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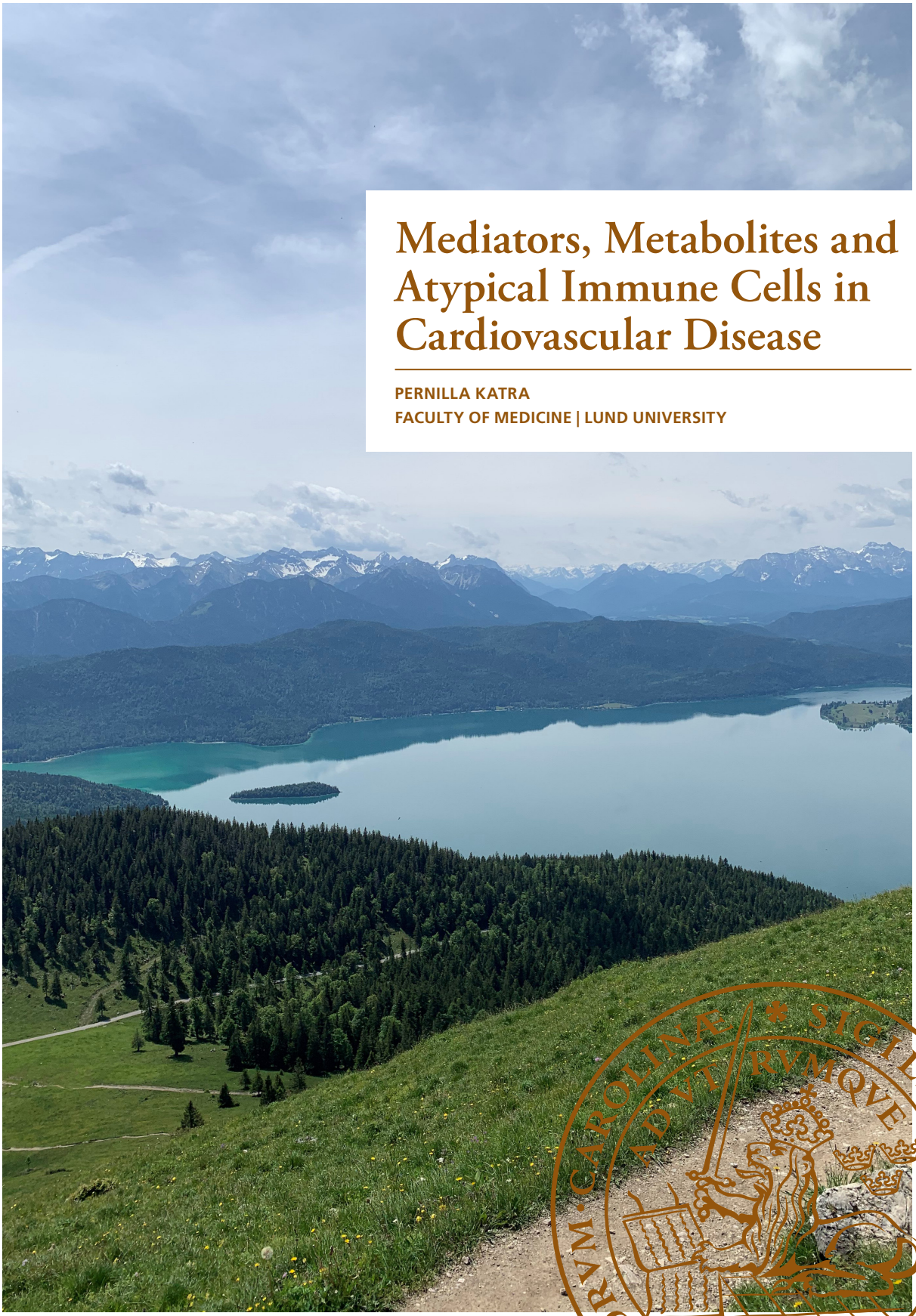

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FACULTY OF MEDICINE | LUND UNIVERSITY

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Pernilla Katra

LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on the 11th of October at 09.00 in Segerfalksalen, Sölvegatan 19, 223 62, Lund.

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Abstract:

Cardiovascular disease (CVD) remains, despite intense research, the most common cause of death worldwide. The main underlying cause of CVD is atherosclerosis, which is characterised by chronic inflammation. CVD and atherosclerosis have a complex aetiology, which necessitates research into multiple areas in order to gain insight into the pathogenesis and find novel markers that could aid in identifying individuals at risk. In this thesis, I have investigated mediators, metabolites and atypical immune cells and how they affect atherosclerosis and risk of coronary events. To this end we have used both mouse models and a clinical cohort.

In *Paper I* we investigated associations between the leukocyte guiding chemokines CCL21 and CCL19 and incident coronary events in the general population-based Malmö Diet and Cancer cohort. We found that high plasma levels of CCL21, but not CCL19, had an independent association to incident coronary events. High levels of CCL19 were on the other hand associated with both incident heart failure and mortality.

In *Paper II* we investigated the effect of drinking water supplementation with α-ketoglutarate or glutamine on atherosclerosis development and plaque composition. Our main finding was that glutamine, an important fuel source for immune cells, caused increased development of atherosclerosis in male mice. These mice also had larger accumulation of cells, including neutrophils, in the adventitia surrounding the aorta.

In *Paper III* we investigated $CD21^{low}$ age-associated B cells (ABCs), previously identified in atherosclerotic plaques. We evaluated their clonality, differentiation potential and effect on atherosclerosis development. In humans we investigated if $CD21^{low}$ ABCs were associated to incident coronary events. We found that $CD21^{low}$ ABCs were clonally expanded and could differentiate into plasma cells in vivo. $CD21^{low}$ ABCs also aggravated murine atherosclerosis and high numbers of circulating $CD21^{low}$ ABCs were associated with incident coronary events in humans.

In *Paper IV* we investigated if, as suggested by animal studies, invariant natural killer T (iNKT) cells have an association to incident coronary events in humans. However, high numbers of these lipid-specific cells did not have an association to incident coronary events. Furthermore, we identified a subpopulation of iNKT cells that were $CD4^{-}CD8^{-}$ which had an independent, inverse association to incident coronary events.

In conclusion, this thesis illustrates the multifaceted contributions of inflammatory mediators and cells in CVD and atherosclerosis, while also providing novel insights into this important research field.

Key words: Immunology, metabolism, cardiovascular disease, atherosclerosis, age-associated B cell, invariant natural killer T cell, coronary event, immunometabolism

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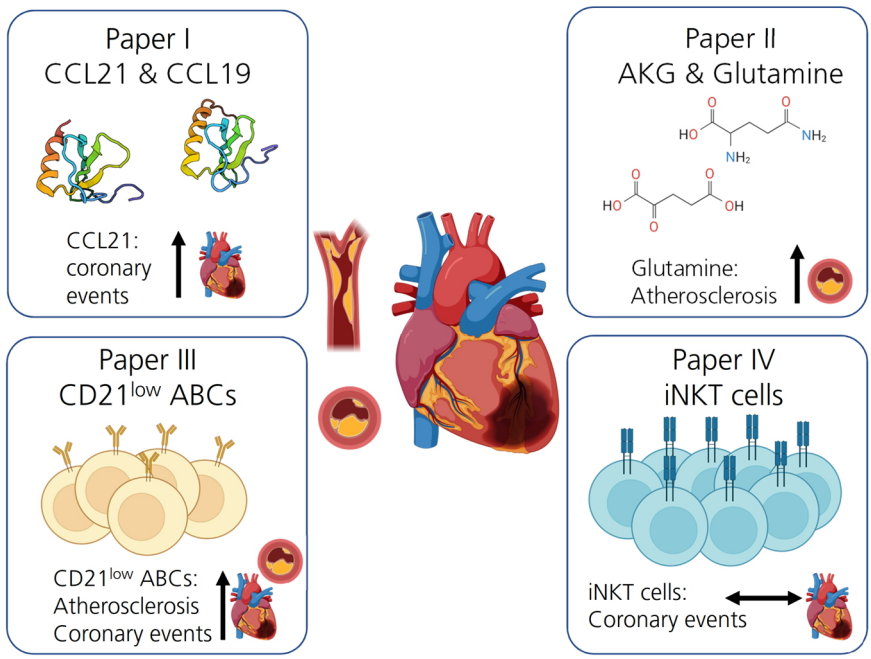
Abstract

Cardiovascular disease (CVD) remains, despite intense research, the most common cause of death world-wide. The main underlying cause of CVD is atherosclerosis, which is characterised by chronic inflammation. CVD and atherosclerosis have a complex aetiology, which necessitates research into multiple areas in order to gain insight into the pathogenesis and find novel markers that could aid in identifying individuals at risk. In this thesis, I have investigated mediators, metabolites and atypical immune cells and how they affect atherosclerosis and risk of coronary events. To this end we have used both mouse models and a clinical cohort.



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In conclusion, this thesis illustrates the multifaceted contributions of inflammatory mediators and cells in CVD and atherosclerosis, while also providing novel insights into this important research field.

Populärvetenskaplig Sammanfattning

I min avhandling har jag undersökt hur immunförsvaret påverkar hjärt-kärlsjukdom. I de fyra artiklarna som är med i avhandlingen har jag arbetat med flera komponenter av immunförsvaret och analyserat dels hur de påverkar hjärt-kärlsjukdom i möss men också i människor. Mina resultat kan förhoppningsvis i framtiden bidra till att det finns fler och bättre behandlingar, men också att vi kan identifiera personer som befinner sig i riskzonen tidigare.

Hjärt-kärlsjukdom, som inkluderar till exempel hjärtinfarkt är väldigt vanligt, bara i Sverige har 2 miljoner människor någon form av hjärt-kärlsjukdom. Det är också den vanligaste dödsorsaken i Sverige och i hela världen. Tack vare intensiv forskning så har behandlingarna blivit bättre och bättre men fortfarande drabbas många. Vi behöver helt enkelt mer kunskap om hur hjärt-kärlsjukdom utvecklas över tid och vilka faktorer som ökar risken att drabbas.

Ateroskleros, som också kallas åderförkalkning eller åderförfettning är en av huvudorsakerna bakom hjärt-kärlsjukdom. I ateroskleros ansamlas fettmolekyler, kolesterol, i blodkärlens väggar. Över lång tid ansamlas mer och mer kolesterol, och även immunceller. Detta området, som vi kallar ett plack, blir inflammerat och växer. Till slut kan placket brista, och skapa en blodpropp som täpper igen blodkärlet. Om detta händer i ett av blodkärlen i hjärtat, så orsakar det en hjärtinfarkt. Då får hjärtat inte tillräckligt med syre och näring, och kan inte slå som det ska länge. Om man inte får behandling så kan det vara dödligt.

I *Artikel I* undersökte vi två närbesläktade protein, CCL21 och CCL19, som guidar immuncellers migration till olika delar av kroppen. För att immunförsvaret ska fungera på ett bra sätt så är det viktigt att immunceller befinner sig på rätt plats vid rätt tillfälle och CCL21 och CCL19 är två proteiner som bidrar till det. Vi analyserade nivåerna av dem i blodet hos personer som var med i studien Malmö Kost Cancer. Det visade sig att personer som hade höga nivåer av CCL21 hade större risk att få en hjärtinfarkt. Detsamma gällde inte CCL19, vilket är intressant eftersom CCL19 och CCL21 är väldigt lika till strukturen och har liknande funktioner.

I *Artikel II* undersökte vi hur metaboliterna α-ketoglutarat och glutamin påverkar hur ateroskleros utvecklas i möss. Vi såg att mössen som vi gav glutamin fick mer ateroskleros, det vill säga, deras plack blev större. Därefter undersökte vi innehållet i placken, till exempel hur många immunceller av olika typer som fanns. Vi tittade

också på hur mycket bindväv, kollagen, som fanns. Om det finns fler immunceller i ett plack finns det oftast mer inflammation, vilket påverkar plackets stabilitet negativt. Kollagen, å andra sidan, är en positiv, stabiliserade faktor som kan förhindra att placket brister. Intressant nog så hittade vi inga skillnader i plackinnehåll, och än så länge vet vi inte exakt varför glutamin gjorde att mössen utvecklade mer ateroskleros.

I *Artikel III* undersökte vi en typ av immuncell, B cell, som ökar i antal ju äldre man blir, och därför kallas åldersassocierad B cell. Vi såg att när vi gav möss dessa celler, så fick mössen mer ateroskleros. Hos människor såg vi att personer som hade många åldersassocierade B celler hade större risk att få en hjärtinfarkt. B celler ger upphov till plasmaceller, som producerar antikroppar, vilket sker till exempel efter en vaccination. Vi såg att åldersassocierade B celler också utvecklades till plasmaceller som producerade antikroppar. I våra framtida studier kommer vi undersöka dessa antikroppar mer, och särskilt ta reda på om de bidrar till att ateroskleros utvecklas.

I *Artikel IV* undersökte vi ytterligare en sorts immuncell, som kallas iNKT cell. Det är en speciell sorts cell som har egenskaper från flera olika typer av immunceller. iNKT celler har protein på sin yta som kan känna igen fettmolekyler, vilket gör dem intressanta att undersöka i ateroskleros. Vi var också intresserade av iNKT celler eftersom tidigare studier i möss har sett att de bidrar till utvecklingen av ateroskleros. Men i vår studie i människor såg vi att personer som fick en hjärtinfarkt inte hade fler iNKT celler i blodet. Det var snarare så att de som inte fick en hjärtinfarkt hade fler iNKT celler, vilket skulle kunna innebära att iNKT celler har en skyddande effekt. Men det kan vi inte säga med säkerhet, utan vi måste gör fler studier för att ta reda på vad iNKT celler har för roll i människor.

Sammanfattningsvis så visar resultaten från våra studier att mediatorer, metaboliter och immunceller är påverkade och själva kan påverka utvecklingen av hjärt-kärlsjukdom. Resultaten från våra studier viktiga pusselbitar som kan hjälpa framtida forskning och i förlängningen bidra till bättre behandlingar mot hjärt-kärlsjukdom.

Papers Included in the Thesis

Paper I

"Plasma levels of CCL21, but not CCL19, independently predict future coronary events in a prospective population-based cohort".

Katra, P, Hennings, V, Nilsson, J, Engström, G, Engelbertsen, D, Bengtsson, E and Björkbacka, H, Atherosclerosis, vol. 366, pp. 1-7, 2023

Paper II

"Glutamine supplementation leads to increased atherosclerosis and adventitial cell accumulation in Apoe$^{-/-}$ mice".

Katra, **P**, Andersson, L, Palmer, S, Schiopu, A, Spégel, P, Engelbertsen, D, Bengtsson, E and Björkbacka, H. Manuscript, 2024

Paper III

"Age-associated CD21^{low} B cells aggravate murine plaque development and are associated with coronary events in humans".

Smit, V*****, **Katra, P***, de Mol, J*****, Engels, S.W.M, Tomas, L, Andersson, L, Bernabé Kleijn, M.N.A, Schiopu, A, Bengtsson, E, Bot, I, Engelbertsen, D, Kuiper, J, Björkbacka, H$^{\#}$ and Foks, A.C$^{\#}$. Manuscript, 2024

Paper IV

"Invariant natural killer T cells and incidence of first-time coronary events: a nested case-control study".

Tomas, L*****, **Katra, P***, Badn, W, Andersson, L, Nilsson, J, Schiopu, A, Engelbertsen, D, Gonçalves, I, Bengtsson, E and Björkbacka, H, European Heart Journal Open, vol. 3, pp.1–9, 2023.

*Shared first authorship $^{\#}$Shared senior authorship

Papers not Included in the Thesis

"LAG3 regulates T cell activation and plaque infiltration in atherosclerotic mice"
Mulholland, M*, Kritikou, E*, **Katra, P**, Nilsson, J, Björkbacka, H, Lichtman, A.H, Rodriguez, A[#] and Engelbertsen, D[#]. JACC: CardioOncology. 2022 Dec;4(5):635-645.

"*Atherosclerosis: cell biology and lipoproteins"*
Katra, P and Björkbacka, H. Current Opinion in Lipidology, 2022;33(3):208-210.

"Plasma levels of the interleukin-1-receptor antagonist are lower in women with gestational diabetes mellitus and are particularly associated with postpartum development of type 2 diabetes"
Katra, P, Dereke, J, Nilsson, C and Hillman, M. PLoS ONE. 2016;11(5):e0155701.

*Shared first authorship [#]Shared senior authorship

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Abbreviations

ABC	Age-associated B cell
AKG	α-ketoglutarate
APO E	Apolipoprotein E
AUC	Area under the curve
BCR	B cell receptor
CE	Coronary event
CCL	C–C motif chemokine ligand
CCR	C–C motif chemokine receptor
CD	Cluster of differentiation
CRP	C-reactive protein
CVD	Cardiovascular disease
DN	Double negative
Gln	Glutamine
HDL	High density lipoprotein
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IHC	Immunohistochemistry
iNKT cell	Invariant natural killer T cell
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
MDC	Malmö Diet and Cancer
MDC-CV	Malmö Diet and Cancer – Cardiovascular
MHC	Major histocompatibility complex

NK cell	Natural killer cell
PCSK9	Proprotein convertase subtilisin/kexin type 9
ROC	Receiver operating characteristics
SMC	Smooth muscle cell
TCA cycle	Tricarboxylic acid cycle
TCR	T cell receptor
Th cell	T helper cell
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
Treg	Regulatory T cell

Cardiovascular Disease

Cardiovascular disease (CVD) is the most common cause of death in many countries (Figure 1)(Timmis et al., 2020). This includes the European Union where 32.7% of all deaths in 2020 were due to CVD (Eurostat, 2023). World-wide the mortality due to CVD, including **ischemic heart disease**, which composes the largest proportion of CVD, has decreased with 34.9% between 1990 and 2022. However, there are substantial differences between countries, and several are no longer experiencing a decrease (Mensah George et al., 2023, Arroyo-Quiroz et al., 2020). In the USA it is estimated that the decreased mortality is mainly attributable to improved risk factor profiles, including lowered cholesterol and blood pressure (Ford et al., 2007).

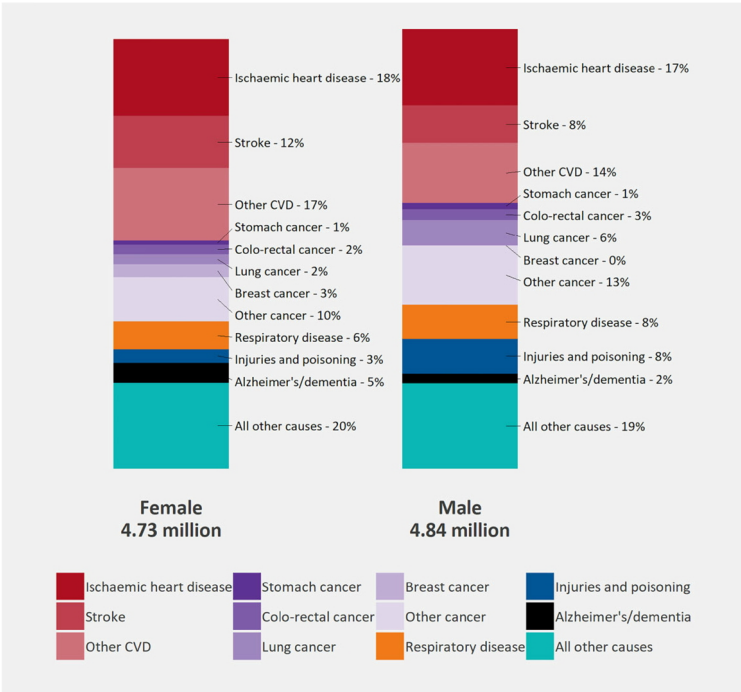


Figure 1: Most common causes for death for men and women in *European Society of Cardiology* member countries. Reprinted by permission of Oxford University Press on behalf of the European Society of Cardiology. Timmis, A et al., European Society of Cardiology: Cardiovascular Disease Statistics 2019. European Heart Journal, 2020, 41, 1, 12-85, doi:10.1093/eurheartj/ehz859.

