cells have been shown to be decreased in patients with established ASCVD.[481,507-509] Decreased circulating cell numbers could hint towards an anergic state in which the iNKT cells might have lost their potential protective function, similar to the decreased levels and impaired iNKT cell function in obesity. This hypothesis is, however, in contrast to most results of the mouse studies of experimental atherosclerosis. Nonetheless, in line with a hypothesised protective function, our group has previously found that high levels of circulating NKT-like cells are associated with a lower incidence of coronary events. This association might, however, have been confounded by blood lipid levels.[510] A major limitation of the previous study is the analysis of NKT-like cells, defined as $CD56^+$ and IFN-γ^+. It is known that conventional T cells can also express CD56 and only a small subset of human iNKT cells expresses CD56, which is also poised for iNKT1 responses.[511-514] Indeed, in our previous study less than 2.0 % of the NKT-like cells were positive for a staining with α-galactosylceramide-loaded CD1d-tetramers, the gold-standard detection method for iNKT cells.[510]

In ***Paper IV***, we analysed the levels and subset distribution of circulating iNKT cells in the prospective observational Malmö Diet and Cancer Study (MDCS) by staining frozen peripheral blood mononuclear cells with PBS57-loaded CD1d-tetramers, with PBS57 being an analogue of α-galactosylceramide (Figure 19).[515] Intriguingly, we did not find a statistically significant association between total iNKT cells and the incidence of first-time coronary events during up to 17 years of follow-up. This is in stark contrast to the suggested role of iNKT cells from mouse studies because if at all, higher iNKT cell numbers associated (non-significantly) with a lower incidence of coronary events.

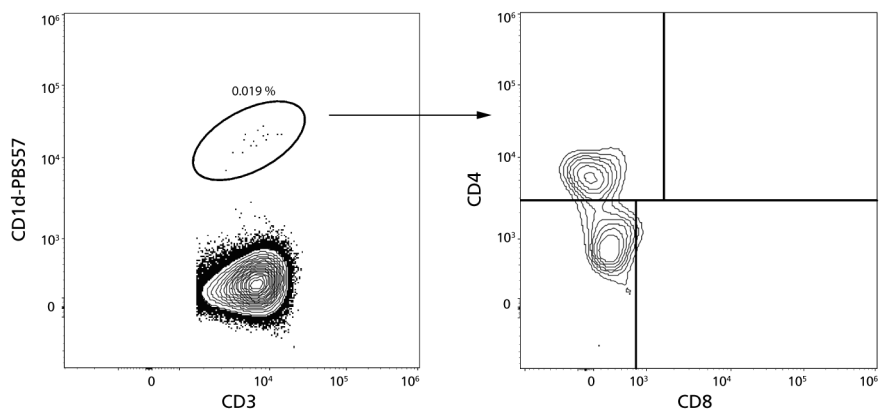


Figure 19. Identification of iNKT cells in the MDCS. After gating out antibody aggregates and doublets, live $CD3^+$ lymphocytes were gated and displayed on a CD3 vs. CD1d-PBS57 plot (left). The diagonal appearance of iNKT cells results from the co-expression of CD3 and the TCR, which is stained by CD1d-PBS57, and increases the confidence of 'true' iNKT cell events. Invariant natural killer T cells were subsequently gated for CD4 and CD8 (right).

As for conventional T cells, the relationship between the iNKT cells with atherosclerosis development is likely to be subset dependent (see *Atherosclerosis – The Immune System in Atherosclerosis*). Thus, we analysed iNKT cell subsets according to CD4 and CD8 staining. Surprisingly, this yielded a statistically significant association of higher levels of double-negative iNKT cells with a lower incidence of first-time coronary events, i.e. hinting towards a protective effect of double-negative iNKT cells. These results are in part surprising, given the large body of evidence from mouse studies for a pathogenic role in atherosclerotic disease. Nonetheless, they are consistent with our previous results of NKT-like cells and other reports of decreased circulating iNKT cell frequencies in patients that have suffered coronary events.[481,507-510]

