Aus dem
Institut für Prophylaxe und Epidemiologie der Kreislaufkrankheiten (IPEK)
Klinikum der Ludwig-Maximilians-Universität München

The role of the epigenetic mark H3K27me3 in T cells during atherosclerosis

Dissertation

zum Erwerb des Doktorgrades der Medizin
an der Medizinischen Fakultät
der Ludwig-Maximilians-UniversitätMünchen

vorgelegt von
Cecilia Assunta Bonfiglio

aus
Mailand

Jahr
2025

Mit Genehmigung der Medizinischen Fakultät der
Ludwig-Maximilians-Universität München

Erstes Gutachten:	Prof. Dr. Christian Weber
Zweites Gutachten:	Prof. Dr. Jürgen Bernhagen
Drittes Gutachten	Prof. Dr. Vigo Heissmeyer

Dekan: Prof. Dr. med. Thomas Gudermann

Tag der mündlichen Prüfung: 11. November 2025

"Scientific knowledge and understanding is a communal achievement, the sum of a multitude of contributions from many different people. Any individual may feel a certain justifiable pride if he knows that he has added one brick to the structure".

C. H. Waddington

1. Affidavit

LMU LUDWIG-MAXIMILIANS-UNIVERSITÄT MÜNCHEN

Dekanat Medizinische Fakultät
Promotionsbüro

Affidavit

Bonfiglio, Cecilia Assunta

Surname, first name

I hereby declare, that the submitted thesis entitled:

The role of the epigenetic mark H3K27me3 in T cells during atherosclerosis

is my own work. I have only used the sources indicated and have not made unauthorised use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.

I further declare that the dissertation presented here has not been submitted in the same or similar form to any other institution for the purpose of obtaining an academic degree.

Munich, January 4th, 2026

Place, Date

Cecilia Assunta Bonfiglio

Signature doctoral candidate

2. Table of Contents

1. Affidavit *2*

2. Table of Contents *3*

3. List of abbreviations *4*

4. List of publications *6*

4.1 Publications included in this work **6**

4.1.1 Paper I 6

4.1.2 Paper II 6

4.2 Additional publications not included in this work **7**

5. Contribution to publications *8*

5.1 Contribution to paper I: Ezh2 shapes T cell plasticity to drive atherosclerosis **8**

5.2 Contribution to paper II: The amino acid homoarginine inhibits atherogenesis by modulating T-Cell function **8**

6. Introduction *9*

6.1 Atherosclerosis: from history to histopathology **9**

6.2 Past and current therapies **11**

6.3 The role of the immune system in atherosclerosis **13**

6.3.1 Innate immune cells 13

6.3.2 Adaptive immune cells 14

6.3.3 Clinical outlook 18

6.4 Epigenetic landscape in atherosclerosis **19**

7. Summary *22*

7.1 Paper I: Ezh2 shapes T cell plasticity to drive atherosclerosis **22**

7.2 Paper II: The amino acid homoarginine inhibits atherogenesis by modulating T-cell function **23**

8. Zusammenfassung *25*

8.1 Publikation I: Ezh2 shapes T cell plasticity to drive atherosclerosis **25**

8.2 Publikation II: The amino acid homoarginine inhibits atherogenesis by modulating T-cell function **26**

9. Paper I *28*

10. Paper II *30*

11. Bibliography *32*

12. Acknowledgments *39*

3. List of abbreviations

APC	Antigen presenting cell
ApoB100	Apolipoprotein B100
ApoE	Apolipoprotein E
ASCVD	Atherosclerotic cardiovascular disease
CAD	Coronary artery disease
CEA	Carotid endarterectomy
ChIP	Chromatin immunoprecipitation
CVD	Cardiovascular disease
DCs	Dendritic cells
cDCs	Conventional dendritic cells
pDCs	Plasmacytoid dendritic cells
EZH2	Enhancer of zeste homolog 2
H3K27me3	Histone 3 lysine 27 trimethylation
HA	L-homoarginine
hs-CRP	High-sensitivity C-reactive protein
IFN-γ	Interferon- γ
IL-	Interleukin
Ig	Immunoglobulin
iNKT	Invariant NKT cells
LDL	Low-density lipoprotein
LDL-C	LDL-cholesterol
(ox)LDL	Oxidized low-density lipoprotein
MI	Myocardial infarction
Plzf	Promyelocytic leukemia zinc finger
PRC2	Polycomb repressive complex 2
qPCR	Quantitative polymerase chain reaction
scRNA-seq	Single cell RNA sequencing
STEMI	ST-elevation myocardial infarction

TCR	T cell receptor
Th	T helper cells
TGF-β	Transforming growth factor
Treg	Regulatory T cells
TNF-α	Tumor necrosis factor-α
Zbtb16	Zinc Finger And BTB Domain Containing 16

4. List of publications

4.1 Publications included in this work

4.1.1 Paper I

Cecilia Assunta Bonfiglio*, Michael Lacy*, Vasiliki Triantafyllidou, Floriana Maria Farina, Aleksandar Janjic, Katrin Nitz, Yuting Wu, Venetia Bazioti, Irem Avcilar-Kücükgöze, Yonara Freire Soares Marques, Markus Joppich, Mahadia Kumkum, Katja Röß, Anuroop Venkateswaran Venkatasubramani, Axel Imhof, Wolfgang Enard, Lars Maegdefessel, Menno de Winther, Christian Weber, Donato Santovito, Esther Lutgens[#], Dorothee Atzler[#]. Ezh2 shapes T cell plasticity to drive atherosclerosis. *Circulation*, 2025 May 13;151(19):1391-1408, DOI: 10.1161/CIRCULATIONAHA.124.072384. Epub 2025 Feb 7.

4.1.2 Paper II

Katrin Nitz*, Michael Lacy*, Mariaelvy Bianchini, Kanin Wichapong, Irem Avcilar-Kücükgöze, **Cecilia Assunta Bonfiglio**, Roberta Migheli, Yuting Wu, Carina Burger, Yuanfang Li, Ignasi Forné, Constantin Ammar, Aleksandar Janjic, Sarajo Mohanta, Johan Duchene, Johan W M Heemskerk, Remco T A Megens, Edzard Schwedhelm, Stephan Huveneers, Craig A Lygate, Donato Santovito, Ralf Zimmer, Axel Imhof, Christian Weber, Esther Lutgens[#], Dorothee Atzler[#]. The amino acid homoarginine inhibits atherogenesis by modulating T-Cell function. *Circulation Research,* 2022 Sep 30; 131(8):701-712, DOI:10.1161/CIRCRESAHA.122.321094. Epub 2022 Sep 14.

*denotes shared first authorship

[#]denotes shared last authorship

4.2 Additional publications not included in this work

- Elisa Martini*, Marco Cremonesi*, Arianna Felicetta*, Simone Serio, Simone Puccio, Erica Pelamatti, Jasper J.P. van Beek, Vasiliki Papadopoulou, Chiara Catalan, Francesca Fanuele, Desirée Giuliano, Gianluca Basso, **Cecilia Assunta Bonfiglio**, Cristina Panico, Marco Vacchiano, Pierluigi Carullo, Laura Papa , Carla D'Andrea, Naz Tuzger, Sergio Marchini, Paola Magistroni, Silvia Deaglio, Antonio Amoroso, Enrico Lugli, Gianluigi Condorelli, Marinos Kallikourdis. Autoimmune-Like mechanism in heart failure enables preventive vaccine therapy. *Circulation Research*; 2025 Jan 3; 136(1):4-25. DOI: 10.1161/CIRCRESAHA.124.324999. Epub 2024 Dec 4.

- **Cecilia Assunta Bonfiglio**, Christian Weber, Dorothee Atzler, Esther Lutgens. Immunotherapy and cardiovascular diseases: novel avenues for immunotherapeutic approaches. *Quarterly Journal of Medicine*; 2023 Apr 29; 116(4):271-278. DOI: 10.1093/qjmed/hcab207.

*denotes shared first authorship

5. Contribution to publications

5.1 Contribution to paper I: Ezh2 shapes T cell plasticity to drive atherosclerosis

Cecilia Bonfiglio and Dr. Michael Lacy are the first authors of this paper. Bonfiglio and Lacy both worked extensively with the mouse lines included in the paper. Bonfiglio and Lacy generated and analysed the data included in the paper and created the figures. Within the murine studies, Bonfiglio confirmed the cell-specific deletions, helped to characterize the atherosclerotic lesions, and to phenotype the mice via flow-cytometry, gene and protein expression analysis. Bonfiglio participated to the human and murine scRNA-seq analyses. She further established the flow cytometric-based iNKT cell phenotyping. To elucidate the underlying mechanism behind the deletion of Ezh2 in T cells, Bonfiglio performed the chromatin immunoprecipitation experiments. Moreover, she was responsible for the experiments, which clarified a non-canonical role of Ezh2 in T cells. She took part in writing the manuscript together with Dr. Michael Lacy, Prof. Esther Lutgens and PD Dr. Dorothee Atzler.

5.2 Contribution to paper II: The amino acid homoarginine inhibits atherogenesis by modulating T-Cell function

Cecilia Bonfiglio is a co-author of this publication. She accomplished murine studies, including blood withdrawal via cardiac puncture and aortic arch and aorta preparation for immunohistochemical studies. She took part in the flow cytometric staining and analysis as well as the functional T cell assays (e.g., T cell proliferation and migration assays).

6. Introduction

6.1 Atherosclerosis: from history to histopathology

For decades, the role of blood vessels during disease processes remained unclear. A first definition of pathologic coronary arteries was provided by Edward Jenner, when performing the autopsy of the colleague John Hunter, surgeon and physiologist at St. George Hospital, who suddenly deceased after *angina pectoris*. Jenner reported in his autopsy that "*no material disease of the heart, except that the coronary artery appeared thickened*"[1]. He was the first one to describe coronary atherosclerosis in a patient suffering from *angina pectoris*. In the 19th century, the pathologist Rudolph Virchow was the first to postulate that atherosclerosis is "*chronic inflammation induced by cholesterol*"[2]. Later, Russel Ross confirmed the role of a vicious circle of inflammation in atherosclerotic plaques[3]. Nowadays, increasing awareness is suggesting that both cholesterol and a plethora of immune cells infiltrated in atherosclerotic plaques contribute to the establishment of a chronic inflammatory *milieu* at the arterial wall. Atherosclerotic plaques may erode or rupture, ultimately leading to the onset of atherosclerotic cardiovascular diseases (ASCVD), namely ischemic heart disease, stroke and peripheral vascular diseases, among others.