However, the incidence of ischemic heart disease has largely been stagnant and has not decreased by much during the last 20 years in many western countries, (Conrad et al., 2024, Timmis et al., 2020). Additionally, more than 75% of the total CVD burden now occurs in low and middle income countries, which have previously had relatively low prevalence of CVD, demonstrating even more the need for better preventative care and research into the underlying pathology of CVD (Mensah George et al., 2023).

Risk Factors

CVD and ischemic heart disease are multifactorial diseases and there are numerous known risk factors (Figure 2)(Pencina et al., 2009). Some of these are modifiable and can be treated, like high blood pressure and lipoprotein levels, while others are non-modifiable, like age.

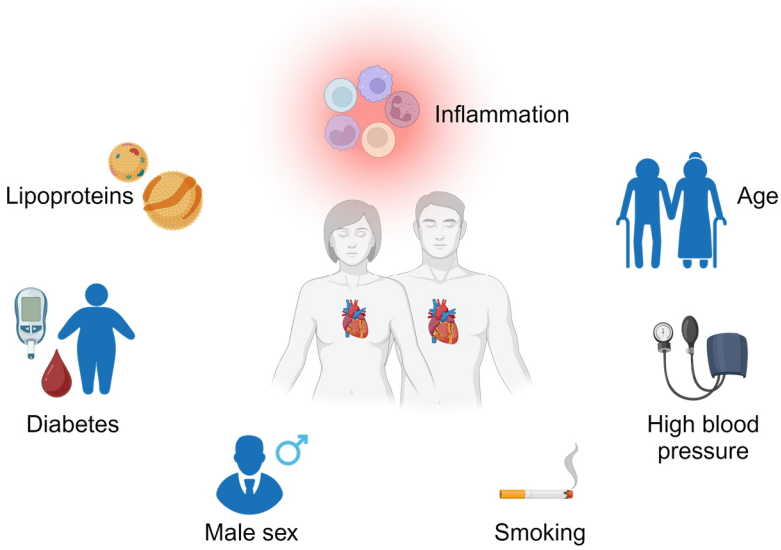


Figure 2: Risk factors for CVD and ischemic heart disease

Aging may be the main risk factor for developing CVD. The increased risk of CVD with age is clear, even though an increasing number of younger individuals are also affected by CVD (Libby, 2021). Age leads to general deterioration of many physiological functions that play roles in the risk of developing CVD. This includes the decreased elasticity of arteries, making them stiffer, which in turn leads to increased blood pressure (The Reference Values for Arterial Stiffness' Collaboration, 2010). Aging also leads to increased dysfunction of the immune system, with changes occurring in all parts of the immune system, including the cells themselves, the lymphoid organs and soluble mediators (Nikolich-Žugich,

2018). Changes in immune cell numbers have been seen in aged mice, including a skewing towards more myeloid cells at the expense of T and B cells, but also expansion of specific subpopulations (Smit et al., 2023). In *Paper III* we delve deeper into this topic when we investigate age-associated B cells.

Male sex is also a risk factor for CVD (Pencina et al., 2009), though it must also be recognised that women are not in any way unaffected by CVD. In the *European Society of Cardiology* member countries (which includes Europe, and some Middle-Eastern, Asian and North African countries) there were 2 million new cases of ischemic heart disease in men and 1.6 million in women in 2017 (Timmis et al., 2020). Men and women also differ in some of the other CVD risk factors, such as smoking and alcohol consumption, though it is possible that the differences in these factors can be attributed to gender, rather than biological sex (Timmis et al., 2020). More considerations in regards to biological sex in research and differences between men and women are discussed in the *Ethical Considerations*.

Lipoproteins are important risk factors for CVD. They can be divided based on size and density into chylomicrons, very low-density lipoprotein, intermediate density lipoprotein, low-density lipoprotein (LDL) and high-density lipoprotein (HDL). These particles carry lipids, including cholesterol, in the circulation (Bhargava et al., 2022). In observational studies LDL and HDL levels show opposite associations to CVD events. High LDL levels are positively associated with CVD incidence and high HDL levels are negatively associated with CVD incidence (Voight et al., 2012, Pencina et al., 2009). However, in a Mendelian randomisation study of 20 cohorts, no causal role for HDL in myocardial infarction was found, while the same study identified a strong causal association for LDL (Voight et al., 2012), in agreement with other evidence (Ference et al., 2017).

Lipoprotein (a), Lp(a), is in part structurally similar to LDL, but also has an apolipoprotein (a) domain, which shares homology with plasminogen (Kamstrup, 2021). Plasma levels of Lp(a) have a strong genetic component and are mostly insensitive to lipid-lowering therapy, leading the *European Atherosclerosis Society* to recommend analysis of Lp(a) in adults, at least once during an individual's lifetime (Kronenberg et al., 2022).

High blood pressure, especially high systolic blood pressure shows a strong association to CVD, while simultaneously being very common, which makes lowering blood pressure an important treatment target (Fuchs and Whelton, 2020). In a meta study including over 600 000 individuals the risks of coronary heart disease and heart failure were decreased by 17 and 28% per 10 mm Hg decrease in blood pressure, respectively, and also demonstrated no lower limit of effectiveness (Ettehad et al., 2016).

Smoking is associated with an earlier onset of CVD by 5.1 years in men and 3.8 years in women, and for smokers the primary CVD event is also more often fatal (Khan et al., 2021). Recently there has been an increase in the use of electronic

cigarettes and vapes. Though these are marketed as being safe, or at least safer than cigarettes, adverse effects have been seen on endothelial function, including lower nitric oxide (NO) secretion, similar to the effect of cigarettes (Mohammadi et al., 2022).

While great strides have been made in terms of managing some of the above risk factors, with the curves for both smoking, LDL and blood pressure trending downward, there have been two- to three-fold increases in **obesity** and **diabetes** in *European Society of Cardiology* member countries over the last 30 years (Timmis et al., 2020). This trend is not only present in high income countries, but increasing even more in middle income countries, although from lower levels (Timmis et al., 2020). The International Diabetes Federation is estimating that 783 million individuals (12.2% world-wide) will have diabetes in 2045 (Sun et al., 2022). Breaking this increase in diabetes is a paramount step in preventing a corresponding increase in CVD incidence, as diabetes patients have 30% increased risk of CVD events, and only 6% of diabetes patients have optimal risk factor control (Wright et al., 2020). However, studies have shown different results as to what residual risk for CVD that patients with optimally managed diabetes and CVD risk factors have. Wright *et al*. reported that diabetes patients still have a 21% higher risk of CVD, while Rawshani *et al*. reported only minimal increased risk compared to diabetes-free individuals (Rawshani et al., 2018, Wright et al., 2020).

However, although the factors above are important risk factors for CVD and ischemic heart disease, the role of **inflammation** and the **immune system** cannot be disregarded. As we will learn in the next section, lipoproteins are necessary for the initiation of atherosclerosis development, but immune cells are also there every step of the way (Libby and Hansson, 2019). Evidence of the causal role of the immune system in CVD, including the role of IL-6 and IL-1β has been shown both by Mendelian randomisation studies and randomised controlled trials (The Interleukin-6 Receptor Mendelian Randomisation Analysis (IL6R MR) Consortium, 2012, Ridker et al., 2017). Inflammation and the immune system are the main topics of this thesis, where we have investigated both soluble and cellular components of the immune system to gain further knowledge about their roles in CVD and atherosclerosis.

The list of risk factors can be made longer, and also includes **ethnicity, physical inactivity, nutrition, alcohol consumption and genetic factors** (Virani et al., 2020).

Treatments

Current pharmacological treatment of CVD is mainly focused on lowering lipids and blood pressure, although lifestyle changes are cornerstones of preventative care. The main lipid-lowering medication are statins, which block the cholesterol synthesis in the liver (Ferri and Corsini, 2020). There are also others, including

monoclonal antibodies targeting proprotein convertase subtilisin-kexin type 9 (PCSK9) (Stewart et al., 2017). Cholesterol lowering therapies have been estimated to be the single most influential factor behind decreases in coronary heart disease mortality, with blood pressure lowering therapies being the second (Ford et al., 2007). There are numerous blood pressure lowering medications, which target multiple organs, mirroring the complex regulation of blood pressure, which includes the kidneys, heart and blood vessels (Oparil et al., 2018).

As the role of inflammation has become increasingly recognised as very important in CVD, clinical trials have investigated the effect of inhibiting various inflammatory pathways (Engelen et al., 2022, Farina et al., 2024). The most well-known is probably the Canakinumab Antiinflammatory Thrombosis Outcome Study (CANTOS), which found that blocking IL-1β with a monoclonal antibody decreased the number of recurrent CVD events, ultimately proving that increased inflammation can lead to CVD events (Ridker et al., 2017). However, targeting the immune system can be difficult due to its complexity, as seen in the increased risk of fatal infections in the CANTOS trial (Ridker et al., 2017).

Atherosclerosis

Atherosclerosis is a chronic inflammatory disorder characterised by the formation of lipid-laden and fibrous lesions within the arterial vessel wall. It is the underlying pathology of many types of CVD, including ischemic heart disease. Here follows a brief overview of the sequential development of an atherosclerotic plaque, while the roles of the individual mediators and cells of specific interest in this thesis are discussed in detail in the following sections.

A normal artery consists of several layers: facing the lumen is the intima, a normally thin layer covered by endothelial cells (Figure 3). Then the media, containing smooth muscle cells (SMCs), elastic fibres and collagen. Lastly, the outermost layer, the adventitia, that contains extracellular matrix (ECM) proteins, micro vessels that supply the artery with oxygen and nutrients, as well as nerve endings and resident cells (Libby et al., 2019).

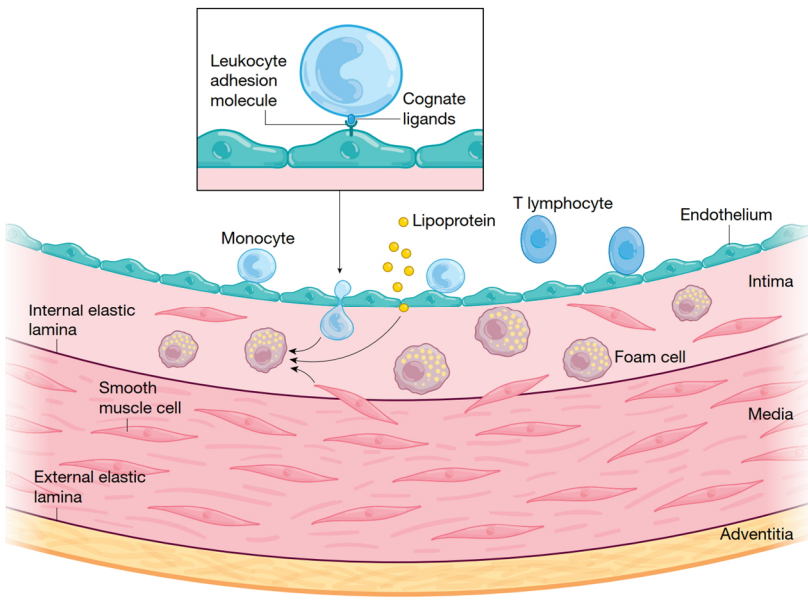


Figure 3: Initiation of atherosclerosis
Overview of an artery and cells in the early phase of atherosclerosis development (Libby, 2021) Reproduced with permission from Springer Nature.

Atherosclerotic plaques typically start forming at arterial sites with disturbed blood flow, which occurs in curves and branching points in the vascular system. Here, the forces exerted on the endothelial cells are altered, causing oscillatory shear stress (Tamargo et al., 2023). This can induce numerous changes in the endothelial cells, including the upregulation of adhesion molecules, such as ICAM-1 (Figure 3) (Nagel et al., 1994).

Endothelial dysfunction causes increased permeability of the artery and leads to the accumulation of LDL cholesterol in the intima, and formation of an atherosclerotic plaque can be initiated (Figure 3) (Mundi et al., 2018). Once inside the intima the LDL particles can be modified by oxidation. The expression of adhesion molecules on the endothelium allow for the extravasation of monocytes and other immune cells into the intima, and once there, monocytes differentiate into macrophages which phagocytose the modified LDL particles (Libby et al., 2019). In the presence of large amounts of LDL, the macrophages turn into so called foam cells, that contain large intracellular lipid droplets. SMCs that have migrated from the media can also give rise to foam cells, at least in mice, apart from their normal role in ECM protein production (Wang et al., 2019). Other immune cells are also recruited into the plaque, including neutrophils, T and B cells, though these also reside in the adventitia were they can over a longer period of time give rise to tertiary lymphoid organs that orchestrate the immune reaction (Moos et al., 2005, van Leeuwen et al., 2008).

With time the plaque grows larger and the cells within the plaque divide and continue to secrete inflammatory mediators, both pro- and anti-atherogenic, further driving the recruitment of more cells (Figure 4) (Libby, 2021). This inflammatory milieu impairs cellular functions, and macrophages that would normally clear dead cells from its surroundings by efferocytosis can no longer do so properly.

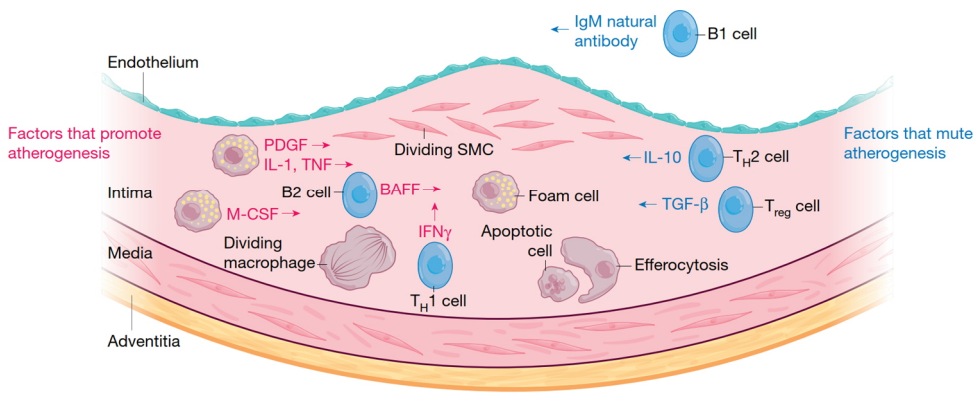


Figure 4: Progression of atherosclerosis Illustration of the processes occuring in a progressing plaque (Libby, 2021) Reproduced with permission from Springer Nature.

This leads to the accumulation of dead necrotic cells, that form the necrotic core of the plaque (Yurdagul et al., 2018). The atherosclerotic plaque may cause a clinical cardiovascular event, such as a myocardial infarction or stroke, in two ways. The classic model is the rupture of a fatty plaque, with a large necrotic core, covered by only a thin layer of collagen, the fibrous cap. If the fibrous cap ruptures, it can lead to thrombus formation and vessel occlusion (Quillard et al., 2017). Alternatively, a thrombus can also form by erosion of the plaque, which exposes the inside of the plaque, triggering coagulation (Quillard et al., 2017). The plaques that undergo erosion differ in their composition compared to the thin capped rupture prone plaques; they are more fibrous, with a thicker cap, less often lipid rich and calcified (Jia et al., 2013). Notably, women more often have plaques that cause coronary thrombosis by erosion than men (Farb et al., 1996).

Interestingly, the characteristics of carotid plaques have seemingly changed over time. In the time period 2002-2011 van Lammern *et al.* reported a shift from lipid-rich plaques with a high percentage of calcification and macrophages towards a more stable plaque phenotype. This change in plaque composition also coincided with increased prescription of statins and a concomitant decrease in plasma cholesterol levels (van Lammeren et al., 2014). This overall improvement in plaque composition over time illustrates the efficiency of the current treatment regimes. Still, many individuals suffer from a first or recurrent cardiovascular event, so it is also evident that that new approaches need to be researched (Nilsson, 2017).

Immunology in Cardiovascular Disease

A functional immune system is an essential part of a healthy life and dysfunction or disruption to it can cause or worsen all manner of diseases, from autoimmune diseases like type 1 diabetes to cancer and CVD (Figure 5). Currently, the importance of the immune system in CVD and atherosclerosis is clear, but prior to the 1980s there had not been much interest in immunology research within this field. Although it was suggested that immune cells participate in atherosclerosis already in 1856 by Rudolf Virchow, the prevailing theory about atherosclerosis development included the involvement of lipids and SMCs (Hansson and Jonasson, 2009). However, with the identification of macrophages and T cells in human plaques (Jonasson et al., 1985), the first steps were taken towards the vast array of knowledge that we now have about the role of the immune system in atherosclerosis, from the localisation of different cells, determination of their phenotypes, effector functions, and the pro- and anti-atherogenic consequences.

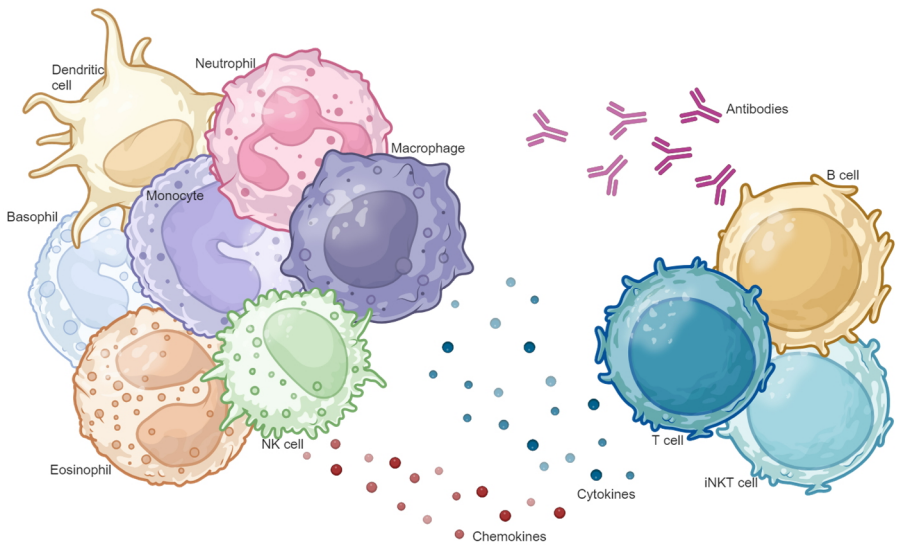


Figure 5: Selection of cells and soluble mediators of the innate and adaptive immne systems

Innate Immunity

The innate branch of the immune system are the first responders against invading pathogens. The innate immune cells (Figure 6) can recognise conserved structures expressed by pathogens; pathogen associated molecular patterns (PAMPs), but also endogenous structures; danger associated molecular patterns (DAMPs) with their pattern recognition receptors (Carpenter and O'Neill, 2024). Recognition of these structures causes activation of the cell, leading to an inflammatory response and activation of the adaptive branch of the immune system. Even though activation of the innate immune system does not create an immunological memory in the same sense as the adaptive immune system, innate cells exhibit a type of memory termed trained immunity. After an initial exposure to a PAMP or DAMP, a later re-exposure will induce a stronger response, for example in cytokine production (Bekkering et al., 2016). This is mediated by epigenetic modifications and is also associated with immunometabolic changes (Riksen and Netea, 2021, van der Heijden et al., 2017).