Patients with the autoimmune disease systemic lupus erythematosus also have decreased levels of circulating iNKT cells, similar to obesity and ASCVD. A recent report in lupus patients has provided evidence for a protective role of iNKT cells in patients with subclinical atherosclerotic disease that have not experienced an atherosclerotic cardiovascular event in the past. These cells were characterised as avid producers of the iNKT2 cytokines IL-4 and IL-13 and had the potential to induce reparatory macrophages. In contrast, iNKT cells of patients with previous atherosclerotic events lost their protective phenotype and became unresponsive to proliferative stimuli, i.e. anergic.[516] Of note, also the patients examined in ***Paper IV*** had no previous atherosclerotic cardiovascular events, except for 10 individuals that had experienced a stroke or undergone either percutaneous coronary intervention or coronary artery bypass grafting without a preceding myocardial infarction.

Thus, the results of ***Paper IV*** question the notion, generated by numerous mouse studies, that iNKT cells are a pro-atherogenic cell type. Future studies will have to elucidate whether a protective effect of iNKT cells can be seen in mouse models that do not possess the limitations of $ApoE^{-/-}$ and $LDLr^{-/-}$ mice, like $LDLr^{-/-}$ transplanted with wild-type bone-marrow.

A function of double-negative iNKT cells that might play a role in the potential athero-protection is their role in the defence against pneumococci. A major anti-pneumococcal function of iNKT cells is the provision of B cell help for effective antibody responses,[517-521] which has been reported to be dependent on double-negative iNKT cells.[518,521] Intriguingly, pneumococcal vaccination results in increased antibody titres against oxidized LDL and ameliorates atherosclerosis development in $LDLr^{-/-}$ mice.[522,523] Although it is not clear whether the protective antibody effect in experimental atherosclerosis is mediated in part by iNKT cells, it might be an explanation for our observation of the negative association of double-negative iNKT cells with coronary events.

Limitations

There are a few limitations for the study presented in ***Paper IV***, besides being an observational study, which by default cannot report on causality. The analysed cells were peripheral blood mononuclear cells, which had been stored in liquid nitrogen

since the baseline examination in the early 1990s. Cryopreservation has been shown to alter the subset distribution for certain cell types and we cannot exclude this possibility for our dataset,[524] although we have not observed a change in other studies within the MDCS,[130] including the report in ***Paper V***. Additionally, the analysis of blood samples taken in the early 1990s might constitute a selection bias, because the population and particularly the environmental influences of the immune system might be different in today's society, e.g. through a different medication or changing pathogen burden.
Lastly, iNKT cells are a rare immune cell subset which requires sophisticated methodology for flow cytometric analysis.[525] Our rigorous flow cytometric setup (see ***Paper V***) complies with these requirements. Nonetheless, the use of stored peripheral blood mononuclear cells resulted in a limited sample quantity availability, which in turn leads to a lower statistical precision in the assay. Therefore, we have limited the analysis to samples with at least 25 000 events within the T cell gate, leading to a counting precision for iNKT cells with an average coefficient of variation of 27 %, which is still below the typical assay variation of 30 %.[526]

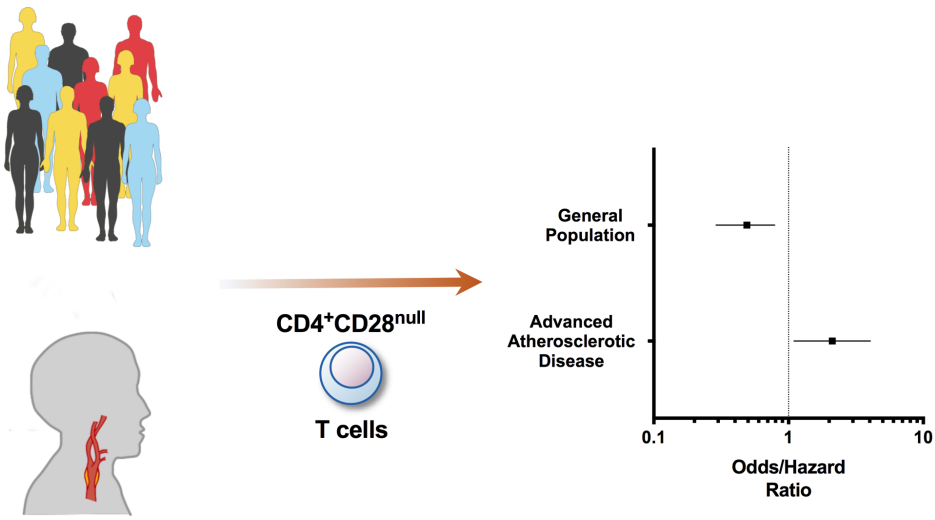
CD4⁺CD28ⁿᵘˡˡ T cells and cardiovascular events