The pathogenesis of atherosclerosis is conceptualized as slowly progressive accumulation of luminal plaques in large- and medium-size vessels. The formation of plaques occurs at anatomically predisposed sites, where the blood flow dynamics are altered, at such as branches or bifurcations. At these sites, in the setting of hyperlipidaemia, atherosclerosis is initiated when endothelial cells start to over-express adhesion molecules. Endothelial cell activation and dysfunction, i.e., the absence of a confluent luminal elastin layer and the exposure of proteoglycans[4], lead to subendothelial retention of low-density lipoprotein (LDL), which gets modified by reactive oxygen species into oxidized-LDL (oxLDL). OxLDL triggers the intimal immune cell infiltration, as endothelial cells express adhesion molecules and secrete chemokines. In addition, oxLDL is antigenic and gets phagocytosed by macrophages, which transform into the so-called 'foam cells' and enhance leukocytes recruitment, such as T cells and B cells. Early plaques, also defined as *fatty streaks* or *intimal xanthomas*, are characterized by the presence of foam cells and few T cells[5]. At this stage the plaques are clinically silent. When the plaques grows, more immune cells infiltrate and vascular smooth muscle cells start

to migrate to the surface of plaques forming a fibrous cap, progressing to the so-called *pathological intimal thickening*[5]. As the lipid accumulation and immune cell infiltration advances, the plaques transform into *fibrous cap atheroma*, which consist in a thin (<65 µm) fibrous cap, covering a necrotic core, or *fibrocalcific nodules*, containing high percentage of extracellular matrix and calcifications[5]. Changes in the extracellular matrix composing the fibrous cap may result into intraplaque haemorrhage, as a consequence of blood infiltration from luminal sites[6] or from the rupture of the vasa vasorum[7]. At later stages athero-progression is associated to thrombus formation. Plaques may undergo erosion, rupture or, more rarely, can transform into calcified nodules[5]. Plaque thrombosis can lead to further growth of the atherosclerotic plaque or occlusion of the artery, ultimately resulting in fatal adverse events (i.e., myocardial infarction (MI), stroke).

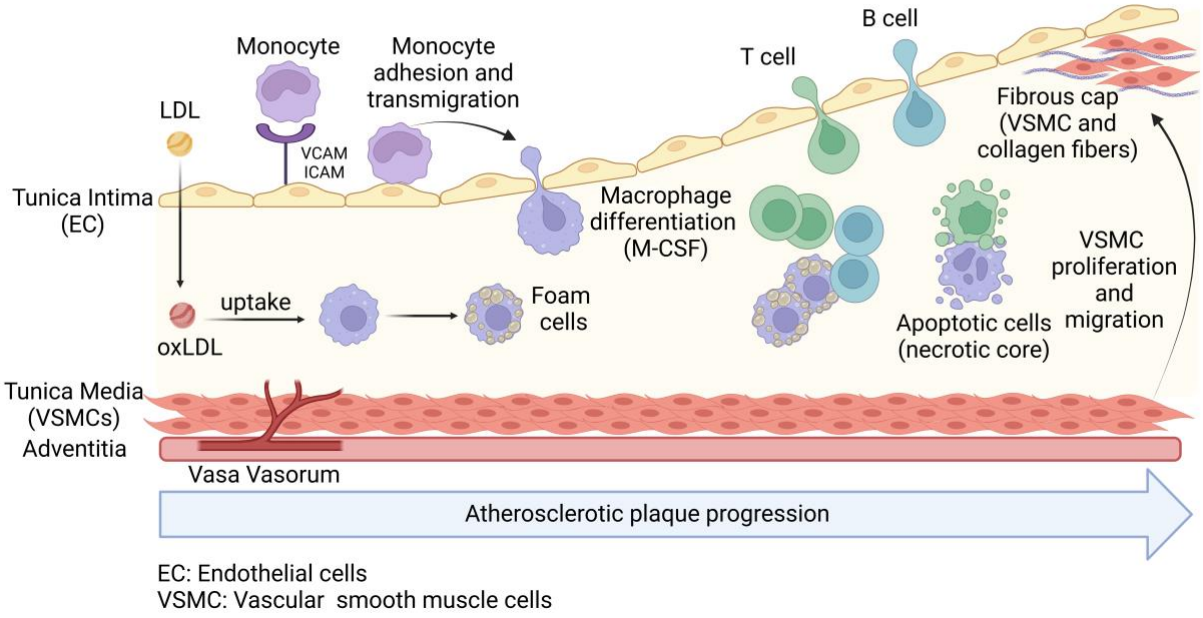

Figure 1: **Atherosclerotic plaque progression.** Upon monocyte infiltration and low-density lipoprotein (LDL) accumulation, initial lesion formation occurs. The LDL undergoes oxidation (oxLDL) and is subsequently engulfed by monocyte-derived macrophages, which transform into foam cells. This process contributes to the progression of lesions into fatty streaks. Subsequent infiltration by additional immune cells, including B and T lymphocytes, is observed. Moreover, vascular smooth muscle cells (VSMC) migrate into the intima, forming a fibrous cap composed of collagen fibers. The apoptotic and necrotic events within immune cells lead to the development of necrotic cores. Adapted from Björkegren *et al* [8]. *Created with Biorender.*

6.2 Past and current therapies

Cardiovascular disease (CVD)-related deaths currently remain the leading cause of morbidity and mortality worldwide[9,10]. The prevalence of obesity, which provide the basis for metabolic syndromes, insulin resistance and diabetes, is considerably increased in the past 50 years[11]. Hence, beside the development of newer, more adequate therapies, a strict control of dietary and lifestyle habits, such as smoking and sedentary lifestyle, are of urgent need.

The *European Society of Cardiology/European Atherosclerosis Society* (ESC/EAS) and *American College of Cardiology/American Heart Association* (ACC/AHA) guidelines suggest aiming for an LDL-cholesterol (LDL-C) level of under 1.8 mmol/L (<70 mg/dL), or a reduction of over 50% in LDL-C from baseline in high-risk patients[12,13]. The use of blood lipid-lowering drugs, such as statins (hydroxyl-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitors) and the more recently developed proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors (alirocumab and evolocumab) has shown promising results in the clinic, with an effective LDL-C reduction and an overall decline of major adverse cardiovascular events by almost 50%[14–16]. Despite their proven efficacy, statins are associated with side effects such as myopathies and myalgia. Over the last decades, several lipid-lowering therapies, used as monotherapy or in combination to statin therapy, have emerged, thus re-evaluating the treatment approach for dyslipidaemia. Among these, ezetimibe, that inhibits the intestinal absorption of biliary and dietary cholesterol, has been shown to improve cardiovascular outcomes in patients affected by acute coronary syndromes[17]. Furthermore, in patients intolerant to statins, bempedoic acid, approved in 2020 by Esperion[18], has demonstrated to be efficacious in reducing major adverse cardiovascular events[19].

However, despite the benefits of the abovementioned lipid lowering strategies, ASCVD still remains a significant health burden, suggesting that targeting the lipid component of atherosclerosis may not be the sole pathway to achieving improved outcomes. In the recent years, substantial evidence has confirmed Virchow's original postulation regarding the crucial role of inflammation during the development and progression of atherosclerosis. In 2017, the pivotal *Canakinumab Anti-inflammatory Thrombosis Outcome Study* (CANTOS) trial demonstrated for the first time that blocking the inflammatory cytokine Interleukin-1β (IL-1β) with the monoclonal antibody canakinumab led to a significant reduction of first recurrent cardiovascular events in patients with prior MI and residual inflammation, defined by a persistently elevated (>2 mg/L) high-sensitivity C-reactive protein (hs-CRP)[20]. In a second

analysis, Ridker et al. further proved that IL-1β blockage was capable of reducing cardiovascular events and all-cause mortality by 31%, but only in patients who achieved on-treatment hs-CRP concentrations below 2 mg/L. Patients who had persistent hs-CRP concentrations of 2 mg/L or above did not benefit from the treatment[21]. Of note, Canakinumab caused undesired effects that required attention: patients receiving the treatment were more susceptible to infections due to neutropenia and experienced a significantly higher number of deaths, primarily from infections or sepsis, compared to the placebo group[20].

Taking a deeper look into potential anti-inflammatory therapies, the *Low Dose Colchicine* trial (LoDoCo)[22], LoDoCo2[23] and COLCOT[24] (*Colchicine Cardiovascular Outcomes Trial*) showed that low doses of colchicine (0.5 mg/day), in combination with other secondary-prevention therapeutic strategies, such as aspirin, clopidogrel and statins, reduced ASCVD risk in patients with stable coronary artery disease (CAD) or recent MI. However, noteworthy not all anti-inflammatory agents demonstrated to be beneficial. In fact, in the *Cardiovascular Inflammation Reduction Trial* (CIRT) low-dose methotrexate (administered twice weekly) to patients with previous MI or 3-vessel disease and diabetes or the metabolic syndrome, failed to improve ASCVD endpoints[25]. Overall, these data clearly show the efficiency of anti-inflammatory treatment, but at the same time emphasize the necessity of developing immune-targeted interventions that can modulate atherosclerosis with greater precision, while ensuring safety, durability, and efficacy. Selectively targeting specific components and pathways of the immune network that drive atherogenesis will demand a thorough understanding of the cellular and molecular mechanisms underlying the disease.

6.3 The role of the immune system in atherosclerosis

The complex inflammatory *milieu* of atherosclerosis involves the interplay of the innate and adaptive immune system. Flow cytometry, mass cytometry and multidimensional single-cell molecular profiling (i.e., single cell RNA sequencing (scRNA-seq)) have provided newer and higher resolution in uncovering the cellular compositions of atherosclerotic plaques and the arterial wall[26–28]. Here, I provide a brief overview of the most abundant innate and adaptive immune cells, orchestrating the chronic inflammation during atherosclerosis. Emphasis will be given to T cells and their epigenetic landscape, which serves as the central focus of the projects included in this dissertation.