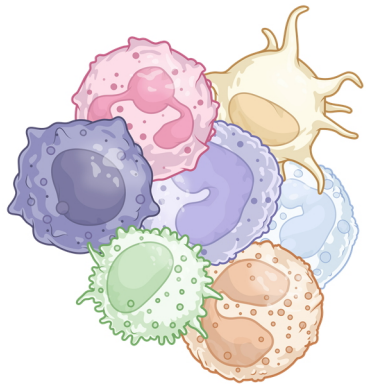


Figure 6: Innate immune cells

Macrophage, neutrophil, NK cell, monocyte, dendritic cell, eosinophil, basophil.

Macrophages are present in atherosclerotic plaques from the very start of the development of fatty streaks and are an integral part of plaque progression, beyond being foam cells (Hou et al., 2023). Macrophages are highly plastic phagocytic cells and are able to adopt a wide range of effector functions, with the classical division of inflammatory M1 and reparatory M2 macrophages thought to be the extremes (Hou et al., 2023).

Neutrophils are also present in plaques, especially in the shoulder regions (Rotzius et al., 2010). Neutrophils have been described as a "double-edged sword", because they can, like macrophages, promote both pro-inflammatory processes but also repair oriented processes (Sreejit et al., 2022). In the early stage of atherosclerosis neutrophils contribute to immune cell recruitment by inducing endothelial dysfunction (Sreejit et al., 2022). On the other hand neutrophil depletion can impair cardiac healing after MI, due to impaired macrophage efferocytosis (Horckmans et al., 2017). Additionally, neutrophils can contribute to SMC death and plaque instability by neutrophil extracellular trap release, NETosis, the process in which neutrophils release their nuclear content, creating a potent tissue damaging reaction (Silvestre-Roig et al., 2019).

Adaptive Immunity

B cells and **T cells,** the immune cells that comprise the adaptive immune system, both possess receptors that recognise specific antigens with high precision, which allows them to initiate immune responses to almost all imaginable structures and create a life-long immunological memory (Figure 7) (Lam et al., 2024). The receptors, the B cell receptor (BCR) and T cell receptor (TCR) are created by gene rearrangement of variable (V), diversity (D) and joining (J) segments, which leads to the creation of BCRs and TCRs with a wide range of specificities (Flajnik and Kasahara, 2010). B cells can also undergo somatic hypermutation which leads to further modifications of the BCR, and a chance for even higher affinity (Flajnik and Kasahara, 2010). The adaptive immune response is slower than that of the innate immune cells, but can in turn create an immunological memory, which makes the response upon re-exposure to the same antigen quicker and stronger (Lam et al., 2024).

Figure 7: Adaptive immune cells
A T cell, B cell and an iNKT cell

B cells

B cells are the precursors of plasma cells that secrete antibodies, the soluble version of the BCR, which makes up one part of the humoral immune response. The BCR can have different immunoglobulin (Ig) subtypes, IgM, IgD, IgG, IgE and IgA, which imparts different functions on the antibodies when secreted from plasma cells (Lam et al., 2024). After activation the B cell can switch the Ig type of the BCR, according to cytokine signals present in the environment (Stavnezer and Schrader, 2014). B cells also act as professional antigen presenting cells and support the activation of T cells via antigen presentation, co-stimulation and cytokine secretion (Romero-Ramírez et al., 2019).

The net-effect of B cells on atherosclerosis development in mice is protective, as transfer of B cells into splenectomised mice reduces atherosclerosis development (Caligiuri et al., 2002). Further research into B cell subsets revealed that B2 cells, which comprises the majority of B cells, are pro-atherogenic (Kyaw et al., 2010), while the B1a and regulatory B cells are anti-atherogenic, via mechanisms involving IgM and IL-10 secretion, respectively (Kyaw et al., 2011, Strom et al., 2015). In human CVD and atherosclerosis B cells have been identified in the adventitia surrounding the vessel, together with plasma cells (van Dijk et al., 2015). In this paper van Djik *et al.*, identified the B cells and plasma cells in the more advanced plaques, including those that had ruptured, but, interestingly, not in the less advanced plaques, nor in healed plaques (van Dijk et al., 2015). Analysis of

circulating cells has shown that high numbers of memory B cells had an inverse, protective association to recurrent cardiovascular events in individuals following carotid endarterectomy (Meeuwsen et al., 2017).

Age-associated B cells

As indicated by the title of this thesis a part of my work, presented in *Papers III and IV*, has been focused on atypical immune cells. The first of these atypical cells are **age-associated B cells (ABCs)** which we investigate along with other B cell populations in *Paper III*. As indicated by the name these cells are associated with aging and increase in number with increasing age, but are also increased in autoimmunity (de Mol et al., 2021). In mice, ABCs accumulate progressively with increasing age, and make up between 10-30% of mature B cells in the spleen of 22 month old female mice (Hao et al., 2011). ABCs are a heterogeneous group of cells and have been defined by a range of surface markers and transcription factors by different investigators. The main markers used to define ABCs are $CD21^{low/-}$, $CD11c^{+}$, $CD11b^{+}$, $CD23^{-}$, in combination with expression of the transcription factor T-bet (Figure 8) (Rubtsova et al., 2013, Hao et al., 2011). CD21 is a complement receptor that can act as a co-receptor for the BCR, and reduce the activation threshold of the B cells, especially at low antigen concentration, and can then be cleaved off after activation of the B cell, indicating that $CD21^{low/-}$ B cells have been previously activated (Masilamani et al., 2003, Mongini et al., 1997). Additionally, the transcription factor ZEB2 was recently identified as a key factor driving generation of ABCs in mice, while humans with a haploinsufficiency for *ZEB2* had fewer ABCs defined as $CD11c^{+}$T-bet^{+} (Dai et al., 2024).

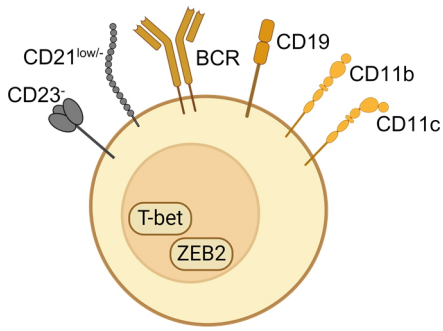


Figure 8: ABC with some commonly used defining surface markers and transcription factors. In grey: down-regulated markers. In yellow: expressed factors

Expansion of similar B cell populations have been described in elderly humans, including $CD11c^{+}$ cells and $IgD^{-}CD27^{-}$ double negative (DN) B cells (Colonna-Romano et al., 2009, Pattarabanjird et al., 2024). $IgD^{-}CD27^{-}$ DN B cells are also increased in autoimmunity and may stem from defective germinal centre reactions (Li et al., 2021). Some $IgD^{-}CD27^{-}$ DN B cells, termed the DN2 subpopulation, are also $CD21^{-}$, indicating that the $IgD^{-}CD27^{-}$ DN B cells overlap with the ABCs (Sanz et al., 2019). So, while there are similarities between human and murine ABCs there are still more potential differences.

Additionally, numerous studies have investigated ABCs (often defined as T-bet^{+} or $CD21^{low}$) in human autoimmunity and chronic infection and found them to be

expanded in these contexts (Doi et al., 2014, Freudenhammer et al., 2020, Marrapodi et al., 2020, Knox et al., 2017). Additionally, the heterogenous nature of the ABCs has also been shown in human cells, for example Freudenhammer *et al.* classified ABCs as naïve, IgM and IgG memory and $CD27^-$IgG (atypical) memory. All four subpopulations had a weaker response to BCR stimulation compared to $CD21^+$ B cells, which has also been reported by Doi *et al*, though only in the $CD21^-CD27^-$ subpopulation (Doi et al., 2014, Freudenhammer et al., 2020). Additionally, expression of the co-stimulatory molecule CD86 was higher in all $CD21^{low}$ populations except the IgM memory subpopulation, though CD69 levels were similar to $CD21^+$ B cells (Freudenhammer et al., 2020).

Conversely, others have investigated the importance of innate stimulus for ABC development. BCR and TLR7 (which binds viral RNA) activation together induces the expression of T-bet, CD11b and CD11c (Rubtsova et al., 2013), while a gain of function mutation in the *TLR7* gene leads to accumulation of $CD11c^+$ B cells and is enough to induce development of systemic lupus erythematosus (SLE) (Brown et al., 2022). In a mouse model of SLE, a B cell specific deletion of T-bet improved kidney pathology and reduced mortality (Rubtsova et al., 2017).

Several studies have shown ABC differentiation and antibody secretion after activation, and Brown *et al.* found that the antibodies had SLE specific DNA and RNA specificity, while Glauzy *et al.* identified clonally expanded $CD21^{low}$ B cells and disease specific antibodies in individuals with Sjögren's syndrome (Glauzy et al., 2018, Rubtsova et al., 2013, Brown et al., 2022). Taken together, these data indicate that the ABCs are able to drive antigen specific autoimmunity. However, influenza vaccination also leads to expansion of antigen specific $CD21^{low}CD27^+$ that express T-bet, illustrating the role of ABCs in a homeostatic, non-chronic response, (Lau et al., 2017). However, this data also raises the question of whether the ABCs that we see driving autoimmunity are the same B cells as the those seen in vaccine responses, or whether we are limited the by the markers used to define ABCs and other B cell populations.

ABCs have been identified in both murine and human atherosclerotic plaques (Smit et al., 2023). ABCs were identified in aortic arches from aged $Ldlr^{-/-}$ mice by single cell RNA sequencing and were characterised by expression of *Itgam* (CD11b), *Itgax* (CD11c), *Tbx21* (T-bet), as well as low expression of *Cr2* (CD21). ABCs also expressed higher levels of H2-Ab1 (MHCII) and co-stimulatory molecules such as *Cd80* than other B cell populations, indicating a role for ABCs as antigen presenting cells in the aorta (Smit et al., 2023). This was also investigated *in vitro,* where ABCs were found to induce stronger activation of T cells compared to follicular B cells, measured by CD69 expression and proliferation of the T cells (Smit et al., 2023). Lastly, $CD11c^+$T-bet^+ ABCs were detected in human carotid plaques by flow cytometry (Smit et al., 2023). In another recent publication Pattarabanjird et al. investigated $CD11c^+$ B cells in individuals with coronary artery disease (CAD), and identified that individuals with severe CAD had more circulating ABCs, here

defined as $CD11c^{+}CD27^{low}IgD^{-}CXCR5^{-}$, as well as DN2 B cells, $CD11c^{+}CD27^{-}IgD^{-}CXCR5^{-}$, compared to those with low CAD severity (Pattarabanjird et al., 2024).

So, while we now know that ABCs are present in atherosclerotic plaques, it still remains unknown if they have a direct effect on atherosclerosis development, and if they share other properties, such as antibody production, with ABCs in autoimmune disorders. Additionally, it is also not known if an increase in circulating ABCs precedes the onset of CVD in humans.

T cells

T cells recognise peptide antigens presented to them on MHC class I and II molecules, and their activation leads to a wide range of effector functions, including cytokine release and cytotoxic functions (Sun et al., 2023). There are many subpopulations of T cells. They can most simply be divided based on their expression of CD4 and CD8 into T helper (Th) cells and cytotoxic T cells. The Th cells can be further divided in Th1, Th2, Th17 and regulatory T cells (Sun et al., 2023). T cells are present both within atherosclerotic plaques, and in the surrounding adventitia (Saigusa et al., 2020). Different effects on atherosclerosis development have been identified, including the pro-atherogenic effect of Th1 cells and the anti-atherogenic effect of regulatory T cells (Ait-Oufella et al., 2006, Buono et al., 2005).

However, the overall effect of broad T cell activation is detrimental in CVD, as indicated by the three-fold increased risk of CVD events in individuals treated with immune checkpoint inhibitors for cancer (Drobni et al., 2020).

Invariant Natural Killer T cells

Invariant natural killer T cells (iNKT cells) are the second atypical immune cell that I have focused on during my PhD studies, resulting in *Paper IV*. iNKT cells develop in the thymus like other T cells, but also express typical NK cell markers, and have NK cell effector functions, thereby combing traits of adaptive and innate immunity(Crosby and Kronenberg, 2018). Upon activation via the TCR, cytokines, or NK cell receptors like NKG2D, iNKT cells will secrete large amounts of cytokines and cytolytic mediators (Figure 9)(Coquet et al., 2008, Kuylenstierna et al., 2011).

iNKT cells, also known as type 1 NKT cells express a semi-invariant TCR, which recognises lipid antigens presented to them on a CD1d molecule, which has structural similarity to MHC class I (Brennan et al., 2013). CD1d however lacks the polymorphic properties of the MHC class I and II molecules, and the inside of the antigen binding groove contains hydrophobic amino acid residues, suitable for lipid interactions (Van Kaer et al., 2016). CD1d is expressed on antigen presenting cells, but also on hepatocytes, intestinal epithelial cells and adipocytes (Van Kaer et al., 2016).

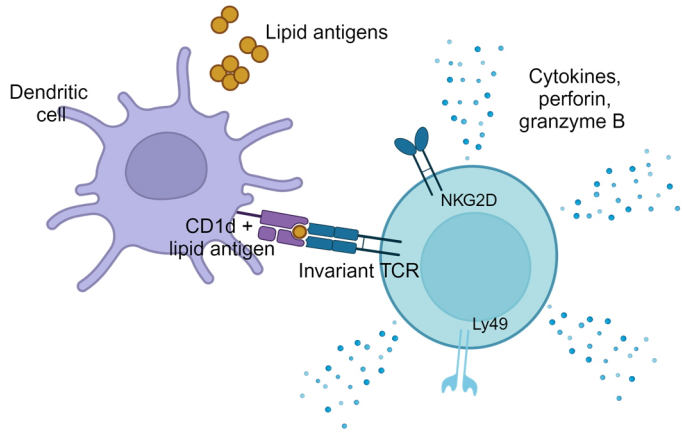


Figure 9: Prototypical activation of an iNKT cell

A dentritic cell presents a lipid antigen on CD1d to an iNKT cell which binds with its TCR, resulting in the release of numerous mediators.

The semi-invariant TCR consists of the Vα14 and Jα18 combined with Vβ2, Vβ7, Vβ8.1, Vβ8.2 or Vβ8.3 in mice, while the equivalent TCR in humans is a combination of Vα24, Jα18 and Vβ11 (Brennan et al., 2013). iNKT cells were first identified in the 1990s and their ability to recognise lipid antigens make them highly interesting to investigate in a lipid-driven disease like atherosclerosis (Lantz and Bendelac, 1994, Dellabona et al., 1994).

Several ligands have been suggested for iNKT cells, including endogenous lipids β-glucosylceramide (βGlcCer), plasmalogen lysophosphatidylethanolamine (plasmalogen lysoPE) and isoglobotrihexosylceramide (iGb3), as well as microbial α-glucosyldiacylglycerol (αGlcDAG), expressed by *Streptococcus pneumoniae* (Brennan et al., 2013). However, the prototypical antigen, used to activate iNKT cells *in vitro* as well as for detection *in vivo* with tetramers, is α-galactosylceramide (αGalCer), expressed by the marine sponge *Agelas mauritianus,* or by its colonising bacteria (Brennan et al., 2013).

Studies of iNKT cells in murine atherosclerosis have mainly shown a pro-atherogenic effect, both in studies of Vα14 transgenic and CD1d knock out mice, as well as in studies where iNKT cells were activated by exogenous stimulation with αGalCer (Nakai et al., 2004, Tupin et al., 2004, Subramanian et al., 2013, Major et al., 2004). Other studies have identified time dependent effects, where plaque size differed early in atherosclerosis progression only, and also found different results between iNKT mouse models (VanderLaan et al., 2019, Aslanian et al., 2005) Additionally, atheroprotective effects of iNKT cells have been reported (van Puijvelde et al., 2009). One reason for these different results can be partly traced to

the atherosclerosis model used - *Ldlr*$^{-/-}$ or *Apoe*$^{-/-}$ mice. ApoE is needed for lipid antigen presentation, as it facilitates the uptake of the lipid antigens and delivers them to endosomes containing CD1d (Elzen et al., 2005). Indeed, van Puijvelde identified no difference in plaque size in *Apoe*$^{-/-}$ mice after αGalCer treatment, whereas *Ldlr*$^{-/-}$ mice had smaller plaques (van Puijvelde et al., 2009).

Another important consideration is the subpopulations of iNKT cells, which have been described in several ways, including similar to that of Th cells (NKT1, NKT2, NKT17, NKTfh and NKT10, or regulatory iNKT cells), which express large amount of the corresponding cytokines upon TCR activation. Although, the comparison to Th cells is lacking, as many iNKT cells to not express CD4 (Engel et al., 2016, Lee et al., 2002, Monteiro et al., 2010, Watarai et al., 2012). Other researchers have also based the subset separation on gene expression in single cell RNA sequencing studies, or based of CD4 (and sometimes CD8), (Lee et al., 2002, Wang et al., 2022, Zhou et al., 2020). Proportion-wise, about 50% of human iNKT cells are CD4^{+}, and 50% are CD4^{-}CD8^{-} DN, and only few are CD8^{+} (Lee et al., 2002) Expression of NK cell markers also vary, including those of the Ly49 family of regulatory surface proteins (Sköld and Cardell, 2000).

A few murine atherosclerosis studies have taken this heterogeneity into account, and identified that CD4^{+}, but not CD4^{-}CD8^{-} DN iNKT cells are pro-atherogenic in an *Apoe*$^{-/-}$ mouse model (To et al., 2009). A follow-up study revealed that the increased plaque size was mediated via a granzyme B and perforin dependent mechanism (Li et al., 2015).

Human data of iNKT cells in atherosclerosis is rather scarce. Kyriakakis *et al*. identified iNKT cells in human atherosclerotic plaques, together with CD1d expressing cells. Isolated iNKT cells from plaques were mostly CD4^{+}, and produced large amount of IFN-γ, TNFα, IL-4 and GM-CSF upon re-stimulation after *in vitro* expansion. Additionally, circulating levels of iNKT cells were lower in the individuals with previous symptomatic cardiovascular events (Kyriakakis et al., 2010). The numbers of CD1d^{+} cells/mm^{2} were higher in more advanced plaques, as classified by the American Heart Association classification system, but there was no difference between individuals with or without symptomatic cardiovascular events (Kyriakakis et al., 2010). In individuals with SLE, that are at increased risk of CVD, iNKT cells from individuals with asymptomatic CVD were more activated and induced an anti-inflammatory M2 response in macrophages. This was not seen in those with symptomatic disease, indicating a protective role for iNKT cells in early atherogenesis, that is lost as the disease progresses (Smith et al., 2016).

However, we still do not know much about iNKT cells and their subpopulations in human CVD, especially functionally, and we also do not know whether iNKT cells mediate CVD risk.

Soluble Mediators

In order for immune cells to know what to do and where to go, they need to receive signals from their environment to instruct them. These mediators, including cytokines and chemokines allow for efficient communication, not only between immune cells, but also with other cell types, including SMCs and endothelial cells (Hughes and Nibbs, 2018).