Specific Aims

- To investigate the association of CD4⁺CD28ⁿᵘˡˡ T cells with first-time coronary events
- To validate the potential of using CD4⁺CD28ⁿᵘˡˡ T cells as a prognostic biomarker

Key Finding

- Higher levels of circulating CD4⁺CD28ⁿᵘˡˡ T cells associate with a lower incidence of first-time coronary events, which is in disagreement with their positive association with cardiovascular events in patients with established ASCVD.



$CD4^+CD28^{null}$ T cells

T cells require several signals to become fully activated, including the engagement of costimulatory surface proteins, of which CD28 is one of the most important ones. The requirement of additional stimulation via CD28, apart from antigen recognition through the TCR, is also a safeguard against autoimmunity and therefore sometimes referred to as immune checkpoint. Functionally, CD28 stimulation modulates the cellular machinery in various ways, including the adaptation of the T cell's metabolism, to support activation and avoid an anergic unresponsive state or apoptosis.[527-529]

Activated T cells decrease their expression of CD28. This down-regulation is a transient effect and CD28 expression returns to initial levels after a few days.[530,531] With increasing age, however, elevated numbers of $CD8^+$ and $CD4^+$ T cells that persistently lack the expression of CD28 ($CD8^+CD28^{null}$ and $CD4^+CD28^{null}$) can be found in humans.[532,533] The loss of the costimulatory molecule CD28 is thought to be a consequence of repeated or continuous antigen exposure and activation, and is indicative of terminal differentiation.[534] In line with this hypothesis, $CD4^+CD28^{null}$ T cells possess a limited, oligoclonal TCR repertoire and show signs of immunosenescence, namely shortened telomeres and cell cycle arrest.[535]

Nonetheless, $CD4^+CD28^{null}$ T cells retain functional properties like the secretion of IFN-γ and TNF-α. Furthermore, they acquire atypical cytotoxic effector functions and have cytoplasmic granules of the cytotoxic molecules perforin and granzyme, which are usually found in natural killer cells or $CD8^+$ but not $CD4^+$ T cells.[534,535] Additionally, $CD4^+CD28^{null}$ T cells become a rogue immune cell because of their resistance to regulation by apoptosis or T_{REG}-mediated suppression.[535] It must not be neglected though, that the analysis of only three cellular markers (CD3|CD4|CD28) yields a quite heterogenous population of cells that might also significantly differ in different individuals, as representatively shown for memory T cell markers in Figure 20.

As shown in the figure, $CD4^+CD28^{null}$ T cells overlap at least in part with terminal effector memory T cells re-expressing CD45RA (T_{EMRA}), a molecule that is usually confined to naïve T cells.[536] T_{EMRA} cells also show intact effector functions, short telomeres and a reversible cell cycle arrest.[537] Importantly, T_{EMRA} cells are among the most prominent examples for oligoclonality and are associated with a process called memory inflation, which denotes the development of expanded memory T cell pools in response to persisting infectious antigens, particularly cytomegalovirus (CMV).[537-539]