6.3.1 Innate immune cells

The heterogenous group of innate immune cells that accumulate in atherosclerotic plaques includes macrophages, dendritic cells (DCs), monocytes, mast cells and neutrophils. More recent research has revealed a role of natural killer cells, as well as innate lymphoid cells[26,29]. As aforementioned, subendothelial accumulation of LDL and oxLDL trigger the expression of chemokines on the arterial luminal wall, with consequent recruitment of classical monocytes, which differentiate into monocyte-derived macrophages and monocyte-derived DCs. Macrophages are, together with T cells, the most abundant immune cell type in human and murine atherosclerotic plaques. They are responsible for lipid uptake and clearance, transforming into 'foam cells'. Plaque macrophages might have pro-inflammatory or anti-inflammatory features. Lipidomic and transcriptomic analyses of lipid-loaded macrophages have revealed an enrichment of anti-inflammatory genes in cells exhibiting foamy characteristics, suggesting that foam cell formation alone is not inherently pro-inflammatory[30,31]. In addition, it has been demonstrated that both conventional DCs (cDCs) and plasmacytoid DCs (pDCs) are found in human and murine arteries, as well as in atherosclerotic lesions. cDCs bear the capacity to activate antigen specific T cells through the major histocompatibility complexes (MHC)-I and -II, promoting tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) production, ultimately resulting in the initiation of the adaptive immune response and promoting their accumulation in atherosclerotic plaques[32]. On the other hand, pDCs are potent producers of type I interferons (IFN-α/β), particularly in response to self-DNA or RNA released by dying cells in the atherosclerotic plaque. They secrete chemokines that attract other immune cells, such as monocytes and T cells, into the plaque,

contributing to lesion growth. Their role within the plaque is controversial. It has been demonstrated that pDCs can interact with regulatory T cells (Treg), favoring the anti-inflammatory IL-10 cytokine production. In fact, antibody mediated depletion of pDCs resulted in aggravated atherosclerosis[33]. Contrarily, another study proposed a pro-atherogenic role of pDCs[34].

In conclusion, both cDCs and pDCs play a role in modulating atherogenesis in mice by influencing T cell activation and adaptive immune responses, partially through antigen-dependent mechanisms. However, the precise pathways through which DCs exert this regulation remain to be fully understood.

6.3.2 Adaptive immune cells

Within the intricate immune network of atherosclerosis, both B cells and T cells are central players. B cell-mediated cellular and humoral immunity significantly influences plaque formation, though its effects remain controversial. Immunoglobulin (Ig) types such as IgM, IgG, and IgE can impact plaque progression, either promoting or mitigating its development[35,36].

Likewise, the role of T cells during the development and progression of atherosclerosis has remained unclear for decades. Histological analysis of *post-mortem* human carotid artery plaques demonstrated that the culprit lesions are characterized by large necrotic cores, a thin fibrous cap and high ratio of macrophages to smooth muscle cells[37]. Accordingly, most of the past research has focused its attention on the role of macrophages on plaque instability[38,39]. However, the advent of more advanced techniques, such as mass cytometry, scRNA-seq and cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq), has allowed a more precise mapping of atherosclerotic plaques. These allowed to discover that T cells represent the largest leukocyte population in atherosclerotic plaques and the number of effector T cells associates with plaque instability[27,40]. $CD4^+$ and, to lesser extent, $CD8^+$ T cells are found in murine atherosclerotic plaques[41] and their recruitment occurs via chemokines and chemokine receptors, namely CC-chemokine receptor 5 (CCR5), CXC-chemokine receptor 3 (CXCR3) and CXCR6. Classically, naïve T cell are primed in secondary lymphoid organs where antigen presenting cells (APC), among which DCs, macrophages and B cells, present peptides from apolipoprotein B100 (ApoB100) of LDL on MHC class molecules. Naïve T cells recognize this

complex through engagement of the T cell receptor (TCR) and co-stimulatory molecules with their ligands, resulting in T cell clonal expansion. Co-stimulatory molecules induce T cells to express transcription factors that favour the differentiation into distinct T helper (Th) phenotypes. Homing receptors promote T cell migration to atherosclerotic lesions, where they secrete effector cytokines. Studies have identified the ApoB100 as antigen driving atherosclerosis. In line, its blocking with antibodies resulted in reduced plaque development both in humans and mice[42,43]. However, recent research is suggesting that atherosclerosis might be considered an autoimmune-like disease, confirmed by TCR clonality and antigen-specific activation, as well as aberrant T cell tolerance[40,41]. Moreover, Fernandez *et al*. demonstrated that plaque T cells are more activated and exhausted compared to their blood counterpart, suggesting potentially loss of T cell functions, favoured by unresolving inflammation within the plaque microenvironment[27].

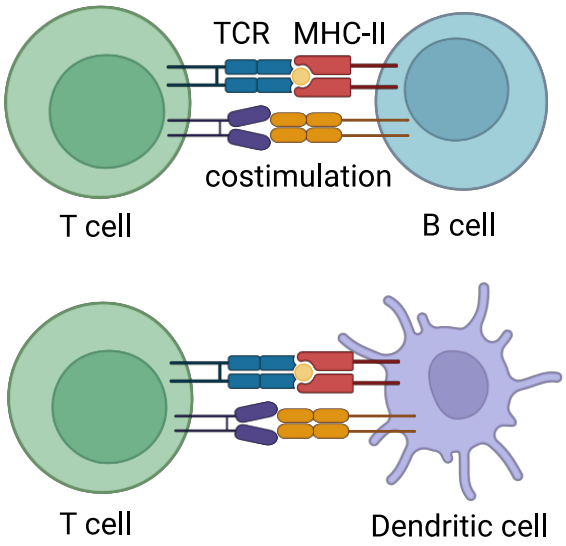

Figure 2: **T cell activation requires antigen presentation and co-stimulatory signals.** T cell activation involves the recognition of specific antigens. The process occurs in several steps. The antigen is presented on the surface of antigen presenting cells (APC), namely B cells, macrophages or dendritic cells, using the major histocompatibility complex (MHC). MHC class I presents to $CD8^+$ cytotoxic T cells, while MHC class II presents to $CD4^+$ T helper cells. The T cell receptor (TCR) specifically binds to the peptide-antigen-MHC complex on the surface of the APC. A second signal, co-stimulation, is necessary for T cell activation. This occurs through the binding of co-stimulatory molecules on the surface of the APC (such as CD80/CD86) to receptors on the T cell (e.g., CD28). This additional signal is necessary to prevent an incomplete or an inappropriate immune response. Upon successful binding and co-stimulatory signal reception, T cells get stimulated by cytokines produced by the APC or surrounding cells, influencing their differentiation and proliferation. Activated T cells undergo clonal expansion, and differentiate into effector T cells or memory T cells. Adapted from Nitz *et al* [44]. *Created with Biorender.*

$CD4^+$ T cells in atherosclerotic plaques can differentiate into Th cells or Tregs, responsible for the activation or the dampening of the immune response, respectively. Once activated, subsets of $CD4^+$ effector T cells are distinguished based on the transcription factor they express and on the cytokines they produce. Each set of cytokines is optimized for combatting different types of pathogens, e.g., bacteria or helminths. In immunology, this differentiation of T cells is defined as polarization. Th cells are distinguished in T helper 1 (Th1), T helper 2 (Th2) and T helper 17 (Th17). Th1 represent the vast majority of T cells in atherosclerotic plaques. They are responsible for the production of high levels of IFN-γ, which further promotes macrophages and T cell recruitment and consequently plaque growth. The polarization towards Th1 cells is driven by the cytokines IL-12 and IFN-γ, which stimulate Th1 differentiation by activating the transcription factors T-bet, STAT1 and STAT4.

On the other hand, Th2 polarization is favoured by IL-4, which, upon binding to its receptor on Th cells, is able to activate the two transcription factors: STAT6 and GATA-3. The cytokines most typically associated with Th2 cells are IL-4, IL-5, IL-9 and IL-13, and combinations of these cytokines drive B cell proliferation and immunoglobulin class-switching to IgE, eosinophilia (mainly induced by IL-5) and mast cell proliferation (induced by IL-9). Whether Th2 cells are pro-atherogenic or athero-protective remains unclear and the role of IL-4 is still under investigation[45].

In addition, Th17 activate the immune response against extracellular bacteria and fungi. They also have a role in mediating the response in autoimmune diseases[46]. IL-1, IL-6 and IL-23 promote differentiation towards the Th17 phenotype. At the transcriptional level, Th17 differentiation relies on the factors RORγt and STAT3. The role of Th17 in atherosclerosis remains controversial[45,47].

Tregs contribute to maintain tolerance to self-antigens, thereby preventing autoimmunity. Natural Tregs (nTreg) develop in the thymus where they are instructed to recognize “self” antigens. Another type of Tregs, known as inducible Tregs (iTreg) can be generated in the periphery in the presence of transforming growth factor-β (TGF-β) or IL-10.

Tregs are generally viewed to be athero-protective in mouse models in the early stages of atherosclerosis, whereas they only play a minor role in advanced atherosclerosis[48]. Depletion of Tregs aggravates atherosclerosis[49]. Contrarily, increase in Tregs number in the plaque limit the atherosclerotic pathology[50,51].

$CD8^+$ T cells are key player in cell-mediated immunity, found both in murine and human atherosclerotic plaques[27,28,52]. Although lower in number in early plaques, they appear to be the dominating cell types in advanced human lesions. Once activated they induce apoptosis through the release of cytotoxins (e.g., perforins), and they secrete cytokines such as IFN-γ and TNF-α.

Although more rarely found, invariant natural killer T (iNKT) cells can accumulate in atherosclerotic plaques. They recognize a variety of lipid antigens presented by the atypical MHC class I molecule CD1d. They respond rapidly upon activation, producing type 1, type 2 or type 17 prototypical cytokines. Differently from T helper cells, iNKT get polarized in the thymus into iNKT1, iNKT2 or iNKT17 subsets, and they are capable of producing the typical cytokines of Th cells, namely IFN-γ (iNKT1), IL-4, IL-5, IL-9, IL-13 (iNKT2), and IL-17 (iNKT17). The role of iNKT cells during atherosclerosis is however under debate[53,54].