Cytokines

Cytokines regulate communication between cells, and in so doing participate or have an have an effect on many biological processes. Cytokines can be classified into several groups, for example based on their effects, into chemotactic, (chemokines, see below) or into pro- and anti-inflammatory cytokines (Dinarello, 2007). Cytokines also affect polarisation of immune cells, such as IL-4 and IL-13 directing the development of M2 macrophages, Th2 cells and BCR class switching into IgE, leading to a coordinated activation against helminths (Heeb et al., 2020).

Chemokines

Chemokines, a subgroup of cytokines, guide the migration of immune cells in a specific manner, determined by the chemokine and the chemokine receptor expressed by the immune cells. Chemokines and are essential in homeostasis as well as in inflammation (Hughes and Nibbs, 2018). Chemokines can be classified based on their N-terminal amino acid sequence into the CC, CXC, C3XC and XC motif families, where C is cysteine and X is any other amino acid (Döring et al., 2024). Chemokine receptors include a family of G protein-coupled receptors (GPCRs) as well as a group of atypical chemokine receptors (AKCRs). Often the receptors bind more than one chemokine, creating a complex network (Döring et al., 2024).

My work has been focused on the two chemokines **CCL21** and **CCL19,** (Figure 10), which is presented in *Paper I.* Both chemokines bind to the receptor CCR7, but also AKCR4 (Bastow et al., 2021, Friess et al., 2022, Yan et al., 2019). CCL21 and CCL19 are expressed within the T cell zone of lymph nodes and in high endothelial venules, guiding the chemotaxis of B cells, T cells and dendritic cells into lymph nodes (Luther et al., 2000b, Willimann et al., 1998, Jørgensen et al., 2018). Because of their roles in guiding dendritic cells and T cell interactions in lymph nodes, they are

Figure 10: Protein structures of CCL21 and CCL19 Structures were retrieved from the Protein Data Bank (www.rcsb.org)

often called homeostatic chemokines. They also share a similar structure, though CCL21 has a C terminal tail peptide, which allows it to interact with glycosaminoglycan (GAG) chains (Hromas et al., 1997). GAG binding lets CCL21 act as an immobilised chemotactic (haptotactic) signal for dendritic cells, that can cleave off the tail section from CCL21. This creates a soluble gradient and enables recruitment of more cells (Schumann et al., 2010). This "tailless" CCL21 is more similar to CCL19, which is a 10-100 times more potent activator of chemotaxis than full length CCL21 (Ricart et al., 2011). Accordingly, "tailless" CCL21 is also a more potent chemotaxis activator than full length CCL21 (Moussouras et al., 2020). CCL19 induces internalisation of CCR7 upon binding, as a regulatory mechanism, preventing further activation, at least short term (Bardi et al., 2001). Intracellular signalling pathways are also activated differently by CCL21 and CCL19. In human dendritic cells, THP-1 macrophages and aortic SMCs CCL21 provided stronger activation of ERK1/2 and CCL19 created a stronger AKT activation (Hjortø et al., 2016, Xuan et al., 2015, Halvorsen et al., 2014). Taken together, despite their similarities, CCL21 and CCL19 exert different effects on intracellular signalling and chemotaxis of immune cells. In THP-1 macrophages CCL21, but not CCL19 stimulation, lead to increased binding of acetylated LDL and expression of the LOX-1 receptor for oxidised LDL (Halvorsen et al., 2014). Conversely, CCL19 increased proliferation of SMCs and the expression and activity of MMP1 (Halvorsen et al., 2014).

In murine atherosclerosis deletion of the CCL21 and CCL19 binding receptor *Ccr7* have shown both pro- and anti-atherogenic effects. Luchtefeld *et al.* reported reduced atherosclerosis, and lower plasma levels of IFN-γ. Additionally, the *Ccr7*$^{-/-}$ *Ldlr*$^{-/-}$ mice had more T cells within both the plaques and adventitia, possibly due to impaired egress, and notably fewer T cells in the mesenteric lymph nodes (Luchtefeld et al., 2010). Wan *et al.* reported increased atherosclerosis in both the aortic arch and aortic root. There were more T cells in the lesions, more CD4^{+} T cells in the whole aorta and markedly fewer in the lymph nodes (Wan et al., 2013). Additionally, medial SMCs in aged mice express both CCL21 and CXCL13, directing recruitment of immune cells to the adventitia. This drives the formation of tertiary lymphoid organs, though CXCL13 may be the essential mediator for this and not CCL21 (Gräbner et al., 2009, Luther et al., 2000a).

CCL21 and CCL19 have been detected in human carotid plaques, together with CCR7^{+} cells, which consisted mostly of T cells (Damås et al., 2007). Both chemokines are also increased in individuals with CAD, and single nucleotide polymorphisms in the *CCL21*, *CCL19* and *CCR7* genes were associated with increased risk of CAD in two Chinese populations (Cai et al., 2014). Others have also investigated the links between CCL21, CCL19 and different types of CVD to determine their suitability as biomarkers. In individuals with acute coronary syndrome (ACS) higher plasma levels of CCL21 were associated with increased risk of major adverse cardiovascular events (MACE), both in short-term (3 months)

and long-term (in median 98 months) follow-up. The authors also found that high CCL19 levels were associated with heart failure (Caidahl et al., 2019). In stroke patients, both CCL21 and CCL19 showed similar associations to increased mortality risk (Che et al., 2023). Additionally, high CCL21 levels predicted both cardiovascular and all-cause mortality in individuals with heart failure and improved the net reclassification index for both outcomes (Ueland et al., 2013). CCL21 could possibly have a direct pathological effect in heart failure, as higher levels of CCL21 have been identified in the left ventricular myocardium from patients, and levels decreased during treatment with a left ventricular assist device (Yndestad et al., 2012).

However, there is still a knowledge-gap and lack of studies investigating CCL21 and CCL19 in a prospective cohort, that is, without disease at the initiation of the study, which we attempted to rectify in *Paper I.*

Immunometabolism

Immunometabolism is a recent field in immunology research that investigates the many effects of metabolic changes and adaptations that occur in immune cells in response to environmental stimuli. The energetic demands of immune cells vary greatly in different settings and metabolic reprogramming, the shift in utilisation of different metabolic pathways, is essential in maintaining immune function (Ketelhuth et al., 2019). Apart from meeting energetic and biosynthetic demands, it is also increasingly recognised that many metabolites have immunological properties, such as the antimicrobial actions of itaconate, and the pro-inflammatory effect of succinate (Patil et al., 2019).

A resting immune cell produces ATP from glucose first by glycolysis, generating 2 ATP and 1 acetyl-CoA molecule, which then enters the tricarboxylic acid cycle (TCA cycle) inside the mitochondria, followed by the electron transport chain. These processes generate in total 36 ATP molecules per glucose molecule (Figure 11) (O'Neill et al., 2016). However, cell activation by a pro-inflammatory stimulus will induce metabolic reprogramming, including in T cells and macrophages. This generates a shift towards glycolysis for ATP production, and a

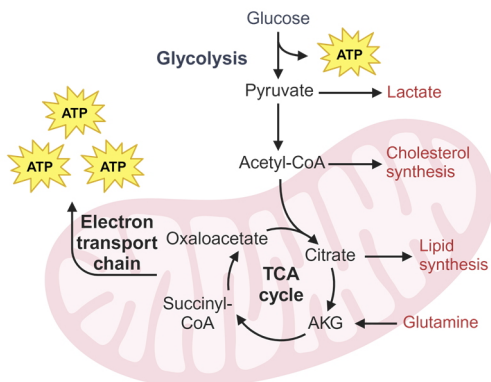


Figure 11: Overview of major metabolic pathways. Glycolysis takes place in the cytosol, and the TCA cycle and electron transport chain take place inside the mitochondria.

downregulation of the TCA cycle (Wang et al., 2011, Jha et al., 2015). This allows the immune cells to quickly, though less efficiently, produce ATP and mobilise intermediates needed for the specific effector functions of the cell, such as expansion and efferocytosis (O'Neill et al., 2016).

Metabolic reprogramming is not only a binary state between resting and activated cells but also varies between cells of different phenotypes that are performing different effector functions. An example is the utilisation of arginine in macrophages, separated into the prototypical M1 and M2 phenotypes (Ley, 2017). Both macrophage phenotypes use large amounts of arginine, M1 macrophages to produce cytotoxic NO and citrulline, while M2 macrophages produces urea and ornithine, which is needed in repair functions, such as collagen synthesis (Ley, 2017).

Atherosclerotic plaques contain numerous immune cells of different phenotypes and activation states (Figure 12). On one hand there are cells that exhibit a catabolic

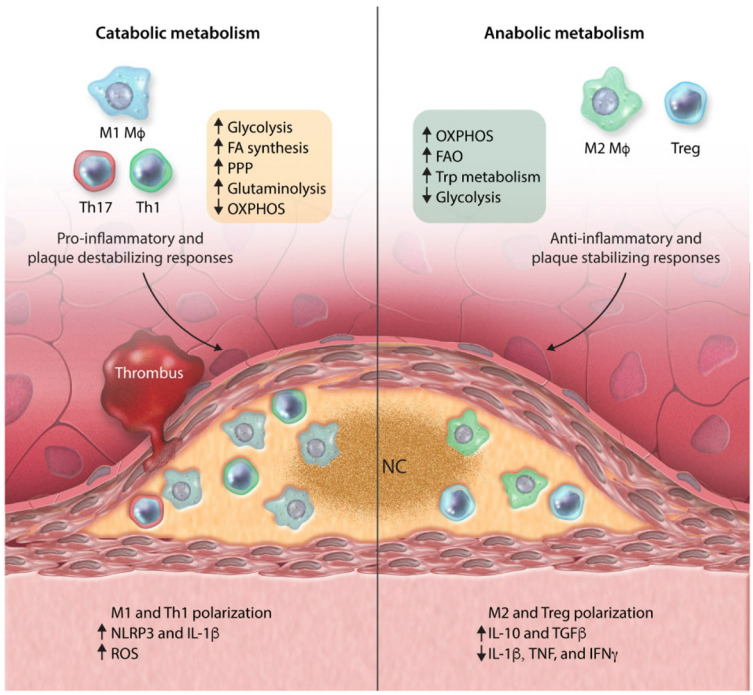


Figure 12: Atherosclerotic plaque illustrating the metabolic reprogramming in different cells

Left: M1 macrophage, Th1 and Th17 cells with a catabolic metabolism, fueling their pro-inflammatory and plaque destabilising effector functions. Right: M2 macrophage and Treg with an anabolic metabolism, supporting their anti-inflammatory and plaque stabilising effector functions. Image reprinted by permission of Oxford University Press on behalf of the European Society of Cardiology. Ketelhuth *et al.* Immunometabolism and atherosclerosis: perspectives and clinical significance: a position paper from the Working Group on Atherosclerosis and Vascular Biology of the European Society of Cardiology. Cardiovasc. Res. 2019, 115, 9, 1385-1392,10.1093/cvr/cvz166.

metabolism, characterised by increased use of glycolysis and fatty acid synthesis, which includes pro-inflammatory M1 macrophages and Th1 cells (Macintyre et al., 2014, Jha et al., 2015). On the other side there are cells with an anabolic metabolism, who utilise oxidative phosphorylation and fatty acid oxidation, including reparatory M2 macrophages and regulatory T cells (De Rosa et al., 2015, Macintyre et al., 2014, Jha et al., 2015).

Our group has previously shown that high-risk human carotid plaques display a different metabolism compared to stable plaques (Tomas et al., 2018). The high-risk plaques were from individuals with symptomatic CVD, including recent stroke or transient ischemic attack. These plaques had more often upregulation of genes involved in glycolysis, higher lactate levels, as well as different expression of numerous lipids compared to plaques from individuals without CVD symptoms (Tomas et al., 2018). These data open the possibilities of targeting the metabolic reprogramming of high-risk plaques to be able to change their metabolic profile and ultimately make the plaques less likely to cause a CVD event.

In *Paper III,* we investigate the metabolites glutamine and α-ketoglutarate (AKG) and study effects on atherosclerosis development in mice, and we also analyse immune cells in the plaques and in the circulation.

Glutamine

Glutamine is the most abundant amino acid in the circulation and an important energy source for immune cells (Figure 13) (Newsholme et al., 2023). Glutamine is an important metabolite and is involved in numerous processes, including as a nitrogen carrier, a substrate for the synthesis of the antioxidant glutathione, and as a substrate for synthesis of numerous amino acids (Newsholme et al., 2023).

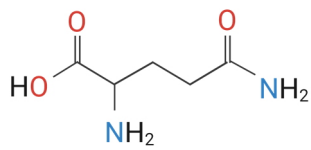


Figure 13: Chemical structure of glutamine

In addition to changes in glycolysis and lipids, our group has also identified that high-risk plaques contains less glutamine, suggesting that these plaques had a greater utilisation of glutamine metabolism (Tomas et al., 2018). Furthermore, glutamine has an important role in macrophage efferocytosis, an energy demanding process in which the macrophage engulfs and clears apoptotic cells. The process limits further inflammation and is an essential pro-resolving process (Schilperoort et al., 2023). In the absence of glutaminase 1 (GLS1), which catalyses the deamination of glutamine into glutamate, mice develop larger plaques characterised by larger necrotic cores, due to impaired efferocytosis (Merlin et al., 2021). In a recent study, Zhang *et al.* found that daily intra peritoneal injections of glutamine led to decreased development of atherosclerosis in male mice (Zhang et al., 2024). Additionally, glutamine has also been shown to increase uptake of LDL in

peritoneal macrophages (Rom et al., 2017). In clinical studies higher expression of *GLS1* was detected in stable human carotid plaques, and had a strong positive correlation to the presence of $Arg1^+$ macrophages (Merlin et al., 2021). Conversely, the glutamate ammonia ligase (GLUL), that catalyses the formation of glutamine from glutamate, the reverse reaction of GLS1, has increased expression in carotid plaques from stroke patients compared to asymptomatic individuals (Saksi et al., 2011). Taken together, these studies highlight an important role for glutamine in atherosclerotic plaques, possibly to fuel the inflammatory needs of the plaque cells.

α-ketoglutarate

α-ketoglutarate (AKG) is a TCA cycle metabolite and can be replenished into the TCA cycle via glutaminolysis, meaning the sequential deamination of glutamine, first into glutamate and then AKG (Figure 14) (Gyanwali et al., 2022). In addition to its role in the TCA cycle, AKG can also act as an antioxidant and reduce ammonia build-up (Liu et al., 2018). Interestingly, AKG is an important epigenetic regulator as co-factor in a family of histone demethylases (Tsukada et al., 2006).

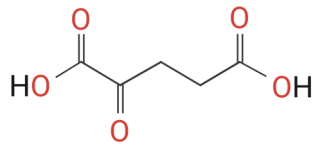


Figure 14: Chemical structure of AKG

In macrophages AKG orchestrates the polarisation to a reparatory M2 phenotype after glutamine stimulation as a co-factor to the histone demethylase Jmjd3, leading to the expression of typical M2 associated genes such as *Arg1* and *Ym1* (Liu et al., 2017). Another role of AKG that is of interest in atherosclerosis is as a co-factor for prolyl hydroxylases, that hydroxylate proline residues in collagen fibres, directly influencing and increasing the stability of the fibres (Rappu et al., 2019).

Individuals with peripheral artery disease have lower plasma levels of AKG compared to controls, while aged mice fed Ca-AKG had improved arterial elasticity (Niemiec et al., 2011). Additionally, mice fed Na-AKG had increased activity of glutathione peroxidase, but decreased activity of superoxide dismutase, and interestingly Ca-AKG did not have the same effect (Niemiec et al., 2011). Both these enzymes catalyse reactions that remove hydrogen peroxide and its tissue damaging oxidative effects. Both arterial stiffness and oxidative stress increases with age, and studies in *Drosophila* and mice have shown that AKG supplementation increases lifespan (Asadi Shahmirzadi et al., 2020, Su et al., 2019). Notably, AKG supplementation decreased inflammation, indicated by a summary of 24 inflammatory mediators, but only in female mice. Additionally, IL-10 production was increased in T cells from female mice treated with AKG *in vitro* (Asadi Shahmirzadi et al., 2020).

To summarise, while there is some evidence that glutamine may lead to decreased atherosclerosis development, the effect of AKG is not known, as are any potential effects on plaque composition and immune cell infiltration.

Aims of the Thesis

Overarching Aim

Investigate mediators, metabolites and atypical immune cells and how they affect atherosclerosis and risk of coronary events, using both mouse models and clinical cohorts, with the long-term goal to prevent or improve the treatments for patients with CVD.

Specific Aims

Paper I

- Investigate associations between the chemokines CCL21 or CCL19 and incident coronary events in the general population-based Malmö Diet and Cancer cohort.

Paper II

- Investigate the effect of drinking water supplementation with α-ketoglutarate or glutamine on atherosclerosis development and plaque composition.

Paper III

- Investigate the clonality and differentiation potential of $CD21^{low}$ age-associated B cells (ABCs).
- Investigate the role of $CD21^{low}$ ABCs in murine atherosclerosis.
- Investigate associations between $CD21^{low}$ ABCs and incident coronary events in humans.

Paper IV

- Investigate if, as suggested by animal studies, invariant natural killer T (iNKT) cells have an association to incident coronary events in humans.

Ethical Considerations

Mouse Studies

Mice are the main animal model used to study atherosclerosis (Wu et al., 2019). Since mice used in medical research are housed in a highly controlled environment, we can adjust most factors, from the food and drink to the specifics of the housing environment to our preferences. The inbred mice strains also lack genetic diversity, which further reduces the variability of study results.

The three Rs; replace, reduce, refine, are core features of the ethical considerations when performing animal experiments since they were introduced in 1959 (Russell et al., 1959) and are included in the process of applying for an ethical permit. Fully replacing mouse models in atherosclerosis studies with *in vitro* experiments is difficult, as the study of the disease requires the complex interaction between many cell types and matrix components. However, certain aspects can be studied *in vitro*, such as cell responses to modified lipids or inflammatory stimuli under different conditions, as a way to fulfil the *replace* R. Including a suitable number of animals in the study, *reduce*, can be difficult to balance. Even though you do not want to have too many animals, you also do not want to have to few and risk not being able to get enough data to draw conclusions from the study. While power calculations help to determine group numbers, it can be difficult to estimate the variation of the data and difference you expect to find (Serdar et al., 2021). You also have to be prepared that some animals may have to be excluded or euthanised during the study. One aspect of *refine* that we spent time on during one of my projects is the cage enrichment, which aims to make the animals less stressed, thus increasing the quality of the data (Musk, 2020). Different types of enrichment include gnawing sticks and houses, but also toys for the mice.

Mice and humans develop atherosclerosis preferentially in different locations in the arterial tree (Lee et al., 2017b). An often-studied site in mice is the aortic root, which is an easily replicable site to quantify atherosclerotic plaques. Humans do not display plaques in this area, a possible reason for this is the drastically different heart rates. Resting heart rate in mice is around 550 beats per minute (BPM), and around 70 BPM in humans, meaning that the blood flow in the aortic root of mice is likely more turbulent, promoting plaque development (VanderLaan et al., 2004). Another factor that is different between mice and humans in terms of atherosclerosis is that

while male sex is associated with earlier atherosclerosis development in humans, it is not in mice (Sinning et al., 2011). Instead female mice develop atherosclerosis earlier than their male counterparts (Getz and Reardon, 2024).