Cytomegalovirus and $CD4^+CD28^{null}$ T cells

$CD4^+CD28^{null}$ T cells have repetitively been shown to associate with CMV seropositivity, i.e. the existence of a previous CMV infection.[534,535,540] This might be a partial explanation of the association of age and elevated $CD4^+CD28^{null}$ T cell levels, since the probability of infection probably rises with increasing age.[541,542] At the same time, associations with CMV have to be interpreted with caution, given the high prevalence of CMV seropositivity, which lies between 45 – 80 % in Europe.[543]

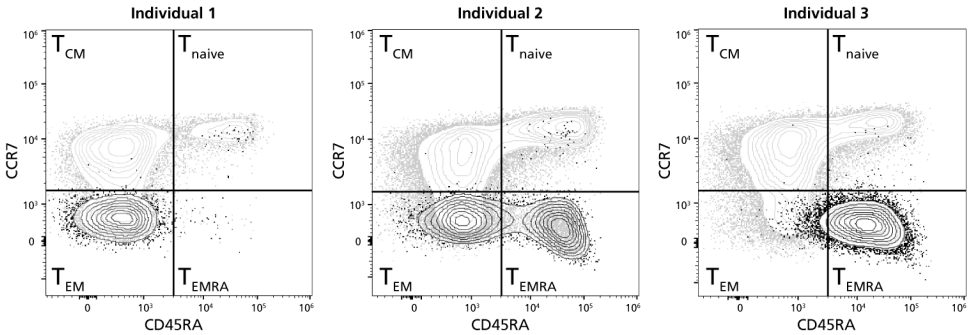


Figure 20. Heterogeneity in CD4⁺CD28^{null} T cells. The figure shows density plots of memory T cell phenotyping in samples from three different individuals from the MDCS. The dark black cell clusters are CD4⁺CD28^{null} T cells, which are overlaid onto the density plots for the complete CD4⁺ T cell subsets. T_{CM} = central memory T cells, T_{EM} = effector memory T cells.

Mouse studies provide another line of evidence for the relationship of CMV and CD4⁺CD28^{null} T cells. Laboratory mice do not harbour any CD4⁺CD28^{null} T cells, whereas 'wild' mice contain a subset of CD27⁻ T cells, which probably represent CD4⁺CD28^{null} T cells (see also *Methodological Considerations – Mouse Studies*).[315,535] Importantly, a recent study reported substantial CD4⁺CD28^{null} T cell frequencies only in CMV seropositive individuals but not in seronegative people.[542] Not surprisingly, cytomegalovirus-reactive T cells have predominantly a CD28^{null} phenotype and a recent small trial found that anti-viral therapy in CMV seropositive patients reduced the frequency of CD4⁺CD28^{null} T cells.[542,544,545]

CD4⁺CD28^{null} T cells have been found to be associated with several autoimmune diseases, in particular rheumatoid arthritis.[535,540] Interestingly, CMV DNA can be found in the joints of patients with rheumatoid arthritis and it has been suggested that CMV, via CD4⁺CD28^{null} T cells, might play a role in the maladaptive immune response in autoimmune and chronic inflammatory diseases.[540] Interestingly, endothelial cells, which constitute the luminal layer in vessels, are a preferential infection target of CMV and viral DNA can be found in atherosclerotic plaques.[546,547]

The only known antigen stimulating CD4⁺CD28^{null} T cells besides CMV proteins is heat-shock protein 60, which is released during increased cellular stress.[535,540] Heat-shock protein 60 is implicated as a potential autoantigen in atherosclerosis and antibody titres, as well as T cell reactivity to human heat-shock protein 60 are associated with more severe ASCVD.[548] Importantly, antibodies targeting heat-shock protein 60 also recognise proteins from CMV.[549] It is tempting to speculate that the antibody response against heat-shock protein 60 is a misguided immune reaction originally targeting a pathogenic invader (CMV), given the exquisite regulation of antigen-specificity in the adaptive immune system. Indeed, CMV infection seems to associate with the risk of atherosclerotic cardiovascular events, although the reported risk ratio is weak.[547,550]