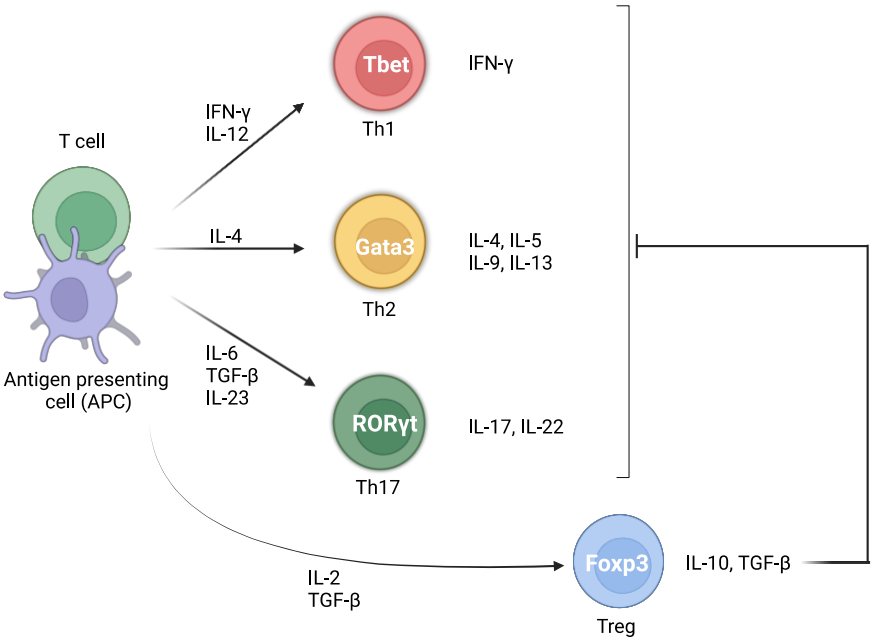

Figure 3: **Schematic overview of T cell polarization.** T cell polarization refers to the differentiation of naïve T cells into distinct subsets, each with specialized functions that respond to various pathogens and environmental cues. This process is largely influenced by the cytokine microenvironment during T cell activation and plays a crucial role in determining the nature of the immune response. Adapted from Cellular and Molecular Immunology, Abul K. Abbas, Andrew H. Lichtman, Shiv Pillai (8th edition), Saunders, 2015. *Created with Biorender.*

6.3.3 Clinical outlook

Despite the lack of effective therapies targeting the aforementioned cell types, the field of immunotherapy in atherosclerosis is rapidly advancing. As T cells constitute the largest leukocyte population within human atherosclerotic plaques, they represent a critical target for developing more precise and effective therapies. Findings from the LILACS study have demonstrated that administering low-dose IL-2 in patients with stable ischemic heart disease and acute coronary syndrome is safe. This selectively promoted Treg expansion without causing significant adverse effects[55]. These findings suggest that low-dose IL-2 therapy has the potential to modulate immune responses and reduce inflammation, providing a promising avenue for future therapeutic strategies aimed at improving outcomes in ASCVD. However, further studies are needed to assess its long-term efficacy and clinical benefits.

A second study, the *Rituximab in Patients With Acute ST-Elevation Myocardial Infarction study* (RITA-MI) has deemed B cell depletion with the monoclonal antibody rituximab to be safe and effective after ST-elevation MI (STEMI)[56]. This study represents the first evidence of safety of Rituximab in patients after MI, and serves as the foundation for the ongoing trial RITA-MI2 tiral. This phase 2b randomized double-blind placebo-controlled clinical trial aims at assessing the impact of B cell depletion with the CD20 monoclonal antibody rituximab on left ventricular dysfunction and cardiac remodeling following acute MI.

Atherosclerosis is further being recognized as possessing autoimmune features, though it lacks the classical features to define an autoimmune disease. The chronic inflammatory nature of atherosclerosis and the role of the immune system in its progression underscore its autoimmune-like characteristics. It is currently unknown whether the autoimmune component of atherosclerosis can be addressed targeting anti-inflammatory cytokines and pathways; however, vaccination and/or immunomodulation might provide a future antigen-specific therapy, avoiding weakening the host defence[45].

To sum up, taming inflammation through targeted modulation of the innate and adaptive immune system is an attractive, yet challenging therapeutic alternative, given the complexity and abundance of inflammatory mediators in atherosclerosis.

6.4 Epigenetic landscape in atherosclerosis

The term “epigenetics” was introduced in 1942 by the embryologist Conrad Waddington, who defined it as “the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being”[57,58]. In particular, epigenetic marks are of paramount importance for maintaining genomic stability. The role of epigenetics in cardiovascular disease (CVD) has gained increasing significance over the last years[59,60]. Epigenetics mainly affects CVD progression by regulating the function and expression levels of CVD-related genes. This regulation occurs through mechanisms such as DNA methylation, histone modification, and noncoding RNA regulation.

Histone methylation and acetylation are the most important post-translational modifications, which occur at the N terminus of lysine and arginine. The most common histone modifications include, among others, histone (H)3 lysine (K)4, H3K9, H3K27, and H3K79 methylation, as well as H3K9, H3K14 and H3K27 acetylation. Depending on the methylated (me) or acetylated (ac) residues, these modifications may result into transcriptional activation (e.g., H3K4me3; H3K79me2,3; H3K9ac; H3K27ac) or repression (e.g., H3K9me2,3 and H3K27me2,3). In this project, we concentrate our attention on the repressive epigenetic mark H3K27me3. The addition of mono-, di- or tri-methyl groups at the H3K27 residue is mediated by the polycomb repressive complex 2 (PRC2). PRC2 is fundamental to maintain adequate embryonic stem cell fate specification[61,62]. The PRC2 is composed by several subunits: the enhancer of zeste homolog 2 (EZH2) or its paralogue EZH1, which has a SET domain and represents the catalytic subunit of the complex. Further components are the suppressor of zeste 12 (SUZ12), the embryonic ectodomain development (EED), the Histone-binding protein RBBP4, and the accessory protein JARID2 (jumonji and (A+T)-rich interaction domain-containing protein 2)[63,64].

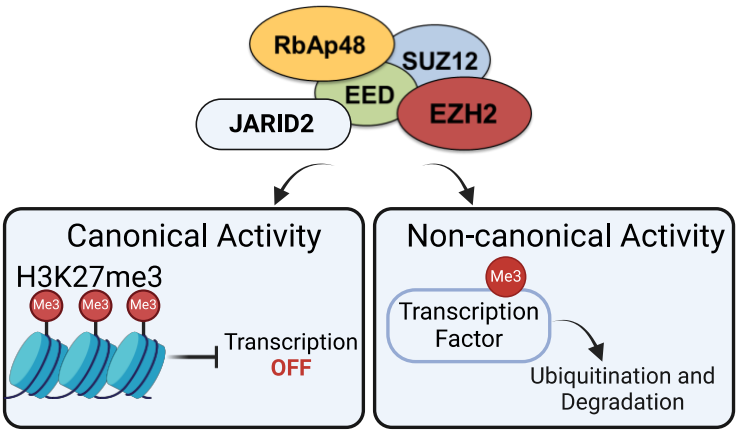

Figure 4: **The polycomb repressive complex 2 (PRC2) and EZH2.** PRC2 is key in regulating genes responsible for directing T cell activation and polarization by mediating the T cell transcriptome via methylation of the H3K27 epigenetic mark. EZH2 represents the catalytic subunit of PRC2. Its functions have been mostly attributed to its canonical H3K27 methylation activity. Recent evidence is suggesting that EZH2 can directly interact with transcription factors, in a chromatin-independent fashion. *Created with Biorender.*

EZH2 plays a key role in the development, activation and differentiation of immune cells, in particular macrophages[65,66] and T cells[67], to dynamically regulate immune responses and tissue homeostasis. During atherosclerosis, EZH2 and H3K27me3 tightly control the expression of T cell polarization-determining transcription factors and cytokines, resulting in maintenance of specific immune cell responses. It has been demonstrated that the repressive epigenetic mark H2K27me3 is essential for lineage commitment and plasticity in developing effector T cells as well as iTregs and nTregs[68]. EZH2 gain-of-functions and loss-of-functions mutations, along with its overexpression, are closely linked to cancer development, making EZH2 a compelling target for novel cancer therapies. The pursuit of EZH2-specific inhibitors has seen significant progresses, yielding promising preclinical ourcomes[69]. Notably, in 2020, the *Food and Drug Administration (FDA)* granted accelerated approval for an EZH2 inhibitor, tazemetostat (Tazverik), for the treatment of adults and pediatric patients with metastatic or locally advanced epithelioid sarcoma not eligible for complete resection. Tazemetostat is also being explored in clinical trials for the treatment of lymphomas[70]. These encouraging findings may open new avenues for repurposing the drug in addressing ASCVD.

EZH2's catalytic activity can also occur in a non-canonical, chromatin-independent fashion, through direct methylation of proteins, such as talin, a crucial regulatory molecule in cell migration[71] or transcription factors, such as Foxp3 (forkhead box P3)[72] and *Zbtb16* (Zinc Finger

And BTB Domain Containing 16), the gene coding for promyelocytic leukemia zinc finger (Plzf), the iNKT cell lineage defining transcription factor[73]. In the setting of atherosclerosis, Neele *et al*. demonstrated that myeloid-specific EZH2 deficiency in mice results in reduced atherosclerotic development. To date, little is known on the role of EZH2 in T cells during the pathogenesis of atherosclerosis.

Given the urgent need of more targeted immunotherapies to tackle ASCVD, this dissertation includes two potential approaches through which T cells can be targeted to limit atherosclerosis. The first mechanism elucidates the epigenetic role of the enzyme EZH2 in T cells in the setting of atherosclerosis, with the help of T-cell specific $ApoE^{-/-}$ (Apolipoprotein E deficient) EZH2 knockout mouse lines (*Ezh2* $^{flox/flox}$, $Cd4^{cre}$ or $Cd8^{cre}$). We demonstrated that $CD4^{+}$ T cell-specific Ezh2-deficiency induces a type 2 immune response, mediated by Th2, iNKT2 cells, and anti-inflammatory macrophages, resulting in athero-protection. Hence, targeting Ezh2 in T cells might be a promising therapeutic approach in atherosclerosis.

The second part of this dissertation focuses on the endogenous amino acid homoarginine (HA), which is a strong biomarker for cardiovascular outcome and mortality, with low circulating levels associated to higher risk of CVD[74,75]. Dietary supplementation of HA has been shown to decrease atherosclerosis in $ApoE^{-/-}$ mice, with substantial reduction of $CD4^{+}$ T cells infiltrating atherosclerotic lesions, highlighting a potential mechanism by which HA supports CVD health. Specifically, HA inhibits T cell proliferation and impairs the migratory capacity of T cells.

In humans, HA administration daily at dose of 125 mg for 4 weeks has been demonstrated to be safe and well tolerated in healthy volunteers[76]. An ongoing clinical trial (https://www.clinicaltrials.gov; Unique identifier: NCT03692234) is investigating the effects of oral HA administration in patients suffering from ischemic stroke.

In conclusion, HA administration presents an intriguing, cost-effective and accessible therapeutic option for patients suffering from ASCVD.

7. Summary

Despite the recent advances in lipid-lowering therapies, atherosclerosis is the main cause of CVD, which constitutes the global health burden. The underlying chronic inflammation is orchestrated by a plethora of immune cell types. Among these, T cells were discovered to be the most abundant cell type. Depending on their activation and polarization status, T cells can be pro- or anti-atherogenic. Given their multi-faceted nature, they represent an attractive target for novel immunotherapies to combat ASCVD.

In the present dissertation I investigated the role of T cells during plaque development from an epigenetic (Publication I) and metabolic (Publication II) point of view.

7.1 Paper I: Ezh2 shapes T cell plasticity to drive atherosclerosis

In publication I, we investigated the role of the epigenetic enzyme EZH2 in T cells during atherosclerosis. In collaboration with the Munich Vascular Biobank (Technical University, Munich), we analysed the expression of EZH2 in human atherosclerotic plaques collected from patients undergoing carotid endarterectomy (CEA). We found that EZH2 expression is upregulated in advanced atherosclerotic lesions, as compared to early lesions. Moreover, the analysis of publicly available single-cell gene expression data from patients who also underwent a CEA showed that EZH2 is predominantly expressed in plaque T cells.