The controlled environment that we house the mice in is great for reducing the effects of unknown parameters on our data, but it does not really mimic the real world in terms of many factors, including microbiota. This has been suggested to be one reason behind the sometimes poor translatability between murine and human studies and clinical trials (Mak et al., 2014). In an attempt to address this, Rosshart *et al*. created the so-called "wildling mice", that have the microbiota of a wild mouse, which is a lot more diverse than that of a laboratory mouse (Rosshart et al., 2019). The authors found that the wildling mice displayed immune responses that differed from those of standard laboratory mice, but recapitulated the results from two failed clinical trials in humans (Rosshart et al., 2019).

So, while mice are an integral part of medical research, the models and methods can still be improved, for example in order to increase the translatability into human studies.

Human Studies

Studies in humans have the advantage that humans are ultimately the species in which we want to treat CVD. One ethical aspect, crucial in human research, that is not really applied in animal research, is consent. The concept of informed consent and its implementation in clinical research is outlined in the Declaration of Helsinki. This is one of the cornerstones of research ethics in humans, initially published in 1964 by the World Medical Association, and updated several times, most recently in 2013 to account for changes in healthcare and research (Goodyear et al., 2007, World Medical Association, 2013). In the declaration it is stated that informed consent must be gathered in such a way that it is not given under duress and also that the physician or researcher must make sure that the individual is able to give consent (World Medical Association, 2013). In Sweden all studies also need to be reviewed by the Swedish Ethical Review Authority, which ultimately decides whether a study is approved.

Additionally, we are also, fortunately, more limited in terms of what type of experiments and what type of biological material we can gather from humans. It is more difficult to study developing plaques in humans, as surgery is usually only performed on advanced and obstructive plaques. However, there are multiple imaging techniques that can give detailed information about plaque characteristics (Dweck et al., 2016). A more recently developed method to investigate vascular inflammation is based on imaging of the perivascular fat tissue by computerised

tomography. This non-invasive method has shown promising results in identifying high-risk, inflamed plaques (Antonopoulos et al., 2017).

Biological Sex in Research

There are also ethical considerations concerning the choice of which sex to include in studies and how sex can affect your results, interpretation and conclusions.

Male and female mice

Many atherosclerosis studies do not include both male and female mice, despite well-known differences in plaque development, lipids and immunity (Caligiuri et al., 1999, Klein and Flanagan, 2016), although many scientific journals now at least encourage authors to include both sexes. Based on 61 papers published in *Arteriosclerosis, Thrombosis, and Vascular Biology* in 2017-2018, 25 papers included both sexes, 26 males only, 9 females only and 1 did not report sex (Wu et al., 2019). In our group we have historically often used female mice in atherosclerosis studies because they develop plaques faster than the male mice, which reduces the time and resources needed for the experiment (Man et al., 2020). But in recent years we have started to consistently include both male and female mice, specifically to be able to discern sex differences, as we do in *Paper II.*

In *Paper III* we used only female mice, not necessarily to shorten the study duration but rather because the $CD21^{low}$ ABCs that we needed to isolate for the transfer experiment are quite rare, and the female mice have more of them than the male mice. So, by using female mice, we needed to use fewer animals, though we lost the insight into sex differences. Another factor is that male mice tend to be more territorial and fight more, which we experienced in the animal study in *Paper II*, where we had to euthanise some animals.

Even though male mice have smaller plaques than female mice, at least at a younger age, they appear to have plaques with more inflammation, indicated by the larger number of $CD45^+$ leukocytes in the aorta compared to female mice (Moss et al., 2019). Others have also reported more $CD4^+$ and $CD8^+$ T cells in male mice (Hernández-Vargas et al., 2006). The same study reported more MOMA-2^+ cells in female mice, although the opposite, or no difference have been reported by others (Hernández-Vargas et al., 2006, Man et al., 2020).

Men and women

In human clinical trials, women have been, and are still, underrepresented. In light of the thalidomide tragedy in the 1950's, exclusion of women in general and pregnant women in particular from clinical trials is perhaps easy to understand, though there has also been an assumption that findings from men would be the same

in women (Kim and Scialli, 2011, Thomas and Braus, 1998). However, the absence of data from women is also dangerous and can exclude women from medical advances due to lack of evidence. In 1993 the USA passed legislature requiring inclusion of women (and ethnic minorities) in clinical trials (Mastroianni et al., 1994), but women are still underrepresented relative to disease prevalence (Sosinsky et al., 2022). In phase 1-3 clinical trials in the USA between 2016-2019 trials in the cardiovascular field included 41.9% women, compared to 49% of CVD patients being women, and in the psychiatry field 42% in the trials were women, compared to 60% of the patients (Sosinsky et al., 2022). Another, world-wide study, demonstrated sex-bias, fewer women, in 7 out of 11 disease categories, including CVD, while there was only female overrepresentation in one disease category. Additionally, the authors found that female representation had not significantly increased between 1993 and 2018 (Feldman et al., 2019).

Of course, there are research studies where it can be appropriate to exclude one sex. For example, if you were to study gestational diabetes, a form of diabetes that occurs during pregnancy, having men in the control group would have confounding effects on the results, and make interpretation difficult (Cho et al., 2009).

In atherosclerosis and CVD, which prior to the 21st century were considered as diseases that primarily affect men, sex differences are gaining more and more attention (Wenger, 2023). This can be illustrated by a recent special issue in the journal *Atherosclerosis* and call to action by the *European Atherosclerosis Society,* but more work remains to elucidate differences between disease in men and women, and, importantly, the clinical application of these differences (Osto et al., 2023, Roeters van Lennep et al., 2023).

Men start to develop atherosclerosis at a younger age than women, indicated by the intima media thickness (IMT) of the carotid arteries. At age 35 men have a higher IMT than women, but at age 74 there is no longer any difference (Sinning et al., 2011). Younger women also experience fewer CVD events than men, but in the age range 60-79 women and men have similar incidence of CVD. In the 80+ category women have more CVD events than men, showing the increased CVD incidence in women after menopause (Virani et al., 2020). The protection in younger women is at least partly mediated by estrogen and the estrogen receptors, which have numerous effects, including on the vasculature, oxidative stress and fibrosis (Iorga et al., 2017). However, hormone therapy has failed to protect against non-fatal MI or other CVD outcomes in post-menopausal women, despite improvements in lipid levels (Hulley et al., 1998).

Additionally, characterisation of coronary plaques by imaging does not show a clear difference in plaque vulnerability between men and women across studies, though it seems that there is an age-associated increase in vulnerability in women, but not in men (Gurgoglione et al., 2023). By histological analysis of carotid plaques, men were found to have a larger infiltration of cells and more neovascularisation

(Wendorff et al., 2015). Others have reported increased collagen and SMC content in plaques from women, especially in asymptomatic individuals (Hellings et al., 2007).

In terms of immunity men and women differ both in the numbers of different immune cells, but also in the strength of the immune response. This confers different susceptibility to infections, but also affects vaccine responses (Giefing-Kröll et al., 2015). Vaccination generally produces a stronger reaction in women, resulting in higher antibody titres, including after vaccination against influenza and hepatitis A and B (Cook, 2008).

Sex differences can be mediated both genetically, by the X chromosome, or lack thereof, as well as by the sex hormones estrogens, progesterone and testosterone (Klein and Flanagan, 2016). Estrogen mainly has immuno-enhancing effects and directly regulates T cells, including the IFN-γ expression in Th1 cells, and TNF-α production in neutrophils and macrophages (Khan and Ansar Ahmed, 2016). Additionally, women are disproportionally affected by autoimmune diseases (Billi et al., 2019). In male mice testosterone mediates a protective effect against autoimmune encephalomyelitis, via reduction of T cell activation, providing one explanation for why men are less prone to develop this specific autoimmunity (Dunn et al., 2007)

To conclude, sex differences in CVD, atherosclerosis and immunity are very complex, and far from fully elucidated, and based on an interplay of sex, sex hormones and age-related changes in these factors across the life-time (Giefing-Kröll et al., 2015). But, despite these challenges it is unethical to exclude either sex from research and medical advances.

Key Methods

The Malmö Diet and Cancer cohort

In *Papers I, III and IV* we analysed plasma and peripheral blood mononuclear cell (PBMC) samples from the Malmö Diet and Cancer cohort (MDC; Figure 15). The MDC is a prospective population-based cohort based on individuals from the city of Malmö. The study was initiated in 1990 when all individuals born between 1926 and 1945, later expanded to 1923-1950, were invited to participate in a study with the overarching aim of investigating diet, lifestyle and their connections to the development of cancer (Berglund et al., 1993, Engström et al., 2000). Soon after the initiation of the study the investigated outcomes were expanded to also include different forms of CVD. Between 1991 and 1994 every other individual that was included in the MDC was offered to participate in a sub-study focused on CVD outcomes – MDC-CV, which came to include 6103 individuals. This sub-cohort forms the base of our cohorts in this thesis. All recruited individuals filled in an extensive questionnaire regarding their occupation, family, lifestyle and diet. They also underwent a physical examination, which included ultrasound measurement of the IMT of the right carotid artery, and left blood samples for isolation of PBMCs and plasma (Hedblad et al., 2000). The individuals were then followed over time and data on multiple end points, including different types of CVD and mortality, were retrieved through a number of Swedish registries based on International classification of diseases (ICD) codes (Engström et al., 2000).

In the papers included in this thesis we have used the MDC-CV cohort in two different ways. First, in *Paper I*, we used almost the whole cohort (N=4636) for the analysis of CCL19 and investigation of associations to several outcomes, including CVD (Figure 15). Using this design, it is possible to investigate multiple outcomes, since we have not made any pre-selection of individuals with a specific outcome.

Second, in *Papers I, III* and *IV* we created nested case-control cohorts based on the MDC-CV, in order to study associations between CCL21, B cell- and iNKT cell populations - specifically to coronary events (CE) (Figure 15). A CE was defined as a fatal or non-fatal myocardial infarction or ischemic heart disease, according to the ICD9 codes 410, 412 and 414, which also correspond to the ICD-10 codes I21, I22,

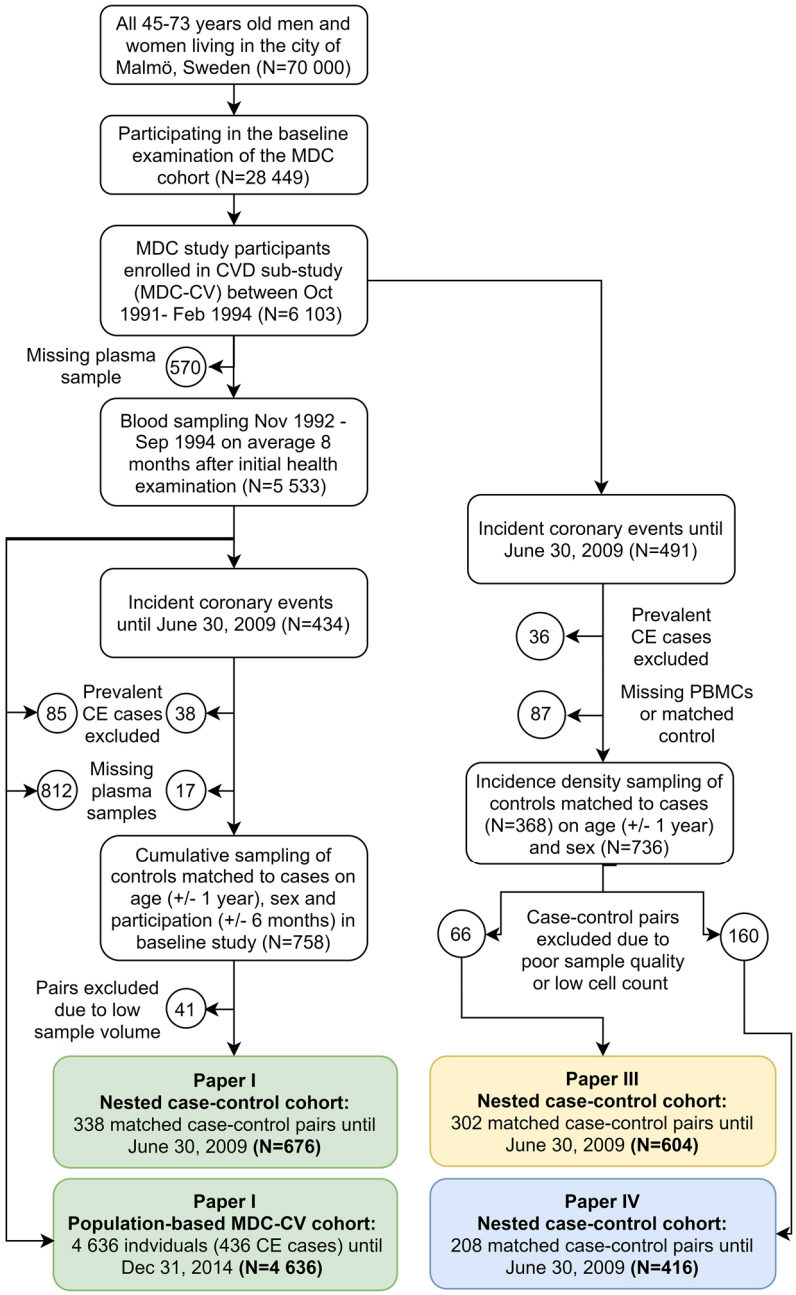


Figure 15: Study design of the Malmö Diet and Cancer study

Samples from the Malmö Diet and Cancer study (MDC) were used in *Paper I*, where plasma samples were analysed (left arm) and in *Papers III* and *IV*, where circulating immune cells were analysed (right arm).

I23 and I25. The nested case-control cohorts were created by identifying individuals who had a CE during the follow-up period. These individuals were then matched to a CE-free control based on age, sex and time of inclusion in the cohort. This design allows for analysis of a specific outcome in a smaller sample than the whole cohort, while maintaining a lot of the statistical power, saving time and resources, though it limits the outcomes we are able to study (Biesheuvel et al., 2008, Essebag et al., 2005). In *Paper I* the matching was performed by cumulative sampling, meaning that a case was matched to a control that remained CE-free throughout the entire follow-up period. In *Papers III* and *IV* the matching was performed by incidence density sampling, which matches a case to a control which at the time-point when the case had a CE, was itself CE-free. This means that an individual can be a control as well as a case and a control may be matched to several cases, as long as it remains CE-free. It has previously been shown that by using cumulative sampling the relative risk of an outcome can be overestimated, whereas when matching is done by incidence density sampling it is not overestimated (Wang et al., 2009). However, the relative risk was only overestimated when there was a significant association is the underlying cohort, not otherwise (Wang et al., 2009).

The aim of the MDC was to recruit a cohort that was representative of the general population, to be able to generalise results to the whole population, including those who did not participate. In the whole MDC cohort the participation rate was approximately 40% (Dahlin et al., 2018). An important factor to consider is the proportion of men and women and whether one sex is over- or underrepresented. In the background population of Malmö, 57.5 % were women within the age-range that was recruited to the MDC, and in the MDC-CV the proportion of women was 57.9% (Dahlin et al., 2018). So, in terms of sex, the MDC-CV cohort is very similar to its background population. Several papers have investigated some other aspects of the generalisability of the whole MDC cohort. Firstly, Manjer *et al.*, investigated how the recruitment strategy affected the characteristics of individuals that responded and consented to be included in the MDC. They compared active recruitment (a personal letter of invitation) with passive recruitment (a general community directed invitation in public places) and found that individuals who responded to passive recruitment had a lower incidence of cancer and mortality as well as more favourable lifestyle factors compared to those who responded to the active recruitment attempts (Manjer et al., 2002).

In another paper Manjer *et al*. compared cancer incidence and mortality in MDC participants (N=28 098) compared to non-participants (N=40 807). They found that the cancer incidence and mortality were higher in non-participants than participants during and after the study recruitment period (Manjer et al., 2001). Taken together, this study illustrates that the MDC cohort is not representative of the whole population of Malmö in terms of cancer incidence and mortality. This could possibly also affect the generalisability of results from investigations of other outcomes from the MDC cohort, including those from our studies. This highlights an important

factor - that findings should be reproduced in other cohorts, not only because cohorts may not be generalisable to its own population, but also that populations differ across different countries and continents.

From our studies we can only draw conclusions about associations between our exposure (like CCL21 levels) and our outcome (having a CE). We concluded that this association is independent of several CVD risk factors that could confound our findings. However, we are not able to determine if having high levels of CCL21 is actually causing individuals to have a CE. One reason for this is that there could still be other factors confounding our results. There are several alternative study designs that can be of use to overcome these unknown confounders and help in determining causality, including randomised controlled trials (RTCs), and Mendelian randomisation studies (Davies et al., 2018, Bovbjerg, 2020). RTCs are typically used for drug trials, and participants are randomised into groups where only the exposure (drug or placebo) differs between them. If the group assignment is truly random, that will account for all confounding factors, known and unknown (Bovbjerg, 2020). However, RTCs are not feasible for the type of studies that we have performed. Mendelian randomisation could, on the other hand, be of use to investigate if the mediators and cells that we have investigated have any causal role for having a CE. This type of study utilises known genetic variants that affect the exposure variable, for example a single nucleotide polymorphism that affect the plasma levels of CCL21, such as those described by Cai *et al.* (Cai et al., 2014). Your genetic variant is randomly assigned and fixed since conception and should because of this not be affected by physiological factors after conception (Sanderson et al., 2022). So, in this way the Mendelian randomisation studies can overcome the risk of confounding and help in analysis of causation. In the end though, results from Mendelian randomisations studies are not perfect, as they rely on several assumptions (Sanderson et al., 2022), so the results also have to be verified in other cohorts.

Mouse Models of Atherosclerosis

There are several different mouse models that can be used to study atherosclerosis in mice. All have been genetically modified in different ways to induce hypercholesterolemia, since wildtype mice are resistant to developing atherosclerosis due to naturally low cholesterol levels, combined with higher HDL levels (Oppi et al., 2019). In *Paper II* we used ApoE knock out mice (*Apoe*$^{-/-}$) to study atherosclerosis development. *Apoe*$^{-/-}$ mice lack the gene encoding for the ApoE protein, which is required for the clearance of chylomicrons and VLDL particles (Zhang et al., 1992). Another model, the LDL receptor knock out (*Ldlr*$^{-/-}$), used in *Paper III*, utilises the lack of the LDL receptor to induce

hypercholesterolemia, due to decreased uptake of LDL particles in the liver (Ishibashi et al., 1993). While both models are prone to hypercholesterolemia, they have some differences in their lipid profiles (Maeda, 2011). Additionally, *Apoe*$^{-/-}$ mice will become hypercholesterolemic even on normal chow diet, and even more so when fed a high cholesterol diet (Zhang et al., 1992). *Ldlr*$^{-/-}$ mice, on the other hand, have only slightly increased cholesterol levels when fed a normal chow diet, and need to be fed a high cholesterol diet in order to develop atherosclerosis in a shorter time-frame (Ishibashi et al., 1993). There are also other factors to consider, depending upon the specific design of the experiment. For example, *Apoe*$^{-/-}$ mice are less suitable for bone marrow transfer experiments, because donor cells also have to be from mice of *Apoe*$^{-/-}$ background, since transfer of ApoE expressing bone marrow cells will decrease atherosclerosis development due to macrophage ApoE expression (Fazio et al., 2002).