$CD4^+CD28null$ T cells in ASCVD

Human atherosclerotic plaques contain $CD4^+CD28^{null}$ T cells with limited TCR clonality.[547,551,552] Furthermore, increased frequencies of $CD4^+CD28^{null}$ T cells can be found in the blood of patients with myocardial infarction, unstable angina or ischaemic stroke and decline concomitantly with decreasing ASCVD severity.[553-555] Importantly, increased frequencies of $CD4^+CD28^{null}$ T cells have been shown to be associated with the incidence of recurrent coronary events or ischaemic stroke.[464,465,556] Even more, in a small subset of 60 diabetic individuals without established atherosclerotic disease, $CD4^+CD28^{null}$ T cells associated with incident first-time atherosclerotic cardiovascular events during a 3-year follow-up.[465] Consequently, $CD4^+CD28^{null}$ T cells represent an interesting potential biomarker of disease and they have been suggested as therapeutic targets.[465,553,557] The reported studies, however, relied on a relatively small number of study participants, which increases the risk of misleading conclusions (see also *Methodological Considerations – Human Studies*). Thus, in ***Paper V*** we set out to validate these findings in 400 individuals of the MDCS, representing a general population without pre-existing ASCVD.

To our surprise, we found that lower levels of circulating $CD4^+CD28^{null}$ T cells associated with a higher rate of incident first-time coronary events. It is important to note, however, that this association was only significant for coronary events occurring more than 9 years after the baseline and held true just for very high $CD4^+CD28^{null}$ T cell frequencies. In line with these findings, we did not detect an association of $CD4^+CD28^{null}$ T cells with ultrasonography-measured carotid plaque size (intima-media thickness) or the progression thereof during 16 years of follow-up. Although our results contradict the study of 60 diabetic individuals, it does not necessarily so with the other studies of recurrent disease. The immune effector functions might differ in individuals without overt ASCVD (MDCS) and in those with established ASCVD.

To evaluate this possibility, we turned to the blood samples of patients that had been operated because of extensive carotid atherosclerosis (CPIP), in which we had measured $CD4^+CD28^{null}$ T cells as well. In this population of patients with pre-existing ASCVD, we could verify the association of increased circulating $CD4^+CD28^{null}$ T cells with future cardiovascular events found in previous studies or recurrent atherosclerotic cardiovascular events. Nonetheless, even in CPIP, $CD4^+CD28^{null}$ T cells did not associate with carotid artery stenosis.

The intriguing finding of opposing associations of this heterogenous T cell population with cardiovascular events in individuals with or without established ASCVD might be representative of the different progression states of atherosclerosis affecting $CD4^+CD28^{null}$ T cells, as outlined in the manuscript. They could, however, as well just be the result of analysing a very heterogenous T cell population that contains T cells with various functional properties, from senescent cells to recently activated T cells. We have partly addressed this possibility in ***Paper V*** by staining for alternative costimulatory receptors that have been shown to be transiently upregulated in

$CD4^+CD28^{null}$ T cells following activation.[552] Interestingly, $CD4^+CD28^{null}$ T cells from the MDCS had a higher potential of upregulating the co-stimulatory receptors OX40 and 4-1BB, whereas $CD4^+CD28^{null}$ T cells in CPIP patients showed a higher baseline expression of OX40. The more poised state of CPIP cells to pro-inflammatory responses could be a reason for the opposite risk association in patients with established ASCVD. On the other hand, higher potential to upregulate these co-stimulatory receptors, as seen in MDCS samples, has been shown to occur preferentially in patients with myocardial infarction and unstable angina, compared to samples of healthy patients or patients with stable angina.[552]

Another important factor to consider is that the MDCS was recruited in the early 1990s, which for instance entails a very low statin coverage. Statins are known to possess pleiotropic anti-inflammatory effects and might reduce the frequency of $CD4^+CD28^{null}$ T cells,[464,465,558] which could have affected the conflicting results in the two cohorts.