To investigate and understand the role of EZH2 in T cells during atherosclerosis we generated $CD4^+$ T cell-specific, as well as $CD8^+$ T cell-specific Ezh2-deficient mice, and we backcrossed them on an atherosclerotic $ApoE^{-/-}$ background ($Ezh2^{cd4}$-KO and $Ezh2^{cd8}$-KO) and respective littermate controls ($Ezh2^{cd4}$-wild type(WT) and $Ezh2^{cd8}$-WT). The mice were fed a western-type diet, to promote the development of atherosclerosis. Analysis of atherosclerotic plaques revealed a significant reduction in plaque burden and plaque progression in $Ezh2^{cd4}$-KO, but not $Ezh2^{cd8}$-KO mice, compared to respective controls. ScRNA-seq of splenic T cells, isolated from $Ezh2^{cd4}$-WT and $Ezh2^{cd4}$-KO mice, revealed a reduction of naive $CD4^+$- and $CD8^+$- T cell, Ccl5+ and regulatory T cell populations, but increased percentages of $CD4^+$- and $CD8^+$-memory T cells, and especially iNKT cells in $Ezh2^{cd4}$-KO mice. Flow cytometric analysis identified a shift towards T helper 2 (Th2) effector $CD4^+$ T cells in $Ezh2^{cd4}$-KO mice and confirmed a profound increase in splenic iNKT cells with high expression of Plzf, the characteristic marker of the

iNKT2 subset. In line, *Zbtb16* transcripts were significantly upregulated in the descending aorta of Ezh2^{cd4}-KO mice.

Moreover, we observed a significant increase in the master cytokines of type 2 immune response, namely Il-4 and Il-13, both in the plasma and in the supernatant of CD4^{+} T cells isolated from Ezh2^{cd4}-KO mice, compared to respective controls. Furthermore, we observed a significant upregulation of *Il-4* transcripts in the descending aorta of Ezh2^{cd4}-KO mice compared to controls, suggesting a local role of the type 2 immune response, likely mediated by Th2 and iNKT2 cells.

To dissect the mechanism, we performed bulkRNA -sequencing of CD4^{+} T cells of Ezh2^{cd4}-WT and Ezh2^{cd4}-KO mice. The analysis confirmed a significant upregulation of *Zbtb16*, the gene coding for Plzf, in *Ezh2*-deficient CD4^{+} T cells. H3K27me3 chromatin immunoprecipitation (ChIP) on splenic T cells isolated from both Ezh2^{cd4}-KO and Ezh2^{cd4}-WT mice, followed by qPCR revealed a significant enrichment of H3K27me3 at the transcription starting site of both the *Zbtb16* gene and *Il-4* gene in Ezh2^{cd4}-WT mice, suggesting the loss of repression in Ezh2^{cd4}-KO mice.

To sum up, in this project we demonstrated in human and mice a pivotal role of EZH2 in T cell during the pathogenesis of atherosclerosis. Inhibition of Ezh2 in CD4^{+} T cells drives type 2 immune responses, resulting in an accumulation of iNKT2 and Th2 cells, memory-T cells and anti-inflammatory macrophages that limit the progression of atherosclerosis.

7.2 Paper II: The amino acid homoarginine inhibits atherogenesis by modulating T-cell function

In publication II, we investigated the effect of dietary HA supplementation via the drinking water on atherosclerotic plaque development in mice. The emphasis was particularly on a possible influence of HA on the inflammatory component of atherosclerosis.

Female ApoE$^{-/-}$ mice were administered HA (14 mg/L) via the drinking water, starting two weeks before and continuing throughout a six-week feeding period of Western-type diet. Control mice received standard drinking water. The characterization of the immune response and atherosclerotic plaques of the animals was performed using immunohistochemistry and flow cytometry.

HA supplementation doubled circulating HA levels and significantly reduced atherosclerotic plaque burden in both the aortic root and brachiocephalic trunk. A marked decrease in T cells within atherosclerotic lesions, as well as in the periphery, indicated a T cell–mediated effect, predominantly involving $CD4^+$ T cells. Conversely, other immune cell populations, including macrophages, dendritic cells (DCs), and B cells remained unaffected.

To further investigate the underlying mechanism, we analysed the HA-treated T cells and respective controls using mass spectrometry-based proteomics, functional assays (e.g., proliferation and migration/chemotaxis), and super-resolution microscopy. Proteomic and pathway analysis, along with functional *in vitro* studies, revealed that HA altered the organization of actin cytoskeleton in T cells and enhanced filopodia formation by inhibiting Myh9 (myosin heavy chain 9). Further functional studies also demonstrated that HA inhibits T cell proliferation and significantly impairs T cell migration, mechanisms that are likely contributing to its athero-protective properties.

This study identifies a novel molecular mechanism, through which HA mitigates the formation of atherosclerotic plaques. These findings provide a mechanistic explanation for the clinical and epidemiological data of recent years, which show a positive correlation between HA and cardiovascular health.

8. Zusammenfassung

Trotz der kontinuierlichen Fortschritte in der lipidsenkenden Therapie stellen Atherosklerose-bedingte Herzkreislauferkrankung weiterhin eine globale Gesundheitsbelastung dar. Die zugrunde liegende chronische Entzündung wird durch eine Vielzahl von Immunzelltypen gesteuert. Unter diesen wurden T-Zellen als der am häufigsten vorkommende Zelltyp in humanen atherosklerotischen Plaques identifiziert. Abhängig von ihrem Aktivierungs- und Polarisationsstatus können T-Zellen pro- oder anti-atherogen wirken. Aufgrund ihrer vielseitigen Natur stellen sie ein attraktives Ziel für neuartige Immuntherapien zur Behandlung von atherosklerotischen Herzkreislauferkrankungen dar.

In der vorliegenden Arbeit habe ich die Rolle von T-Zellen in der Pathogenese der Atherosklerose aus epigenetischer (Publikation I) und metabolischer (Publikation II) Perspektive untersucht.

8.1 Publikation I: Ezh2 shapes T cell plasticity to drive atherosclerosis

In Publikation I untersuchten wir die Rolle des epigenetischen Enzyms EZH2 in T-Zellen in der Pathogenese der Atherosklerose. In Zusammenarbeit mit der Munich Vascular Biobank (Technische Universität München) haben wir die Expression von EZH2 in humanen atherosklerotischen Plaques, die von Patienten im Rahmen einer Carotis-Endarteriektomie (CEA) gesammelt wurden, analysiert. Wir fanden heraus, dass EZH2 in fortgeschrittenen atherosklerotischen Läsionen deutlich stärker exprimiert wird als in frühen Läsionen. Die Analyse öffentlich zugänglicher *single cell*-Genexpressionsdaten von Patienten, die ebenfalls einer CEA unterzogen wurden, zeigte darüber hinaus, dass EZH2 überwiegend in Plaque-T-Zellen exprimiert wird.

Um die Rolle von EZH2 in T-Zellen während der Atherosklerose zu untersuchen und zu verstehen, haben wir $CD4^+$ T-Tell-spezifische sowie $CD8^+$ T-Tell-spezifische Ezh2-Knockout Mäuse generiert und diese auf einen atherosklerotischen $ApoE^{-/-}$-Hintergrund Mäuse zurückgekreuzt ($Ezh2^{cd4}$-KO, bzw. $Ezh2^{cd8}$-KO und $Ezh2^{cd4}$-Wildtyp (WT), bzw. $Ezh2^{cd8}$-WT Kontrollen). Die Mäuse wurden mit einer *Western* Diät gefüttert, um die Entstehung der Atherosklerose zu beschleunigen. Eingehende Analysen der atherosklerotischen Plaques zeigten eine signifikante Reduktion der Plaquebelastung und Plaqueprogression in $Ezh2^{cd4}$-KO, jedoch nicht in $Ezh2^{cd8}$-KO Mäusen im Vergleich zu den jeweiligen Kontrollen. Eine scRNA-seq-Analyse der T-Zellen, die aus der Milz von atherosklerotischen $Ezh2^{cd4}$-WT und $Ezh2^{cd4}$-KO

Mäusen isoliert wurden, zeigte eine Reduktion naiver $CD4^+$- und $CD8^+$-T-Zell-, $Ccl5^+$- und regulatorischer T-Zell-Populationen (Treg), sowie einen starken Anstieg an $CD4^+$- und $CD8^+$-Gedächtnis-T-Zellen und insbesondere iNKT-Zellen in $Ezh2^{cd4}$-KO Mäusen. Durchflusszytometrische Analysen zeigten darüber hinaus eine Verschiebung hin zu T-Helfer-2-(Th2)-Effektor-$CD4^+$-T-Zellen in $Ezh2^{cd4}$-KO Mäusen und bestätigten eine deutliche Zunahme der iNKT-Zellen, die eine starke Expression von Plzf aufzeigten, dem charakteristischen Marker der iNKT2-Subpopulation. Entsprechend konnten wir einen signifikanten Anstieg der *Zbtb16*-Transkripte in der Aorta von $Ezh2^{cd4}$-KO Mäusen beobachten.

Weiter konnten wir einen deutlichen Anstieg der charakteristischen Zytokine der Typ-2-Immunantwort, Il-4 und Il-13, sowohl im Plasma als auch im Überstand von $CD4^+$-T-Zellen, die aus $Ezh2^{cd4}$-KO Mäusen isoliert wurden, im Vergleich zu den entsprechenden Kontrollen verzeichnen. Zudem zeigte sich eine signifikante Hochregulation von *Il4*-Transkripten in der Aorta von $Ezh2^{cd4}$-KO Mäusen im Vergleich zu Kontrollen, was auf eine lokale Rolle der Typ-II-Immunantwort hindeutet, die wahrscheinlich durch Th2- und iNKT2-Zellen vermittelt wird.

Um den Mechanismus zu entschlüsseln, Bulk RNA-Sequenzierungsexperimente an $CD4^+$-T-Zellen aus $Ezh2^{cd4}$-WT- und $Ezh2^{cd4}$-KO-Mäusen durchgeführt. Die Analysen bestätigten eine signifikante Hochregulation von *Zbtb16*, dem Gen, das für Plzf kodiert, in Ezh2-defizienten $CD4^+$-T-Zellen. Eine H3K27me3-Chromatin-Immunpräzipitation (ChIP) an T-Zellen aus der Milz von $Ezh2^{cd4}$-KO- und $Ezh2^{cd4}$-WT-Mäusen, gefolgt von qPCR, zeigte eine signifikante Anreicherung von H3K27me3 an der Transkriptionsstartstelle sowohl des *Zbtb16*-Gens als auch des *Il-4*-Gens in $Ezh2^{cd4}$-WT-Mäusen, was auf den Verlust der Repression in $Ezh2^{cd4}$-KO-Mäusen hindeutet.