An alternative model, that was developed more recently, targets another regulator of lipid homeostasis; PCSK9. PCSK9 regulates cell surface expression of the LDL receptor by inducing its internalisation and degradation, thereby decreasing the uptake of LDL from circulation (Li et al., 2007). In this animal model a viral vector is used to induce expression of a mutant PCSK9, with a more than 10-fold increased affinity for the LDL receptor, resulting in hypercholesterolemia, similarly to a that of a *Ldlr*$^{-/-}$ mouse (Roche-Molina et al., 2015). A major advantage of this model is that the transgene can be introduced in a mouse of any genetic background (for example a mouse with a knock-out of a specific gene of interest) and does not require creating a double knock-out mouse, which is very time consuming. However, in order for a successful atherosclerosis experiment the mice need to have sustained hypercholesterolemia, as shown by Roche-Molina *et al.* (Roche-Molina et al., 2015). Unfortunately, we have had no luck with the model yet. In a pilot study we performed, getting sustained high cholesterol levels were more difficult than we had anticipated. At the end of the study only 9 of 25 mice had a cholesterol value of more than 80% of the cholesterol value 3 weeks after virus injection. 12 of 25 mice had values around or below only 200 mg/dl. This is similar cholesterol values to that of a wildtype mouse fed a high cholesterol diet, which is approximately 150-300 mg/dL (Nakashima et al., 1994). There could be several reasons for these low cholesterol values, including that the viral vector did not persist in the mice. Additionally, it also requires technical skill to perform the intravenous injection, which is something to consider before using this atherosclerosis model.

Plasma Analyses

In all the papers included in this thesis we performed analyses of plasma or serum. In *Papers I* and *II* we used enzyme-linked immunosorbent assay (ELISA) and in *Papers I* and *IV* we used proximity extension assay (PEA) for analysis of plasma proteins. In the ELISA antibodies are used to specifically detect the protein of interest, in the sandwich method using two separate antibodies. The primary antibody is used to coat the bottom of the plate wells, then the fluid analysed is added. After this the secondary antibody, specific for a different region of the protein is added. The secondary antibody has a conjugated enzyme (for example horseradish peroxidase), which, after addition of a substrate, catalyses the formation a coloured product. Lastly the absorbance of this product is measured photometrically, which is proportional to the concentration of the protein of interest (Sakamoto et al., 2018).

Like ELISA the PEA utilises antibodies to specifically bind the antigen of interest, but the method of quantification differs. The antibodies used for the PEA have bound DNA tags, which means that the amount of protein can be quantified by qPCR. This allows for multiplexing and analysis of numerous proteins in the same sample, but data obtained is relative, not absolute (Assarsson et al., 2014).

In *Papers II* and *III* cholesterol levels were analysed in the atherosclerotic mice with an enzymatic colorimetric method. The method consists of three steps; first cholesterol esterase cleaves cholesterol esters into cholesterol and free fatty acids. The cholesterol is then oxidised by cholesterol oxidase which also produces H_2O_2, that reacts with a dye and a peroxidase creates a coloured product whose absorbance is directly proportional to the amount of cholesterol in the sample (Allain et al., 1974, Roeschlau et al., 1974, Thermo Scientific, 2012). This method is similar to the method that is used a clinical hospital lab, where the samples from *Papers I*, *III* and *IV* were analysed (Langvad and Ekström, 2020, Nilsson-Ehle et al., 1991).

Histology and Immunohistochemistry

In *Papers II* and *III* we used histology and immunohistochemistry (IHC) to analyse structures and specific cell types in and around atherosclerotic plaques.

Before staining, the tissue must be processed and sectioned, which there are two major ways of achieving, either by snap freezing the tissue in liquid nitrogen or by embedding it in paraffin. To preserve the tissue structure it must also be fixed, paraffin embedded tissue is usually fixed before embedding with formaldehyde (or another cross-linking agent), whereas frozen tissue can be fixed after sectioning with a coagulant like acetone (Sampedro-Carrillo, 2022). The main difference between

these techniques, relevant when working with atherosclerosis, is that lipids are preserved in frozen tissue, but not in paraffin embedded tissue. In frozen tissue you can thus stain for neutral lipids with Oil red O dye to identify plaques and estimate their lipid content as in *Paper III* (Spencer, 2019). However, as we do in *Paper II*, you can still identify the lipid-laden foam cells in plaques by staining for a protein component of lipid droplets, perilipin 2. This allowed us to visualise the foam cells very clearly, both with the IHC stain but also by morphology, since the tissue structure is preserved better in paraffin embedded tissue compared to frozen tissue.

Collagen content is a parameter that is important for analysis of plaque stability. The methods used in this thesis, Sirius red with fast green and Masson Trichrome, function differently. Sirius red, which is dissolved in picric acid stains collagen red by binding with its sulphonic acid groups to basic groups in the collagen fibres (Junqueira et al., 1979). Masson Trichrome is based on dyes with different molecular sizes, which penetrate into different cells and fibres to a different extent, thereby creating a specific staining (Bancroft and Layton, 2019b). Both these methods provide good contrast between the collagen fibres and the background tissue, but, importantly, also stains the background (for example cardiac muscle cells) tissue clearly (green or red, respectively, Figure 16). This is an important consideration for the image analysis and the reason why we did not use the Van Gieson collagen stain, which stains the background a light, yellow colour (Bancroft and Layton, 2019b). For this staining, the objective in our slide-scanner often has trouble setting the focus, which makes the image blurry, requiring re-scanning and making it more difficult to analyse compared to the other two methods.

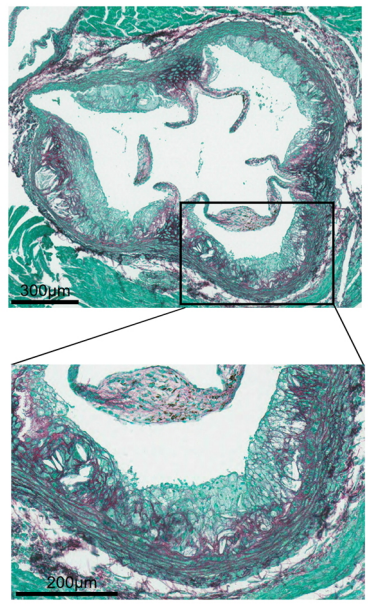


Figure 16: Sirius red with fast green staining for analysis of collagen (red).

While a simple haematoxylin and eosin staining can be used to differentiate many structures and cell types in a tissue (Bancroft and Layton, 2019a), another, more targeted method is to stain for different proteins using IHC (Figure 17). In principle IHC is a similar technique to ELISA and utilises antibodies, the primary being specific for the antigen, the secondary for the FC region of the primary antibody. The secondary antibody is conjugated to an enzyme and addition of a substrate creates a coloured product that can be used for quantification (Sanderson et al., 2019). If the enzyme used is a peroxidase it is important to quench all endogenous

peroxidase activity in the tissue, otherwise addition of the substrate can create artefacts in the stained tissue (Sanderson et al., 2019).

However, before staining the tissue has to undergo the treatment known as antigen retrieval. After fixation with formaldehyde many epitopes appear as "masked" and are undetectable by IHC. The antigen retrieval process will "unmask" the antigen before staining. Though it is not fully known how this occurs it seems that the cross-links that are formed by formaldehyde fixation obscures antigens and prevent antibodies from binding (Shi et al., 2011). There are several antigen retrieval methods, including heat-based antigen retrieval, in which the tissue is placed in a 100°C buffer solution. The time, buffer and its pH can also be varied to achieve the best results (Shi et al., 1991). For our stainings in *Paper II* we used a pH 6 citrate buffer for the perilipin 2, CD68 and CD3 stainings, and a pH 9 Tris-EDTA buffer for the Ly6G staining, but the best method has to be tested for every antibody (Sanderson et al., 2019).

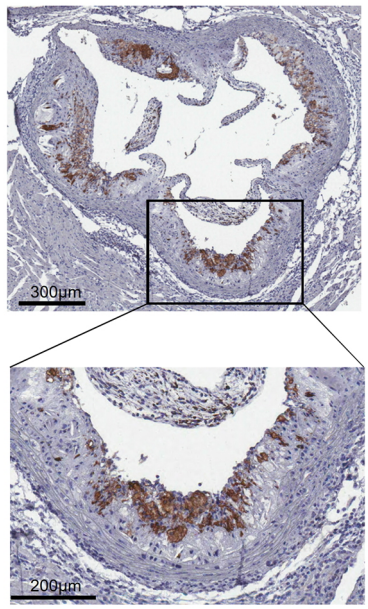


Figure 17: Immunohistochemistry for perilipin 2 for analysis of foam cells (brown).

As with any method, controls are needed when performing IHC, both to demonstrate that the antibody is binding to the correct antigen, but also not to other proteins or structures non-specifically. To account for the specificity of the antibody, a negative control is used, while a positive control shows that the antibody does recognise the antigen. Best practice for the negative control is considered to be using an isotype control, non-specific antibodies of the same isotype, species and concentration as the antibody used, as opposed to just omitting the antibody. That instead controls for unspecific binding of the secondary antibody (Hewitt et al., 2014). A positive control can for example be a different tissue that is known to contain the antigen (Hewitt et al., 2014). When we performed the CD3 stain in *Paper II* the positive control was very valuable in determining that the staining worked, since we found very few CD3 positive T cells in the aortic root. So, we also included sections of spleen, that were processed the same way. The spleen contains many T cells, so we could determine that the staining did work, we just had very few T cells in our aortic root sections.

Flow Cytometry

In all four papers included in this thesis we used flow cytometry to phenotype immune cells. Here, antibodies with conjugated fluorochromes are used to identify proteins expressed on the surface or inside a cell, while the instrument also gives information about cell characteristics. In the flow cytometer used for the majority of our experiments, we can detect 10 markers on an individual cell, which, in *Paper III*, allowed us to define 20 distinct B cell populations. This is truly the strength of the flow cytometry technique, that you can perform a complex phenotyping and quantification of individual cells in a fairly easy manner (Maecker et al., 2012). However, if you are analysing digested tissue, all of the spatial information from the tissue is lost, something which is preserved in sections analysed by IHC or immunofluorescence. But, on the other hand, those techniques are typically more limited in the number of proteins that can be analysed at once, even though there have been advances. (Goossens et al., 2022).

In *Papers III* and *IV* we analysed a large number of samples by flow cytometry, in total more than 600 individual samples, over a period of several months. So, in order to get high quality and reproducible data, we had a rigorous quality assurance programme, intended to optimise the main hardware of the flow cytometer; the lasers, (that excite the fluorochromes), the lenses, mirrors and optical filters (that transmits and focuses the signal) and the photomultiplier tubes (PMTs; that detects the fluorescence signal) (Perfetto et al., 2012). Some of the optimisation only had to be done once, like checking the filters, whereas some was performed daily. This included adjusting the gain (voltage) of the PMTs within a predefined window which was done using standardised beads. This procedure makes sure that the measurement of the signal is very precise, and has a very low day-to-day variation, and importantly, allows us to directly compare the mean fluorescence intensity (MFI) of markers from samples that were run on different days, which would not have been possible otherwise (Perfetto et al., 2012).

Before running an experiment, antibodies should be titrated to find the concentration that gives a good separation between the positive and negative populations, while also not using too much antibody (Brummelman et al., 2019). Additionally, if there is a poor separation of the cell populations regardless, a fluorescence minus one (FMO) control, can be of use make sure that placement of the gate is correct. The FMO is a sample that contains all antibodies except one. By adding all antibodies except the one you are trying to set the gate for the sample will have a similar level of spill-over fluorescence from other channels, which makes it a better control than an unstained cell sample (Roederer, 2002).

After running the cells in the flow cytometer, you gate out the cell populations of interest for analysis. This can be performed in different ways, below is an example of how we gated out B cells from human PBMCs in *Paper III* (Figure 18). First, we

gate on time to make sure that the flow through the cytometer was stable during the analysis, followed by exclusion of antibody dye aggregates. These have very high fluorescence signal, so removal of these facilitates scaling when gating in the later steps. Then cell duplets are excluded based on both size (forward scatter) and granularity (side scatter), because even though cells are supposed to run through one by one, this is not always the case. These quality assurance steps are followed by gating of lymphocytes, based on forward and side scatter, and then $CD19^+$ B cells.

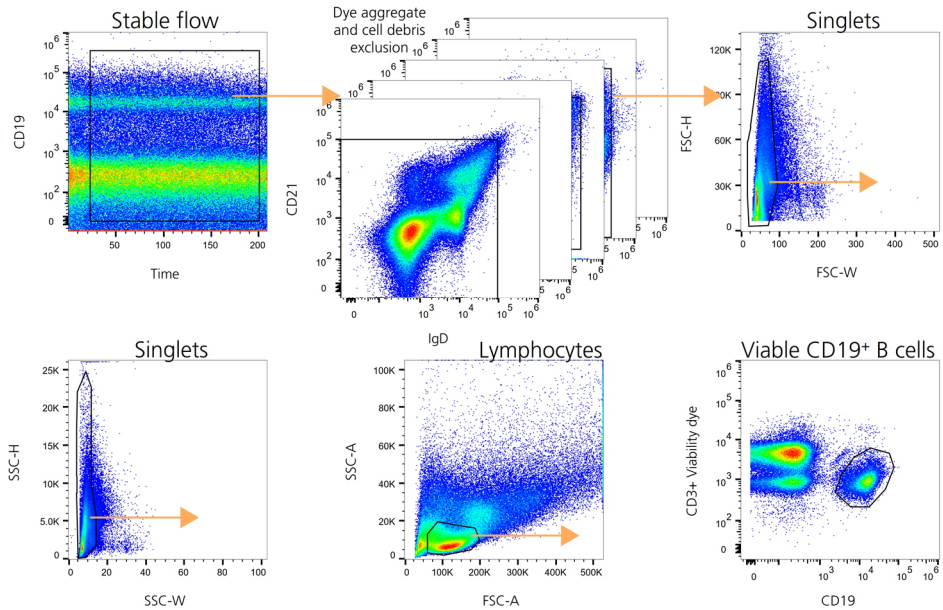


Figure 18: Flow cytometry gating strategy for circulating B cells

Statistics

All of the data generated by the methods described here would not be of as much use to us if we did not have statistical methods to analyse it with and we would not have been able not draw as many conclusions.

Often, we want to know: do the CE cases have more of this cell type, or did the treated mice develop more atherosclerosis? To answer these questions, we use a t-test or, if we have more groups, a one-way ANOVA. Both these tests compare the means between the treatment groups with the null hypothesis that there is no difference. If the result is a small *P* value (usually below 0.05) then the probability is less than 5% to observe the result that we got, if the null hypothesis is true. Since

that is not very likely, we draw the conclusion that there is a difference between the groups (Olsson et al., 2012). If the distribution of the data is non-normal, a non-parametric test, like Mann-Whitney or Kruskal Wallis tests, that ranks the data to reduce the effect of a skewed distribution, is more appropriate to use (Nahm, 2016).

In *Papers I*, *III* and *IV* we used regression analysis. Since the outcomes we investigated were binary we used conditional logistic regression and Cox regression. Both analyses can be used to investigate associations between linear and categorical variables and a binary outcome. The conditional logistic regression is used for matched data, like our nested case-control cohorts (Hosmer et al., 2013). The Cox regression is a type of survival analysis that calculates the association between an exposure and an outcome over a defined time period, and assumes that the effect on the outcome is constant over time (Abd ElHafeez et al., 2021).

To try and account for factors that can confound our results the regression models can be adjusted. For our main outcome, CE, we adjusted for known risk factors, like those defined by the Framingham heart study; age, sex, total and HDL cholesterol, systolic blood pressure, blood pressure-lowering treatment, smoking and diabetes (Pencina et al., 2009), but we also adjusted for some other factors that differed between the CE cases and controls in the papers. For example, in *Paper III* the number of diabetes patients were similar between CE cases and controls, but both plasma glucose and HbA1c values were higher in CE cases, leading us to also adjust for these variables.

It is important to keep in mind, that a non-significant *P* value does not definitely mean that there is no biological difference. Conversely, even if the *P* value if very small, the result might not be very interesting from a biological point of view (Wasserstein et al., 2019). With enough data points in the analysis we can identify significant differences, but the actual difference in the variable can be very small, and is that then interesting? It can be, but not necessarily. For example, in *Paper I*, the CE cases have in median 0.1 mmol/L higher glucose values than the controls, a small difference, but the *P* value is <0.001. But then again diabetes and high plasma glucose are very important risk factors for CVD, so perhaps this small difference is still relevant?

To summarise; statistics and biological effect should be interpreted together before drawing conclusions.

Results and Discussion

Paper I

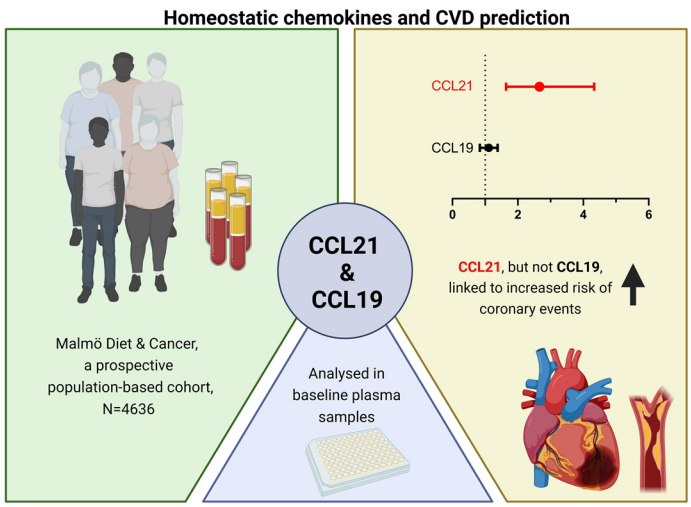
Plasma levels of CCL21, but not CCL19, independently predict future coronary events in a prospective population-based cohort

Specific Aims

- Investigate associations between the chemokines CCL21 or CCL19 and incident coronary events in the general population-based Malmö Diet and Cancer cohort.

Key Findings

- Plasma levels of CCL21, but not CCL19, have an independent association to incident coronary events.
- CCL19 instead have associations to both incident heart failure and mortality.



In *Paper I* we investigated the homeostatic chemokines CCL21 and CCL19. While both had been studied in individuals with established CVD before (Caidahl et al., 2019, Ueland et al., 2013, Yndestad et al., 2012), there were no studies that evaluated CCL21 and CCL19 in a prospective cohort, with individuals that were free of disease at the start of the study. This is also important as we would ideally like to be able to identify individuals at risk before the first coronary event (CE). Also, in a previous paper from our group, associations of the count of a T cell population to incident CE were the opposite in individuals without previous CE and those with recurring events, indicating that we cannot necessarily infer the results from the existing studies about CCL21 and CCL19 onto our cohort (Tomas et al., 2020).

The main question that we were interested in was: given the structural similarities between CCL21 and CCL19, and shared receptor – will they have similar associations to incident CE? And additionally, could either of the two chemokines be a good biomarker? In the study we used plasma samples from individuals in the Malmö Diet and Cancer cardiovascular substudy (MDC-CV), as described in detail in the *Key Methods* section. The analyses for CCL21 were performed in a smaller nested case-control cohort (n=676), while CCL19 was analysed in the entire cohort (n=4636). Both CCL19 and CCL21 were increased in plasma from CE cases, as were many known CVD risk factors, like lipids, smoking and diabetes (Figure 19).