Additionally, we cannot rule out an effect of the CMV status in the individuals, which could unfortunately not be assessed in ***Paper V***. The strong associations of $CD4^+CD28^{null}$ T cells with CMV raise the question whether $CD4^+CD28^{null}$ T cell frequencies are just a surrogate for latent CMV infection, which has been shown to be associated ASCVD. In this case, a differential CMV status in the two cohorts, the MDCS and CPIP, might be responsible for the opposing results. Clearly, determination of the CMV seropositivity in the two cohorts as well as probably future results from CMV vaccination trials would provide valuable information.[559]

In summary, $CD4^+CD28^{null}$ T cells are poor biomarkers of ASCVD development based on our results of ultrasonography-measured plaque progression and carotid artery stenosis. In addition, measurement of $CD4^+CD28^{null}$ T cells does not seem to have a utility as a prognostic biomarker, given the different associations with atherosclerotic cardiovascular events depending on the patient population. Consequently, $CD4^+CD28^{null}$ T cells do not appear to be a viable therapeutical target, unless we acquire a better understanding of their role in ASCVD. Although targeting $CD4^+CD28^{null}$ T cells has been repetitively suggested, our results indicate that this might have adverse effects in individuals without overt ASCVD.

Limitations

Besides the limitations presented in the manuscript, there are additional issues relating to the differential effects in the two cohorts, as outlined above. In particular, the examination of a population that was recruited, and blood samples that were taken in the early 1990s (MDCS) does not necessarily represent today's population, which is sampled in CPIP. One of these differences potentially affecting the comparability is the statin coverage. The different time-points might also be connected to different microbial exposure. Cytomegalovirus is connected to both, $CD4^+CD28^{null}$ T cells and ASCVD, and the lack of information regarding CMV antibody titres represents an additional limitation of this study.

Summary and Outlook

The focus of *Part II – Cellular Traits* was the study of various T cell subsets and their relationship with ASCVD in mice and in humans. The work in ***Paper III*** investigated the role of $CD4^+$ T cells in atherosclerosis development in transgenic mice lacking MHC II. We found an atheroprotective net effect of $CD4^+$ T cells which might be attributable to one of the major regulators of inflammation resolution, the T_{REG} cell. Although mouse studies are valuable tools for the identification of pathogenetic mechanisms, results might not hold true for human-beings (see also *Methodological Considerations – Mouse Studies*). ***Paper IV*** represents an effort to evaluate the translatability of the knowledge of a pro-atherogenic role of iNKT cells gained in mice to the human situation. We were, however, unable to find evidence for the suggested pathogenic role of iNKT cells in human atherosclerotic disease. In contrast, we identified a potential protective effect of double-negative iNKT cells in ASCVD. In the final ***Paper V*** we attempted to validate the feasibility of using $CD4^+CD28^{null}$ T cell frequencies as a predictive biomarker for atherosclerotic cardiovascular events. We found contrasting associations of $CD4^+CD28^{null}$ T cell frequency with atherosclerotic cardiovascular events, depending on prevalence or absence of overt ASCVD. Thus, the usability of $CD4^+CD28^{null}$ T cell frequency as a disease biomarker is limited or even absent.

Thirty years after the first account of activated T cells in human atherosclerotic plaques, the roles of most T cell subsets are still incompletely understood. This can be attributed to the growing acknowledgement of the complexity of T cell subset biology and their plasticity as well as the methodological challenges in analysing some of them, as mentioned in *Invariant natural killer T cells and coronary events*. Progress in the understanding of the mechanisms and the contribution of T cells to atherosclerosis could provide useful information for attempts to dampen the adaptive immune reaction in atherosclerosis.

The work in ***Paper III*** hints towards an overall anti-atherosclerotic effect of $CD4^+$ T cells, and T_{REG} cells are most likely the subset responsible for this effect. This is well in line with other studies in atherosclerosis and other inflammatory diseases and emphasizes their important role in mediating immune homeostasis. [159,560] Given their important role, it is not surprising that T_{REG} cells have been identified as potential therapeutic targets in atherosclerosis and novel anti-atherosclerotic therapies. It will be interesting to see the results of the first ongoing trials attempting to expand T_{REG} cells in humans to counteract ASCVD. [561]