Zusammenfassend konnten wir in dieser Arbeit in Maus und Mensch die entscheidende Rolle von EZH2 in T-Zellen in der Pathogenese der Atherosklerose zeigen. Die Hemmung von Ezh2 in $CD4^+$-T-Zellen fördert eine Typ-II-Immunantwort, was zu einer Akkumulation von iNKT2- und Th2-Zellen, Gedächtnis-T-Zellen und anti-inflammatorischen Makrophagen führt, die die Progression der Atherosklerose begrenzen.

8.2 Publikation II: The amino acid homoarginine inhibits atherogenesis by modulating T-cell function

In Publikation II haben wir den Effekt einer diätetischen Supplementation mit der Aminosäure Homoarginin (HA) über das Trinkwasser auf die Entwicklung atherosklerotischer Plaques

Mäusen untersucht. Besonderer Schwerpunkt lag hierbei auf einem möglichen Einfluss von HA auf die entzündliche Komponente der Atherosklerose. Weiblichen $ApoE^{-/-}$ Mäusen wurde HA (14 mg/L) über das Trinkwasser verabreicht, beginnend zwei Wochen vor und während einer sechswöchigen Fütterungsperiode mit einer *Western* Diät. Kontrollmäuse erhielten standardmäßiges Trinkwasser. Die Charakterisierung der Immunantwort sowie der atherosklerotischen Plaques der Tiere erfolgte mittels Immunhistochemie und Durchflusszytometrie. Die HA-Supplementierung verdoppelte die zirkulierenden HA-Spiegel und reduzierte signifikant die atherosklerotische Plaquebelastung sowohl in der Aortenwurzel als auch im *Truncus brachiocephalicus*. Ein deutlicher Rückgang von T-Zellen innerhalb der atherosklerotischen Läsionen sowie in der Peripherie deutete auf einen T-Zell-vermittelten Effekt hin, der hauptsächlich $CD4^+$-T-Zellen betraf. Im Gegensatz dazu blieben andere Immunzellpopulationen, einschließlich Makrophagen, dendritischer Zellen und B-Zellen, unbeeinflusst.

Um den zugrunde liegenden Mechanismus aufzuklären, haben wir die HA-behandelten T-Zellen und entsprechende Kontrollen eingehend mittels Massenspektrometrie-basierter Proteom-Analyse, funktionellen Assays (z.B. Proliferation und Migration/Chemotaxis) sowie Super-Resolution-Mikroskopie untersucht. Die Proteom- und anschließende Signalweg-Analyse, sowie funktionelle *in vitro* Studien zeigten, dass HA die Organisation des Aktinzytoskeletts von T-Zellen veränderte und die Bildung von Filopodien durch die Hemmung von Myh9 (Myosin IIA) verstärkte. Weiterführende Studien zeigten darüber hinaus, dass HA die T-Zell-Proliferation hemmt und die Migration von T-Zellen signifikant beeinträchtigt, Mechanismen, die vermutlich alle zu HA‘s atheroprotektiven Eigenschaften beitragen.

Zusammenfassend identifiziert diese Arbeit erstmalig einen neuartigen molekularen Mechanismus, durch den HA die Ausbildung von atherosklerotischen Plaques abschwächt. Diese Ergebnisse bieten endlich eine mechanistische Erklärung für die klinischen und epidemiologischen Daten der letzten Jahre, die einen positiven Zusammenhang zwischen HA und kardiovaskulärer Gesundheit zeigen.

9. Paper I

Abstract:

Background: The activation and polarization of T cells play a crucial role in atherosclerosis and dictate athero-inflammation. The epigenetic enzyme EZH2 (enhancer of zeste homolog 2) mediates the H3K27me3 (trimethylation of histone H3 lysine 27) and is pivotal in controlling T cell responses.

Methods: To detail the role of T cell EZH2 in atherosclerosis, we used human carotid endarterectomy specimens to reveal plaque expression and geography of EZH2. Atherosclerosis-prone *Apoe* (apolipoprotein E)-deficient mice with CD (cluster of differentiation) 4^+ or $CD8^+$ T cell-specific *Ezh2* deletion ($Ezh2^{cd4}$-knockout [KO], $Ezh2^{cd8}$-KO) were analyzed to unravel the role of T cell Ezh2 in atherosclerosis and T cell-associated immune status.

Results: *EZH2* expression is elevated in advanced human atherosclerotic plaques and primarily expressed in the T cell nucleus, suggesting the importance of canonical EZH2 function in atherosclerosis. $Ezh2^{cd4}$-KO, but not $Ezh2^{cd8}$-KO, mice showed reduced atherosclerosis with fewer advanced plaques, which contained less collagen and macrophages, indicating that Ezh2 in $CD4^+$ T cells drives atherosclerosis. In-depth analysis of $CD4^+$ T cells of $Ezh2^{cd4}$-KO mice revealed that absence of Ezh2 results in a type 2 immune response with increased Il-4 (interleukin 4) gene and protein expression in the aorta and lymphoid organs. In vitro, *Ezh2*-deficient T cells polarized macrophages toward an anti-inflammatory phenotype. Single-cell RNA-sequencing of splenic T cells revealed that *Ezh2* deficiency reduced naive, $Ccl5^+$ (C-C motif chemokine ligand 5) and regulatory T cell populations and increased the frequencies of memory T cells and invariant natural killer T (iNKT) cells. Flow cytometric analysis identified a shift toward Th2 (type 2 T helper) effector $CD4^+$ T cells in $Ezh2^{cd4}$-KO mice and confirmed a profound increase in splenic iNKT cells with increased expression of Plzf (promyelocytic leukemia zinc finger), which is the characteristic marker of the iNKT2 subset. Likewise, *Zbtb16* ([zinc finger and BTB domain containing 16], the Plzf-encoding gene) transcripts were elevated in the aorta of $Ezh2^{cd4}$-KO mice, suggesting an accumulation of iNKT2 cells in the plaque. H3K27me3-chromatin immunoprecipitation followed by quantitative polymerase chain reaction showed that T cell-Ezh2 regulates the transcription of the *Il-4* and *Zbtb16* genes.

Conclusions: Our study uncovers the importance of T cell EZH2 in human and mouse atherosclerosis. Inhibition of Ezh2 in $CD4^+$ T cells drives type 2 immune responses, resulting in an accumulation of iNKT2 and Th2 cells, memory T cells and anti-inflammatory macrophages that limit the progression of atherosclerosis.

Keywords: EZH2; T-lymphocytes; atherosclerosis; epigenomics; natural killer T cells.

Copyright Disclaimer:

The research presented in this chapter is based on the following published manuscript "*Ezh2 shapes T cell plasticity to drive atherosclerosis*" : (Bonfiglio CA, Lacy M, Triantafyllidou V, Farina FM, Janjic A, Nitz K, Wu Y, Bazioti V, Avcilar-Kücükgöze I, Marques YFS, Joppich M, Kumkum M, Röß K, Venkatasubramani AV, Imhof A, Enard W, Maegdefessel L, de Winther M, Weber C, Santovito D, Lutgens E, Atzler D. Ezh2 Shapes T Cell Plasticity to Drive Atherosclerosis. Circulation. 2025 May 13;151(19):1391-1408. doi: 10.1161/CIRCULATIONAHA.124.072384. Epub 2025 Feb 7. PMID: 39917842; PMCID: PMC12063685.). As required for cumulative dissertations, my specific contribution to the paper is detailed in the 'Author Contributions' section (p. 8) of this thesis, approval from all co-authors has been obtained. No changes have been made to the original article.

10. Paper II

Abstract:

Background: Amino acid metabolism is crucial for inflammatory processes during atherogenesis. The endogenous amino acid homoarginine is a robust biomarker for cardiovascular outcome and mortality with high levels being protective. However, the underlying mechanisms remain elusive. We investigated the effect of homoarginine supplementation on atherosclerotic plaque development with a particular focus on inflammation.

Methods: Female ApoE-deficient mice were supplemented with homoarginine (14 mg/L) in drinking water starting 2 weeks before and continuing throughout a 6-week period of Western-type diet feeding. Control mice received normal drinking water. Immunohistochemistry and flow cytometry were used for plaque- and immunological phenotyping. T cells were characterized using mass spectrometry-based proteomics, by functional in vitro approaches, for example, proliferation and migration/chemotaxis assays as well as by super-resolution microscopy.

Results: Homoarginine supplementation led to a 2-fold increase in circulating homoarginine concentrations. Homoarginine-treated mice exhibited reduced atherosclerosis in the aortic root and brachiocephalic trunk. A substantial decrease in $CD3^+$ T cells in the atherosclerotic lesions suggested a T-cell-related effect of homoarginine supplementation, which was mainly attributed to $CD4^+$ T cells. Macrophages, dendritic cells, and B cells were not affected. $CD4^+$ T-cell proteomics and subsequent pathway analysis together with in vitro studies demonstrated that homoarginine profoundly modulated the spatial organization of the T-cell actin cytoskeleton and increased filopodia formation via inhibition of Myh9 (myosin heavy chain 9). Further mechanistic studies revealed an inhibition of T-cell proliferation as well as a striking impairment of the migratory capacities of T cells in response to relevant chemokines by homoarginine, all of which likely contribute to its atheroprotective effects.

Conclusions: Our study unravels a novel mechanism by which the amino acid homoarginine reduces atherosclerosis, establishing that homoarginine modulates the T-cell cytoskeleton and thereby mitigates T-cell functions important during atherogenesis. These findings provide a molecular explanation for the beneficial effects of homoarginine in atherosclerotic cardiovascular disease.

Keywords: amino acid; atherosclerosis; biomarker; cardiovascular disease; homoarginine.

Copyright Disclaimer:

The research presented in this chapter is based on the following published manuscript "*The aminoacid homoarginine inhibits atherogenesis by modulatin T-cell function*" : (Nitz K, Lacy M, Bianchini M, Wichapong K, Kücükgöze IA, Bonfiglio CA, Migheli R, Wu Y, Burger C, Li Y, Forné I, Ammar C, Janjic A, Mohanta S, Duchene J, Heemskerk JWM, Megens RTA, Schwedhelm E, Huveneers S, Lygate CA, Santovito D, Zimmer R, Imhof A, Weber C, Lutgens E, Atzler D. The Amino Acid Homoarginine Inhibits Atherogenesis by Modulating T-Cell Function. Circ Res. 2022 Sep 30;131(8):701-712. doi: 10.1161/CIRCRESAHA.122.321094. Epub 2022 Sep 14. PMID: 36102188). As required for cumulative dissertations, my specific contribution to the paper is detailed in the 'Author Contributions' section (p. 8) of this thesis, approval from all co-authors has been obtained. No changes have been made to the original article.