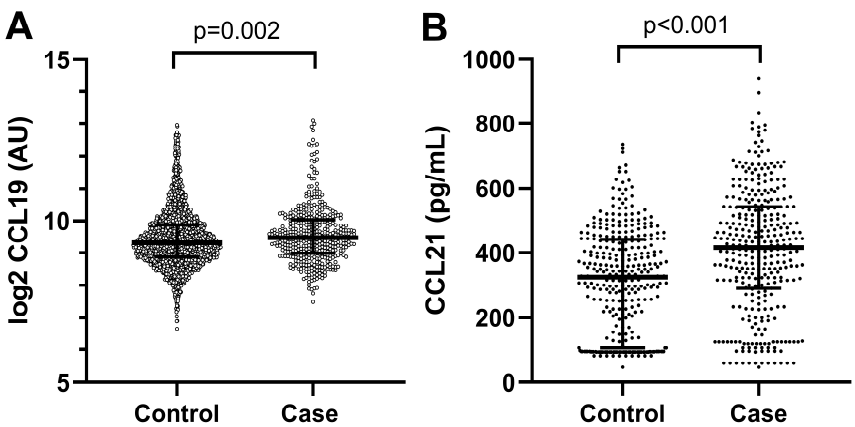


Figure 19: Plasma levels of CCL19 and CCL21 in controls and coronary event cases.

A: CCL19 was measured in the population-based cohort MDC-CV with 436 incident CE cases and 4196 controls. CCL19 levels of each participant are presented as log2 transformed with median and interquartile range (IQR). **B:** CCL21 levels were measured in the nested case-control cohort with 338 age and sex-matched pairs. CCL21 levels of each participant are presented with median and IQR. The data were analysed by Mann-Whitney tests.

Using Cox regression, we found that CCL19 did not have an association to incident CE that was independent of the CVD risk factors defined in the Framingham heart study; age, sex, total cholesterol, HDL, diabetes, use of BP lowering medication, systolic BP and smoking (Pencina et al., 2009). We analysed other outcomes and found that high CCL19 levels were independently associated to incident heart failure, which is in line with the findings of Caidahl *et al*. who found that high levels of CCL19 were associated with development of heart failure in individuals admitted with acute coronary syndrome (Caidahl et al., 2019).

High levels of CCL21, on the other hand, did have an independent association to incident CE (Figure 20). Here, we also standardised the CCL21 values to calculate the OR per standard deviation increase of CCL21, as a complement to the division into tertiles. We also made another, extended, regression model. In that we added more factors to adjust for that we identified as possible confounders in the analyses of the baseline data from the study participants. Consequently, after adjusting for CRP, HbA1c, fasting glucose and eGFR, in addition to the Framingham risk factors, CCL21 still had an association to incident CE.

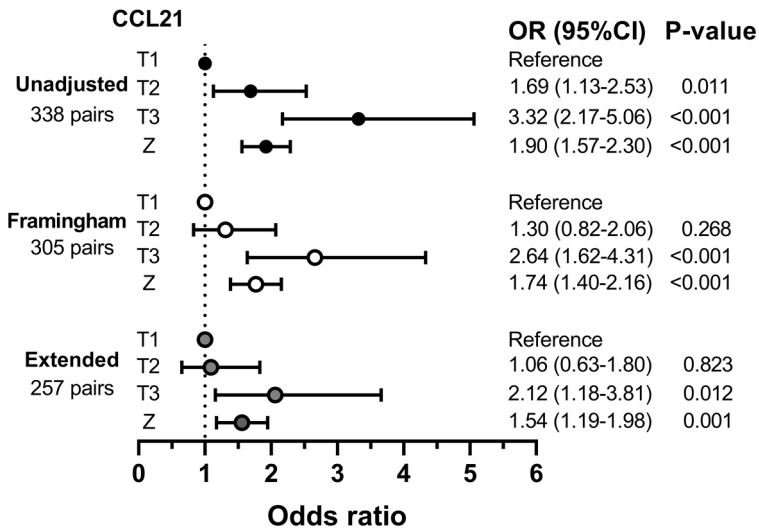


Figure 20: Association between CCL21 and incident coronary events

Conditional logistic regression with odds ratios (OR) and 95% CI for the associations between CCL21 in tertiles (T1-T3) or standardised (Z) and incident CE in an unadjusted model (n=338 pairs, black), a model adjusted for the Framingham risk factors (total cholesterol, HDL, diabetes, use of BP lowering medication, systolic BP and smoking) (n=305 pairs, white) and an extended model adjusted for the Framingham risk factors as above and CRP, HbA1c, fasting glucose and eGFR (n=257 pairs, grey).

We also performed several additional analyses to elucidate if CCL21 could be of use as a complementary biomarker to already known CVD predictors for incident CE. We first used likelihood ratio tests, in which we compared two regression models in order to determine the degree to which they describe the outcome, incident CE. This goodness-of-fit analysis showed that the addition of CCL21 to the Framingham risk factors improved the predictive ability of the regression model for incident CE. We then also performed receiver operating characteristic (ROC) curve analysis, but the increase in the area under the curve after adding CCL21 to the Framingham risk factors was rather small, indicating that CCL21 may not be a good enough candidate as a biomarker for incident CE.

As discussed in the *Introduction,* CCL21 and CCL19 have some key differences in their structures and functions. While we cannot draw any conclusions about the causal role of CCL21 in the pathogenesis of incident CE based on our study, it is tempting to believe that one or more of the structural and functional differences between CCL21 and CCL19 are behind the differing results from our study. Perhaps the most interesting factor is that CCL21 is expressed by medial SMCs in the aorta, while CCL19 seems to not be, which could directly influence immune cells recruited to the atherosclerotic vessel (Gräbner et al., 2009).

Paper II

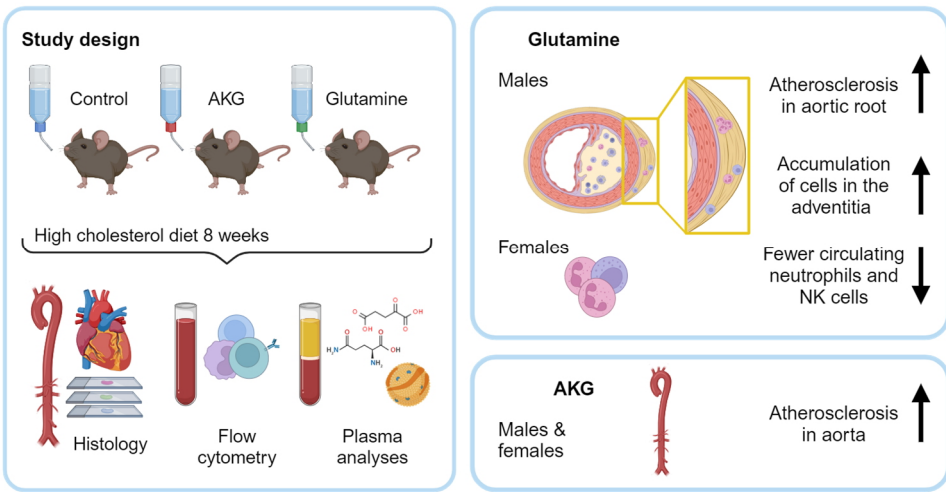
Glutamine supplementation leads to increased atherosclerosis and adventitial cell accumulation in Apoe$^{-/-}$ mice

Specific aims

- Investigate the effect of drinking water supplementation with α-ketoglutarate or glutamine on atherosclerosis development and plaque composition.

Key findings

- Male mice that received glutamine developed larger plaques and had larger accumulation of cells in the adventitia surrounding the aorta.
- Male and female mice that received α-ketoglutarate had a larger plaque burden in the aorta.



In *Paper II* we investigated the metabolites α-ketoglutarate (AKG) and glutamine to determine their effects on atherosclerosis development. As outlined in the *Introduction* we were interested in these metabolites because of reports that showed their roles in macrophage polarisation to a reparatory M2 phenotype (Liu et al., 2017, Jha et al., 2015). This piqued our interest and we initiated the study, where we investigated the effect of dietary supplementation of AKG and glutamine on atherosclerosis development.

In the whole aorta we found that both the male and female AKG groups had more atherosclerosis than their respective control groups. In the aortic root, we analysed 7 slides spanning the aortic root to get a precise estimation of the total plaque volume. There, the result was different, the AKG groups did not have a different amount of atherosclerosis compared to the controls. Instead, the male glutamine group had a larger plaque volume than the control group (Figure 21).

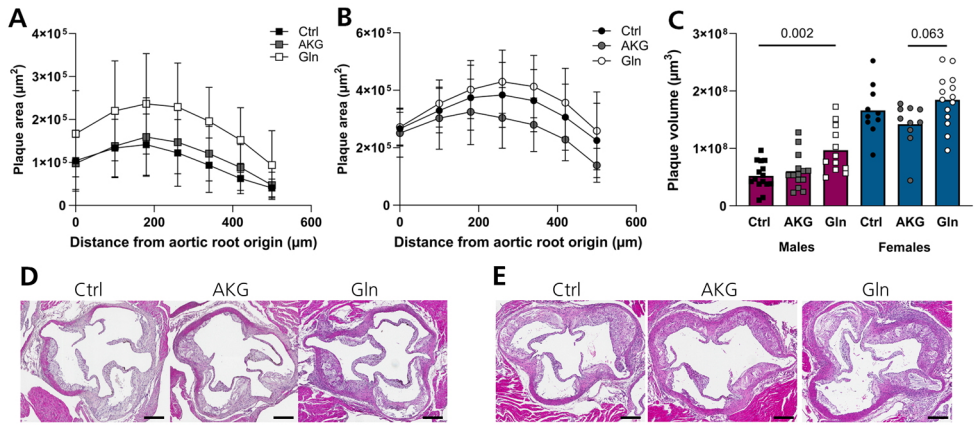


Figure 21: Atherosclerosis in the aortic root

A: Plaque area across the aortic root from male mice of all treatment groups **B**: Plaque area across the aortic root from female mice of all treatment groups **C**: Total plaque volume in the aortic root in the male and female mice, calculated as the area under the curve from B and C. **D:** Representative images of atherosclerotic plaques from males and **E:** females from each treatment group. Scale bar: 300μm.

We then analysed different plaque components, including collagen, foam cells, macrophages and necrosis, but none of these parameters could explain the difference in plaque size. We had hypothesised beforehand that collagen content could be increased in the AKG groups, because AKG is a cofactor for proline hydroxylase which hydroxylates proline residues in collagen, which is necessary for the proper folding of the fibres (Wu et al., 2016). However, to achieve this the AKG would need to make its way into the circulation from the intestine and into the plaques, which highlights a gap in our knowledge, that we are not able to determine where

our supplemented AKG and glutamine ended up or how it was metabolised. This could be addressed by tracing experiments, for example with ^{13}C-labelling.

We also found that the glutamine group had a larger accumulation of cells in the adventitia surrounding the aorta. The structures contained different immune cells, including neutrophils, macrophages, and hardly any T cells. However, these did not account for all of the cells, and the remaining cells could include B cells and dendritic cells but also non-immune cells, such as fibroblasts, which has been reported in previous work (Gräbner et al., 2009). In future experiments we plan to perform stainings to identify more cell types and analyse if there are any differences between the treatment groups. It would also be interesting to stain for CCL21, given other reports of CCL21 expression by medial SMCs (Gräbner et al., 2009).

In another animal study, separate from that presented in *Paper II*, we found more neutrophils in the aortas of the female glutamine mice, which had been digested and then analysed by flow cytometry. By using the whole aorta, we analysed a lot more cells than from a single histology slide, so the data may be more representative. However, the cells from the intima and adventitia are mixed together, so we do not know if the neutrophils were residing in the plaques or adventitia.

In the analysis of plasma metabolites, we found that glutamine supplementation led to an increase in plasma glutamine levels in the male glutamine group. The metabolomic analysis, visualised with a principal component analysis (PCA), showed that the AKG and glutamine mice mostly clustered away from the control mice. However, the AKG and glutamine groups mostly overlapped with each other and did not have differences in any specific metabolites. This was interesting seeing as we observed different results between the groups in regards to many of the parameters that we analysed in the study, even though we found that both groups had increased atherosclerosis, but in different locations.

The data in this manuscript do yet provide a mechanism, and more experiments remain before we have elucidated how glutamine causes increased atherosclerosis.

Paper III

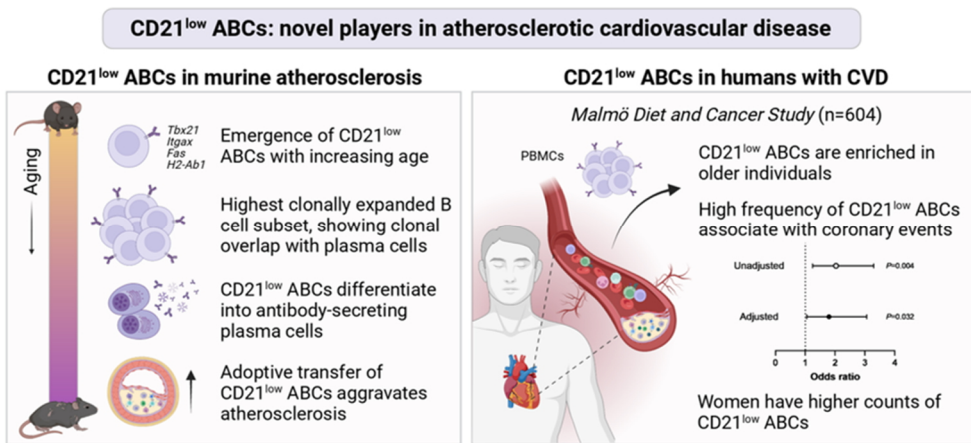
Age-associated CD21^{low} B cells aggravate murine plaque development and are associated with coronary events in humans

Specific Aims

- Investigate the clonality and differentiation potential of CD21^{low} age-associated B cells (ABCs).
- Investigate CD21^{low} ABCs in murine atherosclerosis.
- Investigate associations between CD21^{low} ABCs and incident coronary events in humans.

Key findings

- CD21^{low} ABCs are clonally expanded and differentiate into plasma cells *in vivo*.
- CD21^{low} ABCs aggravate murine atherosclerosis.
- High numbers of circulating CD21^{low} ABCs are associated with incident coronary events in humans.



In *Paper III* we set out to investigate the role of B cell populations in murine atherosclerosis and human CVD, with a specific focus on age-related changes. The paper is a collaboration with a group at Leiden University in The Netherlands, headed by Dr Amanda Foks.

Our collaborators recently identified CD21^{low} ABCs in the aortas of aged atherosclerotic mice and in carotid plaques from CVD patients (Smit et al., 2023), and in this joint project they continued to investigate CD21^{low} ABCs in murine atherosclerosis while we analysed CD21^{low} ABCs and other B cell populations in the MDC-CV cohort. Using this approach, we found evidence suggesting that CD21^{low} ABCs have similar roles in both mice and humans.

We started out by investigating if CD21^{low} ABCs are also expanded in the spleen of aged *Ldlr*$^{-/-}$ mice, by single cell RNA sequencing. We found that the CD21^{low} ABCs was the second largest B cell population in the aged mice, but not in the young mice. By flow cytometry we also identified that CD21^{low} ABCs were expanded in other tissues of the aged mice, including mediastinal lymph nodes, blood and white adipose tissue, but they were especially abundant in the spleen. Additionally, in the single cell RNA sequencing experiment the V(D)J regions of the BCR were sequenced, in order to map B cell clones in different populations. We also analysed whether there was any overlap of clones, which could indicate the differentiation of a B cell clone from one type of B cell into another. We found that the CD21^{low} ABC population had the highest clonal expansion of all B cell populations in the aged mice, and the analysis of clonotypes showed an overlap with the plasma cells. So, based this analysis, it seemed that the CD21^{low} ABCs could differentiate into plasma cells, since these two populations contained cells with the same BCR.

Next, we tested this *in vivo,* by transferring CD21^{low} ABCs into *Ldlr*$^{-/-}$*Rag1*$^{-/-}$ mice. These mice lack all mature T cells and B cells, due to an inability to rearrange their TCR and BCR genes (Mombaerts et al., 1992). By using this mouse model, we could determine if CD21^{low} ABCs will become plasma cells without interference from other cells, as the only B cells the mice have are the ones we transferred. We found that the CD21^{low} ABCs did differentiate into plasma cells, and they produced antibodies both *in vivo,* but also after *in vitro* stimulation. These results are in line with those from Nickerson *et al*. who investigated CD21^{low} ABCs in a mouse model of SLE, which is driven by pathogenic antibodies. Interestingly, the authors also determined that a partial deletion of CD21^{low} ABCs improved renal pathology, analysed histologically (Nickerson et al., 2023). In the same mice we also evaluated atherosclerosis burden and found that transfer of CD21^{low} ABCs increased atherosclerosis in the aortic root, due to more necrosis in the plaques (Figure 22).

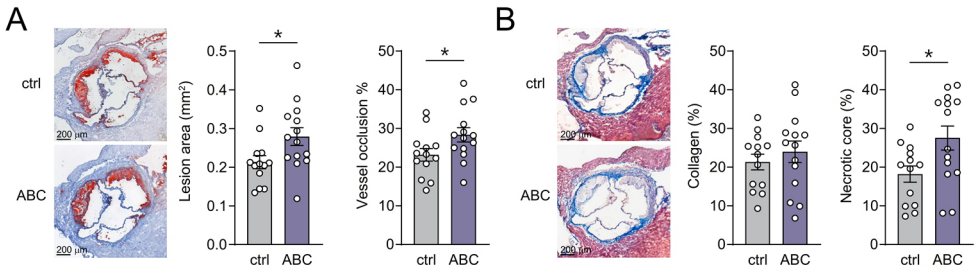


Figure 22: Transfer of CD21^{low} ABCs promotes atherosclerosis in immunodeficient mice

A-B: Cross sections of the aortic root were stained with Oil-Red-O and Masson's trichrome. B: Lesion size and vessel occlusion were quantified. B: Collagen content and necrotic core were measured as percentage of lesion area. Data are from n=13–14 mice per group. The data was analysed by two-tailed t-test or Mann-Whitney test.. Mean ± S.E.M. plotted. *P<0.05

In the human MDC-CV cohort (described in detail in the *Key Methods* section) we analysed circulating B cells from 604 individuals with flow cytometry. We found an expansion of CD21^{low} ABCs in the older individuals, compared to the middle-aged individuals, similar to the results from the mice. We also determined, by clustering our flow cytometry data, and by traditional 2-dimensional gating, that the CD21^{low} ABCs were heterogeneous and consisted of several subpopulations. Based on IgD and CD27 expression we found four populations, IgD^{+}CD27^{-} naïve, IgD^{+}CD27^{+} unswitched memory, IgD^{-}CD27^{+} switched memory and IgD^{-}CD27^{-} DN (or atypical memory; Figure 23). Compared to CD21^{+} cells the memory populations had doubled in size percentage-wise in the CD21^{low} ABCs, while the naïve population was halved in size.

Next, we went on to investigate CD21^{low} ABCs and other B cell populations in the CE cases and controls. The CE cases had higher counts of CD21^{low} ABCs, and in the conditional logistic regression analysis we found an independent association after adjusting for CVD risk factors. But, since the CD21^{low} ABCs are a heterogeneous group of cells, we wanted to see if it was a specific subpopulation of CD21^{low} ABCs that had an association to incident CE. Based on literature and the clustering, it was clear that the CD21^{low} ABCs expressed lower levels of CD24 compared to other B cells. Consequently, we gated out CD24^{+} and CD24^{-} CD21^{low} ABCs from the naïve and DN subpopulations (Figure 23). After performing regression analyses, we identified the CD24^{-} naïve CD21^{low} ABCs as the only subpopulation that had an independent association to incident CE. These cells have also been called activated naïve cells and have been described in SLE as poised for plasma cell differentiation (Jenks et al., 2018).