Whereas ***Paper III*** is based on experimental studies in mice, in ***Paper IV*** we were unable to confirm experimental mouse studies about the pro-atherogenic role of iNKT cells for the human situation. This is important information, particularly given the limitations of previous mouse studies, including the negligence of the various iNKT subsets. We found differential effects of the various iNKT cell subsets, which urges for

a second look into the mechanistic role of iNKT cells and their subsets in atherosclerosis. The results of a potentially protective effect of iNKT cells are intriguing but in line with human and mouse studies of obesity, which is tightly linked to atherosclerosis. [562] The reassessment of the roles of iNKT cells in experimental atherosclerosis in suitable mouse models also bears the potential for broadening our understanding of the immunogenicity of various lipids, given the lipid-specificity of these cells.

Finally, the results presented in ***Paper V*** underscore the importance of replication studies in scientific progress. $CD4^+CD28^{null}$ T cells have repetitively been shown to be elevated in patients with myocardial infarctions and two studies have found an association of $CD4^+CD28^{null}$ T cell frequencies with the risk of suffering a recurrent coronary event. One smaller study even found that $CD4^+CD28^{null}$ T cell frequencies are predictive of first-time atherosclerotic cardiovascular events in diabetic patients without existing ASCVD. Although, in ***Paper V***, we were able to replicate the association of $CD4^+CD28^{null}$ T cells with incident events in patients with pre-existing ASCVD, we found the opposite associations in individuals without overt ASCVD. This raises the question of whether the relationship of $CD4^+CD28^{null}$ T cells with ASCVD is a case of 'guilt by association' or if there are two opposite mechanisms at work – linking $CD4^+CD28^{null}$ T cells with a protective effect in individuals without ASCVD and with a detrimental effect in patients with established ASCVD. Future studies will have to determine what causes the conflicting associations. Until then, however, we should abstain from therapeutically targeting these cells as this might cause serious adverse effects. An intriguing link and potential explanation for the disparate findings is the association of $CD4^+CD28^{null}$ T cells with CMV, which warrants further exploration.

Concluding Remarks

HUMAN LIFE relies on the continuous functionality of the cardiovascular system. Atherosclerosis can severely interfere with the capacity of the cardiovascular system to provide nutrients and oxygen to distant cells. The breakdown of the supply caused by atherosclerotic plaque rupture has life-threatening consequences, like myocardial infarction and stroke, which constitute the major global health burden of the 21st century.

Apolipoprotein B-containing lipoproteins are a causal factor in the initiation of atherosclerosis and connect the disease to the unhealthy modern lifestyle. Another important player in disease development dates back to observations of *Rudolf Virchow*, who identified immune cells in atherosclerotic plaques. In fact, the immune system might be as important for atherosclerotic development as the increased levels of apoB-LPs. Mice that are genetically-engineered to have a defect in the first-responders of the innate immune system hardly develop atherosclerosis, despite substantially increased apoB-LPs. [140,141] Moreover, a large body of evidence culminating in the recent CANTOS trial provides in-human evidence for a causal role of the immune system in atherosclerosis development.

The precise triggers and sequence of events that lead to a pro-inflammatory reaction of the immune system remain obscure. The unhealthy diet and physical inactivity of the Western lifestyle as well as the exposure to noxious substances like tobacco and alcohol are able to activate the immune system, in addition to their effects on apoB-LP levels.[563] Intriguingly, in mummies of humans who lived thousands of years ago (up to 3000 B.C.) aortic calcifications that are indicative of advanced atherosclerosis can be found.[564] These individuals most likely did not have unlimited food access but definitely did not have access to fast food franchises. Some of the examined mummies even stem from hunter-gatherer societies, whose way of life was probably the complete opposite of today's lifestyle. A potential trigger for a pro-atherogenic immune reaction in the mummies could have been the infectious disease burden, which is also a recurring theme throughout this thesis.

Our understanding of the inflammatory process in atherosclerosis has substantially increased since the initial description of inflammatory cells but a considerable knowledge gap remains. The work presented in this thesis is part of the global effort in deciphering the immune system's contribution to atherosclerosis.

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