11. Bibliography

1. Baron J. *The Life of Edward Jenner, MD with Illustrations of His Doctrines, and Selections from His Correspondence*. Cambridge University Press; 1827. doi:https://doi.org/10.1017/CBO9781107281806
2. Virchow R. *Gesammelte Abhandlungen Zur Wissenschaftlichen Medicin / von Rudolf Virchow.* Frankfurt A. M. : Meidinger Sohn & Comp.; 1856.
3. Ross R. The pathogenesis of atherosclerosis: an update. *N Engl J Med*. 1986;314(8):488-500. doi:10.1056/NEJM198602203140806
4. Kwon GP, Schroeder JL, Amar MJ, Remaley AT, Balaban RS. Contribution of macromolecular structure to the retention of low-density lipoprotein at arterial branch points. *Circulation*. 2008;117(22):2919-2927. doi:10.1161/CIRCULATIONAHA.107.754614
5. Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM. Lessons From Sudden Coronary Death. *Arterioscler Thromb Vasc Biol*. 2000;20(5):1262-1275. doi:10.1161/01.atv.20.5.1262
6. Davies MJ, Thomas AC. Plaque fissuring - the cause of acute myocardial infarction, sudden ischaemic death, and crescendo angina. *Br Heart J*. 1985;53(4):363-373. doi:10.1136/hrt.53.4.363
7. Paterson JC. Capilllary rupture with intimal hemorrhage as a cause of pulmonary thrombosis. In: *American Heart Journal*. Vol 18. ; 1939:451-457. doi:10.1016/S0002-8703(39)90671-X
8. Björkegren JLM, Lusis AJ. Atherosclerosis : Recent Developments. *Cell*. 2022;185(10):1630-1645. doi:10.1016/j.cell.2022.04.004
9. Visseren F, Mach F, Smulders YM, et al. 2021 ESC Guidelines on cardiovascular disease prevention in clinical practice. *Eur Heart J*. 2021;42(34):3227-3337. doi:10.1093/eurheartj/ehab484
10. Martin SS, Aday AW, Almarzooq ZI, et al. *2024 Heart Disease and Stroke Statistics: A Report of US and Global Data from the American Heart Association*. Vol 149.; 2024. doi:10.1161/CIR.0000000000001209
11. Blüher M. Obesity: global epidemiology and pathogenesis. *Nat Rev Endocrinol*. 2019;15(5):288-298. doi:10.1038/s41574-019-0176-8
12. Grundy SM, Stone NJ, Bailey AL, et al. *2018*

AHA/ACC/AACVPR/AAPA/ABC/ACPM/ADA/AGS/APhA/ASPC/NLA/PCNA Guideline on the Management of Blood Cholesterol: A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. Vol 139.; 2019. doi:10.1161/CIR.0000000000000625

13. Mach F, Baigent C, Catapano AL, et al. 2019 ESC/EAS Guidelines for the management of dyslipidaemias: Lipid modification to reduce cardiovascular risk. *Eur Heart J*. 2020;41(1):111-188. doi:10.1093/eurheartj/ehz455
14. Ridker PM, Danielson E, Fonseca FA., et al. Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. *Obstet Gynecol Surv*. 2009;64(3):168-170. doi:10.1097/01.ogx.0000344393.60303.0a
15. Sabatine MS, Giugliano RP, Wiviott SD, et al. Efficacy and Safety of Evolocumab in Reducing Lipids and Cardiovascular Events. *N Engl J Med*. 2015;372(16):1500-1509. doi:10.1056/nejmoa1500858
16. Robinson JG, Farnier M, Krempf M, et al. Efficacy and Safety of Alirocumab in Reducing Lipids and Cardiovascular Events. *N Engl J Med*. 2015;372(16):1489-1499. doi:10.1056/nejmoa1501031
17. Cannon CP, Blazing MA, Giugliano RP, et al. Ezetimibe Added to Statin Therapy after Acute Coronary Syndromes. *N Engl J Med*. 2015;372(25):2387-2397. doi:10.1056/nejmoa1410489
18. Markham A. Bempedoic Acid: First Approval. *Drugs*. 2020;80(7):747-753. doi:10.1007/s40265-020-01308-w
19. Nissen SE, Lincoff AM, Brennan D, et al. Bempedoic Acid and Cardiovascular Outcomes in Statin-Intolerant Patients. *N Engl J Med*. 2023;388(15):1353-1364. doi:10.1056/nejmoa2215024
20. Ridker PM, Everett BM, Thuren T, et al. Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease. *N Engl J Med*. 2017;377(12):1119-1131. doi:10.1056/nejmoa1707914
21. Ridker PM, MacFadyen JG, Everett BM, et al. Relationship of C-reactive protein reduction to cardiovascular event reduction following treatment with canakinumab: a secondary analysis from the CANTOS randomised controlled trial. *Lancet*. 2018;391(10118):319-328. doi:10.1016/S0140-6736(17)32814-3
22. Nidorf SM, Eikelboom JW, Budgeon CA, Thompson PL. Low-dose colchicine for

secondary prevention of cardiovascular disease. *J Am Coll Cardiol*. 2013;61(4):404-410. doi:10.1016/j.jacc.2012.10.027

23. Nidorf SM, Fiolet ATL, Mosterd A, et al. Colchicine in Patients with Chronic Coronary Disease. *N Engl J Med*. 2020;383(19):1838-1847. doi:10.1056/nejmoa2021372
24. Tardif JC, Kouz S, Waters DD, et al. Efficacy and Safety of Low-Dose Colchicine after Myocardial Infarction. *N Engl J Med*. 2019;381(26):2497-2505. doi:10.1056/nejmoa1912388
25. Ridker PM, Everett BM, Pradhan A, et al. Low-Dose Methotrexate for the Prevention of Atherosclerotic Events. *N Engl J Med*. 2019;380(8):752-762. doi:10.1056/nejmoa1809798
26. Zernecke A, Winkels H, Cochain C, et al. Meta-analysis of leukocyte diversity in atherosclerotic mouse aortas. *Circ Res*. 2020;127(3):402-426. doi:10.1161/CIRCRESAHA.120.316903
27. Fernandez DM, Rahman AH, Fernandez NF, et al. Single-cell immune landscape of human atherosclerotic plaques. *Nat Med*. 2019;25(3):1576-1588. doi:https://doi.org/10.1038/s41591-019-0590-4
28. Depuydt MAC, Prange KHM, Slenders L, et al. Microanatomy of the Human Atherosclerotic Plaque by Single-Cell Transcriptomics. *Circ Res*. 2020;127(11):1437-1455. doi:10.1161/CIRCRESAHA.120.316770
29. Bonaccorsi I, Spinelli D, Cantoni C, et al. Symptomatic carotid atherosclerotic plaques are associated with increased infiltration of natural killer (NK) cells and higher serum levels of NK activating receptor ligands. *Front Immunol*. 2019;10(JULY):1-9. doi:10.3389/fimmu.2019.01503
30. Kim K, Shim D, Lee JS, et al. Transcriptome analysis reveals nonfoamy rather than foamy plaque macrophages are proinflammatory in atherosclerotic murine models. *Circ Res*. 2018;123(10):1127-1142. doi:10.1161/CIRCRESAHA.118.312804
31. Spann NJ, Garmire LX, McDonald JG, et al. Regulated accumulation of desmosterol integrates macrophage lipid metabolism and inflammatory responses. *Cell*. 2012;151(1):138-152. doi:10.1016/j.cell.2012.06.054
32. Weber C, Meiler S, Döring Y, et al. CCL17-expressing dendritic cells drive atherosclerosis by restraining regulatory T cell homeostasis in mice. *J Clin Invest*. 2011;121(7):2898-2910. doi:10.1172/JCI44925

33. Daissormont ITMN, Christ A, Temmerman L, et al. Plasmacytoid dendritic cells protect against atherosclerosis by tuning T-cell proliferation and activity. *Circ Res*. 2011;109(12):1387-1395. doi:10.1161/CIRCRESAHA.111.256529
34. Sage AP, Murphy D, Maffia P, et al. MHC Class II-restricted antigen presentation by plasmacytoid dendritic cells drives proatherogenic T cell immunity. *Circulation*. 2014;130(16):1363-1373. doi:10.1161/CIRCULATIONAHA.114.011090
35. Sage AP, Tsiantoulas D, Binder CJ, Mallat Z. The role of B cells in atherosclerosis. *Nat Rev Cardiol*. 2019;16(3):180-196. doi:10.1038/s41569-018-0106-9
36. Tsiantoulas D, Sage AP, Mallat Z, Binder CJ. Targeting B cells in atherosclerosis: Closing the gap from bench to bedside. *Arterioscler Thromb Vasc Biol*. 2015;35(2):296-302. doi:10.1161/ATVBAHA.114.303569
37. Finn A V., Nakano M, Narula J, Kolodgie FD, Virmani R. Concept of vulnerable/unstable plaque. *Arterioscler Thromb Vasc Biol*. 2010;30(7):1282-1292. doi:10.1161/ATVBAHA.108.179739
38. Moreno PR, Falk E, Palacios IF, Newell JB, Fuster V, Fallon JT. Macrophage infiltration in acute coronary syndromes: Implications for plaque rupture. *Circulation*. 1994;90(2):775-778. doi:10.1161/01.CIR.90.2.775
39. Libby P, Geng YJ, Aikawa M, et al. Macrophages and atherosclerotic plaque stability. *Curr Opin lipodology*. 1996;7(5):330-335. doi:10.1097/00041433-199610000-00012
40. Depuydt MAC, Schaftenaar FH, Prange KHM, et al. Single-cell T cell receptor sequencing of paired human atherosclerotic plaques and blood reveals autoimmune-like features of expanded effector T cells. *Nat Cardiovasc Res*. 2023;2(2):112-125. doi:10.1038/s44161-022-00208-4
41. Wang Z, Zhang X, Lu S, et al. *Pairing of Single-Cell RNA Analysis and T Cell Antigen Receptor Profiling Indicates Breakdown of T Cell Tolerance Checkpoints in Atherosclerosis*. Vol 2. Springer US; 2023. doi:10.1038/s44161-023-00218-w
42. Asciutto G, Wigren M, Fredrikson GN, et al. Apolipoprotein B-100 antibody interaction with atherosclerotic plaque inflammation and repair processes. *Stroke*. 2016;47(4):1140-1143. doi:10.1161/STROKEAHA.116.012677
43. Kimura T, Kobiyama K, Winkels H, et al. Regulatory CD4+ T cells recognize major histocompatibility complex class II molecule-restricted peptide epitopes of apolipoprotein B. *Circulation*. 2018;138(11):1130-1143.