If we compare the phenotype of the CD21^{low} ABCs between our human and mouse data, the most basic common denominator is that they express low levels of CD21.

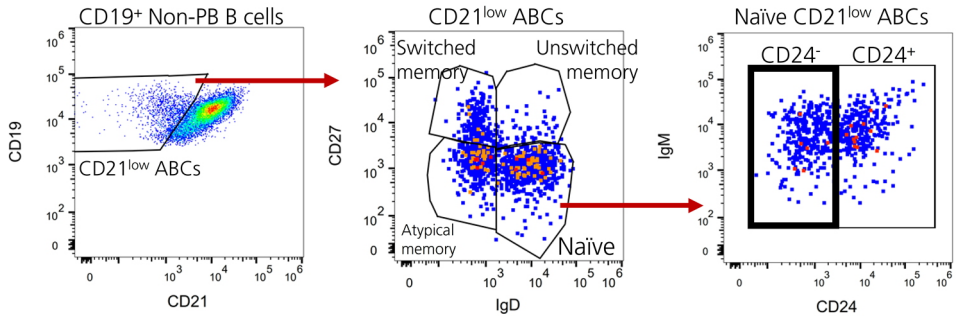


Figure 23: Flow cytometry gating of CD21^{low} ABCs and subpopulations

CD21^{low} ABCs were gated from live CD19^{+} B cells, after excluding CD38^{+}CD24^{-} plasmablasts (PBs) and CD43^{+}CD27^{+} "B1" cells. CD21^{low} ABCs were then further gated into subpopulations based on IgD and CD27. From here activated naïve cells were identified as CD24^{-} naïve CD21^{low} ABCs.

In the mice we characterised the cells first by single cell RNA sequencing and for the transfer experiments the cells were sorted based on several markers, including CD11c and CD11b. Since we did not have CD11c in the flow cytometry panel for the human cohort, we used cells from healthy donors to characterise CD11c expression. Some, but not all CD21^{low} ABCs expressed CD11c, and expression varied between the subpopulations. In the small number of individuals that we performed this analysis in, CD11c expression was most prevalent in the DN CD21^{low} ABC subpopulation. In terms of Ig class, in the mice the vast majority of CD21^{low} ABCs were IgM^{+}, which is in line with the human data, though not quite so many of the human CD21^{low} ABCs were naïve or IgM^{+}. We were not able to verify the increase of the IgG3 subclass seen in the mice in the human cohort as IgG was not included in our panel.

We also looked at sex related differences in B cell populations. In line with previous data (Klein and Flanagan, 2016), we found that women had higher counts of almost all of the B cell populations that we had analysed, including CD21^{low} ABCs, but there was no difference in the percentage of CD21^{low} ABCs (of total B cells) between men and women. We also performed the regression analysis separate for men and women and found that there was an independent association to incident CE for men only, and not for women. However, there were fewer women than men in the cohort, so it is possible that the analysis is underpowered. However, it seems that it is not female sex that drives the association between CD21^{low} ABCs and incident CE.

In the adoptive transfer study, isolated CD21^{low} ABCs were transferred to *Ldlr*$^{-/-}$ *Rag1*$^{-/-}$ mice. From one perspective, this mouse model allows us to study the effect of CD21^{low} ABCs on atherosclerosis in absence of factors that could confound the results. But CD21^{low} ABCs do not operate in a vacuum in a wildtype,

immunocompetent mouse, without interactions with other immune cells. The majority of B cells interact and receive signals from T cells during their lifetime, raising the question of whether we are studying the CD21^{low} ABCs in an artificial system by using the *Rag1*$^{-/-}$ model. We also cannot evaluate if the lack of T cells and other B cells led the CD21^{low} ABCs to act in a way that they would not have otherwise, which could hinder the interpretation of the findings from these experiments. For example, we find that the CD21^{low} ABCs differentiate into plasma cells, and secrete antibodies, which is in line with other studies (Jenks et al., 2018). But would the CD21^{low} ABCs have done that still, and to the same extent in an immunocompetent mouse, or are they expanding more to fill an empty niche? To address this, a new transfer experiment is underway, looking at the transfer of CD21^{low} ABCs into *Ldlr*$^{-/-}$ mice.

Paper IV

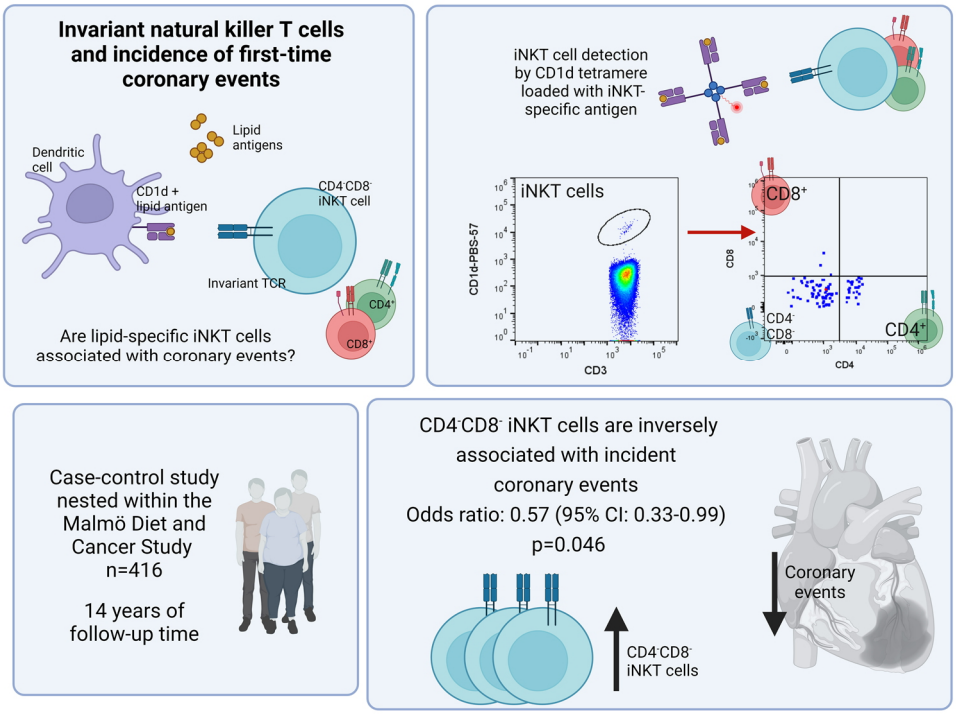
Invariant natural killer T cells and incidence of first-time coronary events: a nested case-control study

Specific aim

- Investigate if, as suggested by animal studies, invariant natural killer T (iNKT) cells have an association to incident coronary events in humans.

Key findings

- High numbers of iNKT cells do not have an association to incident coronary events. However, a subpopulation of iNKT cells that are $CD4^-$ $CD8^-$ double negative have an inverse association.



In *Paper IV* we investigated another kind of atypical adaptive immune cell, iNKT cells. The majority of studies preceding ours indicated that iNKT cells have pro-atherogenic properties in mice (Nakai et al., 2004, Tupin et al., 2004, Major et al., 2004, Aslanian et al., 2005), although there have also been some reports suggesting the opposite (van Puijvelde et al., 2009), but there have not been many studies in human atherosclerosis and CVD.

Our aim was to use PBMC samples from the MDC-CV cohort to analyse associations between iNKT cell counts and incident CE, in order to gain insight into whether iNKT cells could also have a CVD promoting role in humans. Since iNKT cells have a special TCR, that recognises lipid antigens presented on CD1d rather than peptide antigens presented on MHC class I and II, we used tetramers with conjugated fluorochromes for the detection of iNKT cells by flow cytometry. The tetramers consisted of CD1d molecules loaded with PBS-57, an analogue of the iNKT antigen α-GalCer, conjugated to the fluorochrome APC.

We started by looking at total iNKT cells in relation to baseline characteristics, including CVD risk factors and found that women and diabetes patients had more iNKT cells, while there was no difference between CE cases and controls. In the regression analysis we found no association iNKT cells and incident CE, in an unadjusted model, nor after adjusting for CVD risk factors (Table 1). Interestingly, not many CVD risk factors were associated with numbers of iNKT cells.

However, iNKT cells, like other T cells can be split into subpopulations based on expression of CD4 and CD8. We found that the majority, 57%, of iNKT cells were $CD4^{-}CD8^{-}$ DN, followed by 37% $CD4^{+}$ and 6% $CD8^{+}$. After this

Table 1: Associations between iNKT cell counts and incident coronary events, analysed by conditional logistic regression

	iNKT cell tertile	Unadjusted		Adjusted	
		OR (95% CI)	*P*	OR (95% CI)	*P*
Total iNKT cells	1	1 (Reference)		1 (Reference)	
	2	0.71 (0.45-1.13)	0.15	0.70 (0.40-1.21)	0.20
	3	0.81 (0.51-1.29)	0.37	0.74 (0.43-1.27)	0.28
$CD4^{-}CD8^{-}$ iNKT cells	1	1 (Reference)		1 (Reference)	
	2	0.53 (0.32-0.87)	0.012	0.55 (0.31-0.98)	0.043
	3	0.62 (0.38-0.99)	0.046	0.57 (0.33-0.99)	0.046

N in unadjusted model: 208 pairs. N in adjusted model: 181 pairs. Adjustments were made for total cholesterol, HDL, diabetes, BP-lowering medication, systolic BP and current smoking. OR: odds ratio. CI: confidence interval.

separation into three subsets, a different pattern appeared. In the regression analysis we found that the $CD4^-CD8^-$ DN iNKT cells did in fact have an inverse association to incident CE, also after adjustment for risk factors. Not all studies take subpopulation of iNKT cells into accounts but, for example, To *et al.* found that $CD4^+$ iNKT cells, but not $CD4^-CD8^-$ DN iNKT cells promoted atherosclerosis development in adoptive transfer studies (To et al., 2009). The $CD4^+$ iNKT cells promote atherosclerosis via a granzyme B and perforin dependent mechanism (Li et al., 2015) Additionally, $CD4^-CD8^-$ DN iNKT cells express higher levels of inhibitory Ly49 molecules, that recognise MHC class I molecules as a tolerance mechanism (Rahim et al., 2014, To et al., 2009).

Other studies of human iNKT cells, including single cell RNA sequencing have also showed distinct subpopulations, which could be linked to different effector functions (Zhou et al., 2020). iNKT cells have been detected in human plaques, which was combined with lower circulating levels of iNKT cells in individuals with symptomatic CVD, raising the question of whether the cells are extravasating into tissues or if the they are less prone to survive (Kyriakakis et al., 2010).

Lastly, we phenotyped the iNKT cells from a small number of individuals from the MDC-CV cohort. We gated the cells based on the markers CD45RA and CCR7 and found that both $CD4^+$ and $CD4^-CD8^-$ DN iNKT cells were predominately $CD45RA^-CCR7^-$, indicating an effector memory phenotype (Figure 24).

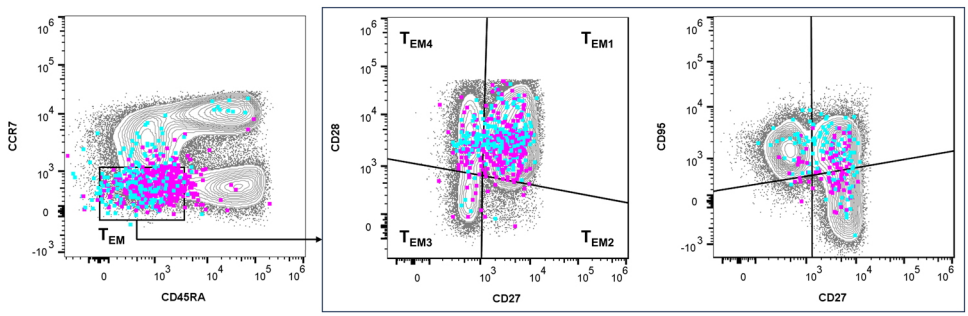


Figure 24: Phenotypic memory markers on iNKT cell subsets

Flow cytometric data were aggregated from 6 individuals from the MDC-CV cohort and gated on CD45RA, CCR7, CD27, CD28 and CD95 expression. Viable $CD3^+$ cells shown as grey contour plots in the background as reference and $CD4^-CD8^-$ DN (magenta) and $CD4^+$ (cyan) iNKT cells overlaid as dot plots. $CD45RA^-CCR7^-$ effector memory T cells (T_{EM}) were further gated as T_{EM}1-4 subsets based on CD27 and CD28 expression.

From the gated $CD45RA^-CCR7^-$ effector memory population we found that both subpopulations were distributed similarly to non-iNKT cells (meaning all other T cells) based on CD27 and CD28 expression, with the majority being $CD27^+CD28^+$, T effector memory 1 cells. Additionally, the $CD4^+$ iNKT cells expressed higher

surface levels of CD95 than the $CD4^{-}CD8^{-}$DN iNKT cells. CD95 (Fas) is used as a marker for memory T cells, including stem cell memory T cells (Figure 24) (Gattinoni et al., 2017). While these data are interesting, and an important part in establishing different functionalities of iNKT subsets, this analysis needs to be performed in a larger number of individuals. It also remains to be seen if the difference in CD95 expression between $CD4^{+}$ and $CD4^{-}CD8^{-}$DN iNKT cells has any functional relevance. But, the results from our study highlight that you cannot always infer results from murine studies onto human biology.

Conclusions and Future Perspectives

In this thesis I have investigated the mediators CCL21 and CCL19, the metabolites AKG and glutamine, and the atypical age-associated B cells and iNKT cells in relation to atherosclerosis development and incident CE.

In *Paper I* we found that the closely related chemokines CCL19 and CCL21 do not share the same predictive abilities for incident CE, and only CCL21 had an association that was independent of CVD risk factors. Despite this, CCL21 may not be appropriate to use as a biomarker, since it contributed to a rather small increase in the AUC from the ROC curve analysis. However, it would still be interesting in future studies to investigate a causal role of CCL21 in CE, which could be done in a Mendelian randomisation study, perhaps using the genetic variants described by Cai *et al.* (Cai et al., 2014).

Given their roles in recruitment of immune cells into various tissues, chemokines and chemokine receptors could be possible targets for therapies in atherosclerosis and CVD (Döring et al., 2024). Although, given the essential homeostatic roles of CCL21 and CCL19, blocking these specific chemokines may prove difficult. In an interesting Phase 1 clinical trial in cancer, dendritic cells were transduced with a viral vector to overexpress CCL21, which were then injected into tumours to provoke an immune reaction. The trial showed a tumour antigen specific response and increased infiltration of $CD8^+$ T cells in 54% of study participants (Lee et al., 2017a). Correspondingly, high CCL19 content in breast cancer tumours predicted better response to immune checkpoint inhibitor therapy and improved survival (Wu et al., 2023). Although these results are very interesting, any kind of similar application in atherosclerosis and CVD would be dependent on also inducing a reparatory anti-inflammatory response, such as recruitment of regulatory T cells.

In *Paper II* we found that dietary supplementation with glutamine increased the atherosclerosis development in male, but not in female mice.

We need to further investigate the mechanism of how glutamine supplementation leads to increased atherosclerosis development. For example, it would be interesting to investigate how the AKG and glutamine is metabolised after ingestion and see whether there is any uptake into the plaques and if there is a sex-specific response. Our targeted metabolomics of plasma metabolites showed some differences in the AKG and glutamine groups compared to the control, but we need to perform further experiments in order to characterise the effect of these differences. Additionally,

this can also include analysing the metabolism of immune cells isolated from mice after AKG or glutamine supplementation, or after *in vitro* treatment, using a flow cytometry based method of analysing cellular metabolism (Argüello et al., 2020).

The different metabolic reprogramming in immune cells with pro- and anti-inflammatory phenotypes open up the possibilities of targeting metabolic pathways in order to promote a more favourable immune cell phenotype. Specific targeting of glutamine metabolism has been tested in cancer. Cancer cells are, as some immune cells, reliant on glutamine. Blocking glutaminase 1, that converts glutamine into glutamate, the first step of glutaminolysis, did however not improve the clinical outcome, despite decreasing proliferation of cancer cells *in vitro* (Biancur et al., 2017, Tannir et al., 2022). Regardless of this, the principle of targeting metabolic enzymes with small compounds can have advantages if we look from an economic and health care perspective, as these are less expensive to manufacture than for example, antibodies. In Sweden the combined costs for CVD were 63.3 billion Swedish kronor in 2017 (Hjalte et al., 2019). In the USA the projected costs in 2035 due to CVD, both direct and indirect, is projected to exceed USD 1000 billion (Virani et al., 2020). So, from this perspective metabolic reprogramming shows promise as a therapeutic target. However, metabolic adaptation, which is what may have happened in the cancer trial, may prove difficult to overcome, given how complicated and interconnected metabolic pathways are. Perhaps multiple pathways would need to be targeted in order to get a favourable, long-term reprogramming of immune cell metabolism, and a stabilising effect on atherosclerotic plaques.

In *Paper III* we identify a pro-atherogenic effect of $CD21^{low}$ ABCs in murine atherosclerosis as well as an independent association between high counts of circulating $CD21^{low}$ ABCs and incident CE in humans.

Furthermore, we also found that $CD21^{low}$ ABCs can differentiate into plasma cells and secrete antibodies in mice. The largest outstanding question in *Paper III* is the specificity of these antibodies, which remains a paramount research question. As discussed in the *Introduction*, other authors have found that antibodies from $CD21^{low}$ ABCs have disease relevant specificities, making it possible that this is also the case in atherosclerosis. This is one of the goals of an EU funded project called *B specific*, where our group at Lund University is one of six centres. *B-specific* is aimed at investigating age associated changes in B cells in relation to atherosclerosis and CVD and possible ways of targeting these cells therapeutically, including with a CAR T cell approach.

In addition, more research and insight into specific subpopulations of $CD21^{low}$ ABCs, and possible differences in their functions is needed. This includes investigations into whether some subpopulations are actively disease promoting and others are not. This requires more phenotyping, for example by flow cytometry or single cell RNA sequencing. In our current study we are somewhat limited by the number of cells. The total $CD21^{low}$ ABC population is rather small in the circulation,

even in the Malmö Diet and Cancer cohort which consists of middle-aged and older individuals. To be able to accurately analyse subpopulations of $CD21^{low}$ ABCs more cells are required.

In *Paper IV* we found that iNKT cells did not have an independent association to incident CE, which is interesting given numerous reports about pro-atherogenic properties of iNKT cells in murine atherosclerosis. Our study does emphasise the importance of separating data from mice and humans, although we cannot provide any causal data in our study about the exact role and effect of iNKT cells in human CVD. Interestingly, we identified one subpopulation of iNKT cells, the $CD4^{-}CD8^{-}$ DN iNKT cells, which had an inverse association to incident CE. The functional differences between different iNKT subpopulations in human atherosclerosis should be the topic of future studies. We also need to investigate whether these subpopulations have any direct effect on atherosclerosis development and the mechanisms of the possible protective effect against CE, and also put more focus on the NK cell specific properties of iNKT cells.

In conclusion, with my work summarised in this thesis, I have added to the vast pool of knowledge about the immune system and its many roles in atherosclerosis and CVD. Although the results from our studies do not provide definitive mechanisms, biomarkers or treatments ready to use in the clinic, we present a large amount of data and insights for us and other researchers to build upon in upcoming studies. In the future, this knowledge about the actions of mediators, metabolites and atypical immune cells in the pathophysiological processes of atherosclerosis and CVD may help us identify new treatment targets or strategies, and ultimately reduce the numbers or improve the lives of individuals living with CVD.

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