doi:10.1161/CIRCULATIONAHA.117.031420

44. Nitz K, Herrmann J, Lerman A, Lutgens E. Costimulatory and Coinhibitory Immune Checkpoints in Atherosclerosis: Therapeutic Targets in Atherosclerosis? *JACC Basic to Transl Sci*. 2024;9(6):827-843. doi:10.1016/j.jacbts.2023.12.007
45. Saigusa R, Winkels H, Ley K. T cell subsets and functions in atherosclerosis. *Nat Rev Cardiol*. 2020;17(7):387-401. doi:10.1038/s41569-020-0352-5
46. Dong C. TH17 cells in development: An updated view of their molecular identity and genetic programming. *Nat Rev Immunol*. 2008;8(5):337-348. doi:10.1038/nri2295
47. Taleb S, Tedgui A, Mallat Z. IL-17 and Th17 cells in atherosclerosis: Subtle and contextual roles. *Arterioscler Thromb Vasc Biol*. 2015;35(2):258-264. doi:10.1161/ATVBAHA.114.303567
48. Spitz C, Winkels H, Bürger C, et al. Regulatory T cells in atherosclerosis: Critical immune regulatory function and therapeutic potential. *Cell Mol Life Sci*. 2016;73(5):901-922. doi:10.1007/s00018-015-2080-2
49. Klingenberg R, Gerdes N, Badeau RM, et al. Depletion of FOXP3+ regulatory T cells promotes hypercholesterolemia and atherosclerosis. *J Clin Invest*. 2013;123(3):1323-1334. doi:10.1172/JCI63891
50. Ait-Oufella H, Salomon BL, Potteaux S, et al. Natural regulatory T cells control the development of atherosclerosis in mice. *Nat Med*. 2006;12(2):178-180. doi:10.1038/nm1343
51. Döring Y, van der Vorst EPC, Yan Y, et al. Identification of a non-canonical chemokine-receptor pathway suppressing regulatory T cells to drive atherosclerosis. *Nat Cardiovasc Res*. 2024;3(2):221-242. doi:10.1038/s44161-023-00413-9
52. Kyaw T, Winship A, Tay C, et al. Cytotoxic and proinflammatory CD8+ T lymphocytes promote development of vulnerable atherosclerotic plaques in ApoE-deficient mice. *Circulation*. 2013;127(9):1028-1039. doi:10.1161/CIRCULATIONAHA.112.001347
53. Nakai Y, Iwabuchi K, Fujii S, et al. Natural killer T cells accelerate atherogenesis in mice. *Blood*. 2004;104(7):2051-2059. doi:10.1182/blood-2003-10-3485
54. Li Y, Kanellakis P, Hosseini H, et al. A CD1d-dependent lipid antagonist to NKT cells ameliorates atherosclerosis in ApoE-/- mice by reducing lesion necrosis and inflammation. *Cardiovasc Res*. 2016;109(2):305-317. doi:10.1093/cvr/cvv259
55. Zhao TX, Kostapanos M, Griffiths C, et al. Low-dose interleukin-2 in patients with

stable ischaemic heart disease and acute coronary syndromes (LILACS): Protocol and study rationale for a randomised, double-blind, placebo-controlled, phase I/II clinical trial. *BMJ Open*. 2018;8(9). doi:10.1136/bmjopen-2018-022452

56. Zhao TX, Aetesam-Ur-Rahman M, Sage AP, et al. Rituximab in patients with acute ST-elevation myocardial infarction: An experimental medicine safety study. *Cardiovasc Res*. 2022;118(3):872-882. doi:10.1093/cvr/cvab113
57. Waddington C. Towards a Theoretical Biology. *Nature*. 1968;218:525–527. doi:https://doi.org/10.1038/218525a0
58. Waddington CH. The epigenotype. *Int J Epidemiol*. 2012;41(1):10-13. doi:10.1093/ije/dyr184
59. Elia L, Condorelli G. The involvement of epigenetics in vascular disease development. *Int J Biochem Cell Biol*. 2019;107(December 2018):27-31. doi:10.1016/j.biocel.2018.12.005
60. Farina FM, Serio S, Hall IF, et al. The epigenetic enzyme DOT1L orchestrates vascular smooth muscle cell-monocyte crosstalk and protects against atherosclerosis via the NF-κB pathway. *Eur Heart J*. 2022;43(43):4562-4576. doi:10.1093/eurheartj/ehac097
61. Boyer LA, Plath K, Zeitlinger J, et al. Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature*. 2006;441(7091):349-353. doi:10.1038/nature04733
62. Lee TI, Jenner RG, Boyer LA, et al. Control of Developmental Regulators by Polycomb in Human Embryonic Stem Cells. *Cell*. 2006;125(2):301-313. doi:10.1016/j.cell.2006.02.043
63. Chammas P, Mocavini I, Di Croce L. Engaging chromatin: PRC2 structure meets function. *Br J Cancer*. 2020;122(3):315-328. doi:10.1038/s41416-019-0615-2
64. Kuzmichev A, Nishioka K, Erdjument-Bromage H, Tempst P, Reinberg D. Histone methyltransferase activity associated with a human multiprotein complex containing the enhancer of zeste protein. *Genes Dev*. 2002;16(22):2893-2905. doi:10.1101/gad.1035902
65. Neele AE, de Winther MPJ. Repressing the repressor: Ezh2 mediates macrophage activation. *J Exp Med*. 2018;215(5):1269-1271. doi:10.1084/jem.20180479
66. Rondeaux J, Groussard D, Renet S, et al. Ezh2 emerges as an epigenetic checkpoint regulator during monocyte differentiation limiting cardiac dysfunction post-MI. *Nat*

Commun. 2023;14(1). doi:10.1038/s41467-023-40186-0

67. Dobenecker MW, Park JS, Marcello J, et al. Signaling function of PRC2 is essential for TCR-driven T cell responses. *J Exp Med*. 2018;215(4):1101-1113. doi:10.1084/jem.20170084
68. Wei G, Wei L, Zhu J, et al. Global Mapping of H3K4me3 and H3K27me3 Reveals Specificity and Plasticity in Lineage Fate Determination of Differentiating CD4+ T Cells. *Immunity*. 2009;30(1):155-167. doi:10.1016/j.immuni.2008.12.009
69. Kim KH, Roberts CWM. Targeting EZH2 in cancer. *Nat Med*. 2016;22(2):128-134. doi:10.1038/nm.4036
70. Duan R, Du W, Guo W. EZH2: A novel target for cancer treatment. *J Hematol Oncol*. 2020;13(1):1-12. doi:10.1186/s13045-020-00937-8
71. Gunawan M, Venkatesan N, Loh JT, et al. The methyltransferase Ezh2 controls cell adhesion and migration through direct methylation of the extranuclear regulatory protein talin. *Nat Immunol*. 2015;16(5):505-516. doi:10.1038/ni.3125
72. DuPage M, Chopra G, Quiros J, et al. The chromatin-modifying enzyme Ezh2 is critical for the maintenance of regulatory T cell identity after activation. *Immunity*. 2015;42(2):227-238. doi:10.1016/j.immuni.2015.01.007
73. Vasanthakumar A, Xu D, Lun AT, et al. A non-canonical function of Ezh2 preserves immune homeostasis. *EMBO Rep*. 2017;18(4):619-631. doi:10.15252/embr.201643237
74. Atzler D, Schwedhelm E, Choe C. L-Homoarginine and cardiovascular disease. *Curr Opin Clin Nutr Metab Care*. 2015;18(1):83-88. doi:10.1097/MCO.0000000000000123
75. Wu Y, Avcilar-Kücükgöze I, Santovito D, Atzler D. Amino Acid Metabolism and Autophagy in Atherosclerotic Cardiovascular Disease. 2024, 14, 1557. *Biomolecules*. 2024;14(12):1557. doi:https://doi.org/10.3390/biom14121557
76. Atzler D, Schönhoff M, Cordts K, et al. Oral supplementation with L-homoarginine in young volunteers. *Br J Clin Pharmacol*. 2016;82(6):1477-1485. doi:10.1111/bcp.13068

12. Acknowledgments

I would like to take this opportunity to express my heartfelt gratitude to everyone who contributed to the success of this work and to my academic development.

First and foremost, my deepest appreciation is extended to the Supervisors of this project. I am profoundly grateful to **Prof. Christian Weber** for his exceptional leadership and insightful guidance, which were instrumental not only for the progress of this project but also for my overall academic growth.
My sincere acknowledgment goes to **Prof. Jürgen Bernhagen** for his supervision of this project and for the enriching discussions during the SFB1123 Retreats over the years.
I am particularly indebted to **PD Dr. Dorothee Atzler** for her outstanding scientific mentorship throughout this project. Her unwavering support has been invaluable for my academic growth and personal development. Her laboratory has been a place of intense effort, rewarding achievements, and occasional disappointments from unsuccessful experiments—all of which contributed significantly to my scientific learning.
I extend my gratitude across the Atlantic to **Prof. Esther Lutgens**, whose excellent co-supervision and constant availability to discuss the EZH2 project were greatly appreciated.

A special note of thanks goes to Floriana, a brilliant colleague and cherished friend. Her expertise in chromatin immunoprecipitation (ChIP) was unparalleled, and her encouragement made this challenging experiment seem attainable.

To the entire AG Atzler team, I am deeply thankful for the collaborative spirit during mouse experiments, lab work, and the many joyful moments we shared—whether at Pizza Lunches, Oktoberfest, or the Weihnachtsmärkte. I owe my gratitude to each and every one of you. Thank you, Michael for introducing me to the EZH2 project; herzlichen Dank, Sigrid, our “Lab Mama”; vielen Dank Katrin, 谢谢 Yuting; ευχαριστώ, Venetia; Obrigada, Yonara. Without our collective efforts, this work would not have been possible.

My special thanks also go to Prof. Santovito and Vasiliki for their invaluable support in deciphering EZH2 sequencing data. Their expertise was of immense significance to this project.

I would also like to that all the co-authors who participated to the EZH2 paper, this work would have never been possible without the great collaborative work.

I would like to express my deepest appreciation to my family—to my parents and to Laura—for their constant encouragement and unwavering belief in me. They have truly been my life coaches. To my Nonna Concetta, thank you for always being just a phone call away, ready to listen to my stories from the Lab.
Finally, my profound gratitude goes to Giacomo, my study partner and older brother, with whom I shared much of this rewarding journey, from preparing our documents for the German Approbation to celebrating each milestone along the way.
A heartfelt thank you is reserved for Stefano, whose steadfast support and presence have given my life deeper meaning.

Thank you all for being an integral part of this chapter of my life. As I continue my scientific journey, the time has now come to embark on my clinical adventure.